PAPER

CRIMINALISTICS

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An STR Melt Curve Genotyping Assay for Forensic Analysis Employing an Intercalating Dye Probe FRET*

ABSTRACT: The most common markers used in forensic genetics are short tandem repeats (STRs), the alleles of which are separated and analyzed by length using capillary electrophoresis (CE). In this work, proof of concept of a unique STR genotyping approach has been demonstrated using asymmetric PCR and a fluorescence resonance energy transfer (FRET)-based hybridization analysis that combines fluorophore-labeled allele-specific probes and a DNA intercalating dye (dpFRET) in a melt match/mismatch analysis format. The system was successfully tested against both a simple (TPOX) and a complex (D3S1358) loci, demonstrated a preliminary detection limit of <10 genomic equivalents with no allelic dropout and mixture identification in both laboratory-generated and clinical samples. With additional development, this approach has the potential to contribute to advancing the use of STR loci for forensic applications and related fields.

KEYWORDS: forensic science, DNA typing, short tandem repeat, fluorescence resonance energy transfer, intercalating dye, fluorophore probe, melt curve analysis

The most discriminatory DNA markers currently used in forensic laboratory analysis are the extensively validated collection of short tandem repeats (STR) comprising the combined DNA index system (CODIS) loci (1). The standard method for the analysis of these loci is primarily capillary electrophoresis (CE), although other options for size separation including mass spectrometry (2) and array-based hybridization (3–5) are under development. A variety of known experimental artifacts arise with CE-based STR genotyping that makes data interpretation challenging for laboratory-trained analysts in a controlled setting.

Developments in other fields for screening both sequence (single nucleotide polymorphisms, SNPs) and size (STRs) DNA variation have the potential to contribute to advancing STR genotyping approaches in forensic science by alleviating or avoiding issues posed by current approaches. The goal of the research detailed in this work focuses on an advancement specifically within the category of hybridization-based detection. Within this category, improvements aimed at discovering and identifying DNA changes can be classified into two major sub-categories: generic DNA intercalator techniques and strand-specific hybridization. Genotyping methods solely using intercalating dyes have primarily been applied for SNP genotyping and have shown a somewhat low-level resolution between amplicons with similar sequence (6). More recent improvements for higher resolution screening have focused on using more proprietary dyes and advances in data analysis (7) and have only recently been tested on di-nucleotide STR loci (8). Although somewhat limited in their ability to resolve many different types of changes in DNA between samples, the major benefit of this hybridization-based approach is the cost savings associated with minimized reagent requirements and reduced design constraints.

Strand-specific methods for improving hybridization-based methods utilize additional nucleic acid reaction components to monitor the progress of amplification reactions using one fluorophore type such as Hybeacons (9) or multiple fluorophore types typically through fluorescence resonance energy transfer (FRET). Hybeacon probes consist of single-stranded oligonucleotides with one or more internal bases labeled with a fluorescent dye. Upon formation of a duplex with its target sequence, the level of fluorescence emission increases because of the disruption of quenching interactions between the fluorophore and nucleobases. Initially used for SNP genotyping, development of Hybeacons for STR typing represents a more complicated approach using multiple additional components and complex analysis strategies (10). The two commonly used types of FRET probes are those using hydrolysis of nucleic acid probes to separate donor from acceptor (i.e., Taqman [11]), and those using hybridization to alter the spatial relationship between donor and acceptor molecules (i.e., Molecular Beacons [12] and dual hybridization probes [13]). The only FRET-based approach that has been recently employed for STR genotyping consists of a dual hybridization probe system (14,15). This requires labeling with two fluorescent molecules which subsequently increases the cost involved in using hybridization-based approaches. Additionally, this method requires the presence of a relatively long stretch of known sequence so that the probe/probe pair can bind specifically in close

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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 or repetitive regions too large to permit effective probe design strategies.

The most optimal approach for screening STR loci would be to combine the reduced cost and ease of use of generic intercalating dyes with the resolution and increased sensitivity of hybridization probes, thus avoiding the artifactual limitations of other approaches. A less sophisticated approach has been demonstrated both by genotyping SNPs with unlabeled probes post-PCR (16) and by inclusion within the amplification reaction (17). This required additional analysis was not able to discriminate all potential alleles and, significantly, was not evaluated as a potential STR typing strategy. An integrated system utilizing FRET between an intercalating dye and a probe labeled with a single fluorophore has been reported previously (18). Howell et al. (18) demonstrated a basic application of the approach for SNP analysis, which showed a dramatic increase in signal intensity when compared with standard intercalating dye and other FRET approaches. The same technology is also specifically useful for studying changes in DNA hybridization (19). Takatsu et al. (20) describe a related approach based on labeled nucleotide incorporation followed by dye fluorophore FRET detection. These studies were not focused on identifying and demonstrating the potential of this approach for genotyping STR loci and its application to forensics and individual identification.

In these studies, proof of concept of an approach for STR typing based on hybridization analysis in which the donor probe is replaced with a DNA intercalating dye has been shown. This approach, termed dye probe fluorescence resonance energy transfer (dpFRET), significantly reduces cost, time to results and assay design, and analysis constraints posed by other approaches. The general dpFRET method is illustrated in Fig. 1 with specific design strategies for STR genotyping depicted in Fig. 2. Briefly, the STR probe is divided into three regions: a "reporter flank," "core repeat region," and "anchor flank." The anchor flank is designed with a higher Tm than the fluorophore-labeled reporter flank that favors hybridization of the anchor region, followed by hybridization of the core repeat region and finally the reporter flank. Upon denaturation, a higher melt signal would be generated for a perfect match versus an imperfect match permitting the correlation of sample allele content with the number of repeats contained within the probe. If the probe were to encounter a mismatch with the template sequence, the result would be imperfect hybridization with the reporter region of the probe resulting in decreased signal intensity because of increased FRET distance and, more importantly, a lower melting temperature because of the reduction in bonding energy of the template/probe complex. A number of different designs were tested for varying lengths of both the reporter and anchor flanks (data not shown). Shorter flanks resulted in partial melt peak separation between a matched and mismatched template. Ultimately, a calculated Tm difference of c. 10–15°C between the reporter and anchor flank sequences proved to be a good indicator for successful probe design for STR genotyping. Additionally, the detection of an amplicon peak by this approach attributed to dye intercalation alone measured at higher wavelengths (620 nm as opposed to 510 nm) is hypothesized to be because of the strong fluorescent signal generated by SYBR Green I whose emission tail end falls within this range (see Fig. 1 "dual signal"). In other words, not only does the probe/template hybrid duplex contribute signal from FRET, but fluorescent signal is also donated by the intercalation of dye by the amplicon. This additional amplicon signal can be used as a qualification of positive amplification in a manner similar to standard intercalating dye melt curve analysis providing an internal control unique to this approach.
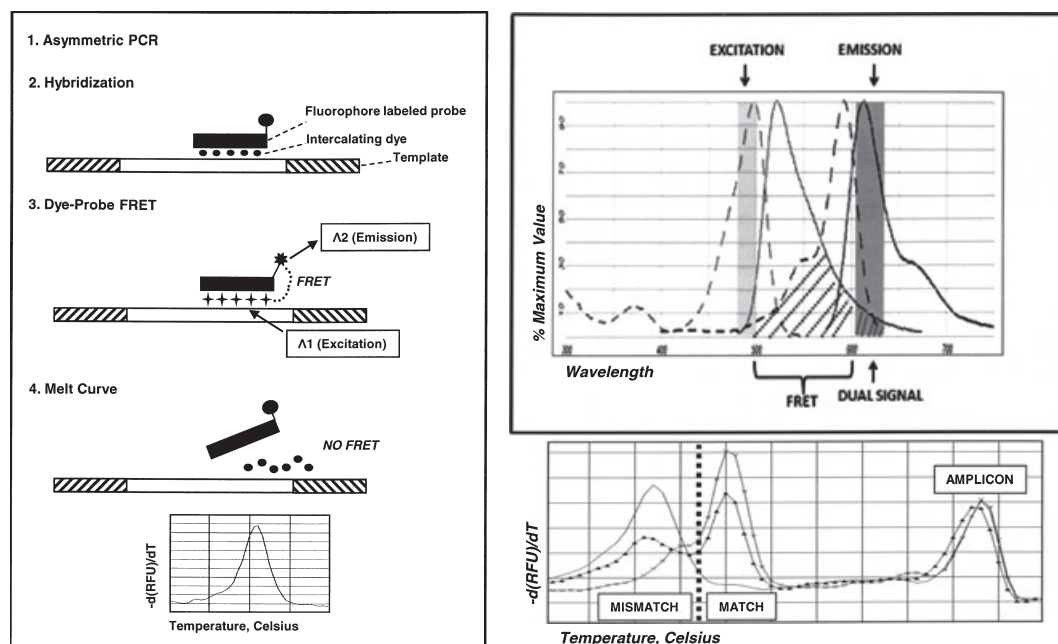

FIG. 1—*dpFRET genotyping strategy. The basic dpFRET protocol (left panel) entails: (1) generation of template for probe hybridization by asymmetric PCR, (2) hybridization of a fluorophore-labeled probe in the presence of a DNA intercalating dye, (3) FRET detection of the interaction between the dye and probe, and (4) a match/mismatch-based melt curve analysis for each allele. Excitation and emission spectra for both the dye and fluorophore attached to the probe are illustrated (top 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 right panel) for dpFRET short tandem repeats genotyping produce either a match probe peak (homozygote—cross), mismatch probe peak (lacks allele—smooth), or both (heterozygote—triangle) with matched and mismatched peaks separated by a dashed line. Amplicon peaks resulting from dual signal donated by the intercalated dye are also shown.*
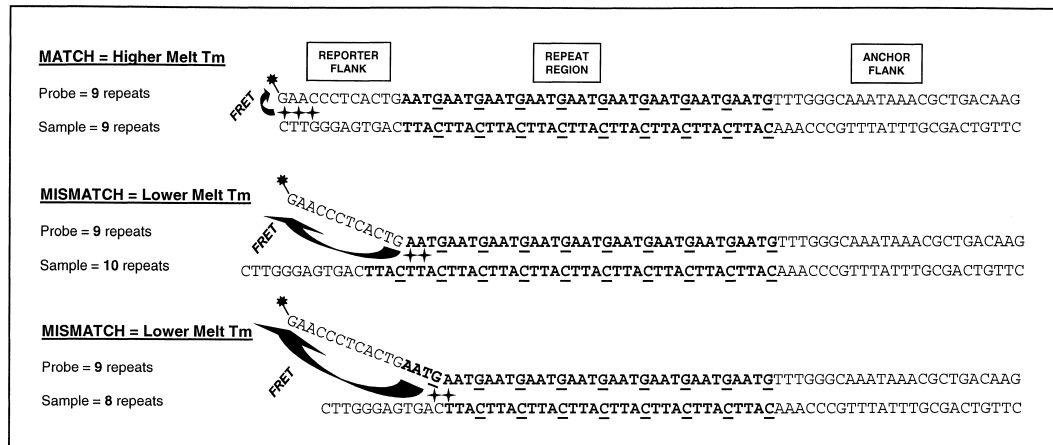
FIG. 2—*dpFRET probe design approach for short tandem repeats genotyping. An example of a TPOX 9 repeat probe sequence is shown theoretically hybridized with sample sequences representing a nine repeat (top), a 10 repeat (middle), and an eight repeat (bottom) template. Imperfect hybridization in the reporter flank region of the probe because of an insertion or deletion of a single repeat unit results in denaturation of the probe/template complex at a lower temperature accompanied by reduced signal strength because of the increase in FRET distance between the dye (plus marks) and fluorophore (star) attached to the probe. The last base in each repeat unit is underlined.*

The purpose of this study was development of dpFRET for STR loci, preliminary determination of the limit of detection, and application to mixed samples for use in forensic analysis. Preliminary data demonstrated that the approach is robust for low copy number detection with no apparent allelic dropout. The results also suggest that this approach has potential to contribute to advancing the use of STRs in forensic analysis by providing the capability to genotype repetitive sequences while alleviating many of the assay design and analysis shortcomings of current approaches.

Materials and Methods

Assay Development—TPOX and D3S1358

Human TPOX and D3S1358 primer sequences from the Power-Plex 16 kit (Promega, Madison, WI) were commercially synthesized (Integrated DNA Technologies, Coralville, IA) and tested against CE-genotyped samples derived from buccal swabs provided by the Johnson County Criminalistics Laboratory (Olathe, KS). Primer sequences included the following: TPOX F (5′-GCACAG-AACAGGCACTTAGG-3′), TPOX R (5′-CGCTCAAACGT-GAGGTTG-3′), D3S1358 F (ATGAAATCAACAGAGGCTTGC), and D3S1358 R (ACTGCAGTCCAATCTGGGT). All amplification reactions were accomplished using a MyCycler thermal cycler (Bio-Rad, Hercules, CA). The primary PCR amplification reaction was supplemented with 1X iQ SYBR Green Supermix (Bio-Rad) and 200 nM of each primer and amplified according to the following rapid thermal protocol: initial denaturation at 98°C for 30 sec followed by 50 cycles of 95°C for 1 sec, 64°C for 15 sec, and 72°C for 3 sec. A portion (8%) of the primary reaction was then added to multiple secondary asymmetric PCR amplifications similar in composition to the primary reaction but supplemented with 1000 nM of template-producing primer. Reactions were amplified for an additional 30 cycles according to the previously mentioned thermal protocol. One secondary asymmetric PCR reaction was carried out for each allelic probe tested. Following amplification, each reaction was supplemented with 5 μM of commercially synthesized allele-specific Texas Red-labeled probe (Integrated DNA Technologies) and melted from 40 to 95°C using a 0.4 degree incremental increase in temperature on an IQ5 real-time PCR platform (Bio-Rad). The emission filter for position two on the instrument was replaced with the Texas Red (620/30M) filter to facilitate FRET detection. Allele-specific probes consisted of the following sequences:

TPOX N [GAACCCTCACTG(AATG)$_N$TTTGGGCAAATAAA-CGCTGACAAG]
D3S1358 17 [TGCATGTA(TCTA)$_1$(TCTG)$_3$(TCTA)$_{13}$TGAG-ACAGGGTCTTGC]
D3S1358 17′ [TGCATGTA(TCTA)$_1$(TCTG)$_2$(TCTA)$_{14}$TGAGA-CAGGGTCTTGC]

The number of TPOX core repeats (N) corresponded with each allele tested. All probes were suspended in 0.1 M EDTA (Promega) to inactivate polymerase and prevent probe extension during hybridization.

Sensitivity and Allelic Dropout—TPOX

Buccal swab samples used for assay development were also used to determine assay sensitivity and potential for allelic dropout. Samples were quantitated using Picogreen and manufacturers' protocols (Invitrogen, Carlsbad, CA) and diluted 10-fold from 5 ng (<1000 genomic equivalent copies) to 50 pg (<10 genomic equivalent copies) in water using 10-fold dilutions. Amplification and melt curve analysis were performed as described earlier.

Mixed Sample Testing—TPOX

Laboratory-generated mixes (1:1 ratio) of human genomic samples derived from buccal swabs were used to determine the potential to detect multiple STR genotypes within a mixed sample. Quantification of the DNA was carried out as described earlier, and 1-ng samples from a homozygote, heterozygote, and an individual lacking a TPOX 8 repeat allele were mixed in different allelic match/mismatch ratios (3:1, 2:2, and 1:3) and tested with a TPOX 8 repeat probe to examine the ability to detect changes in allelic concentrations within a sample.

Following laboratory-generated mixture testing, samples provided by the Dartmouth Hitchcock Medical Center were tested for application to clinical samples. Blinded samples were originally obtained for a previous study on chimerism in bone marrow transplant patients. Multiple cell fractions (monocytes, granulocytes, peripheral blood, and bone marrow) were sampled following clinical treatment

to monitor the success or rejection of the transplanted tissue. Genotypes for the donor, recipient, and cell fractions generated by standard STR genotyping protocols were supplied by Dartmouth Hitchcock Medical Center for comparison to dpFRET STR genotyping. Samples were analyzed using dpFRET as previously described for the TPOX locus, and results compared to those obtained from standard protocols.

Results

Simple STR Locus Testing-TPOX

dpFRET analysis of the TPOX locus was performed on 16 DNA samples provided by the Johnson County Criminalistics Laboratory (JCCL). These samples comprised 14 heterozygotes and 2 homozygotes. Probes recognizing alleles 8–12 and 14 were used. The dpFRET results (Fig. 3) were concordant with the genotype data previously generated by the JCCL using standard CE analysis (Table 1). Matched and mismatched alleles are separated by a dashed line with amplicon peaks included to illustrate specific amplification. Alleles that were known to be present in the samples reacted specifically with the appropriate probe and did not react specifically with the other probes.

Complex STR Locus Testing-D3S1358

dpFRET analysis of the complex STR locus D3S1358 resulted in similar though not identical results. When analyzed by size, complex STR loci can result in the same size profile for alleles that do not contain the same sequence, owing to SNP mutations within or immediately adjacent to the repeat. Increased allelic resolution was seen when analyzed by dpFRET because of subtle sequence differences between the allele probe and the sample allele that were undetectable by CE. This phenomenon will complicate the comparison between dpFRET and standard approaches at loci that exhibit such polymorphisms although subsequent development of micro-variant allele-specific probes should ameliorate this problem. An example of the results generated by dpFRET is provided in Fig. 4 to illustrate this phenomenon. For example, two alleles are listed for D3S1358 for a genotype of 17 in STRBase (http://www.cstl.nist.gov/div831/strbase/str_d3s.htm) labeled as 17 and 17′. Two individuals both typed by CE as homozygotes and containing 17 repeats resulted in differential patterns for a 17′ homozygote (top panels) and a 17, 17′ heterozygote (bottom panels) when analyzed by dpFRET with results confirmed by standard sequencing.
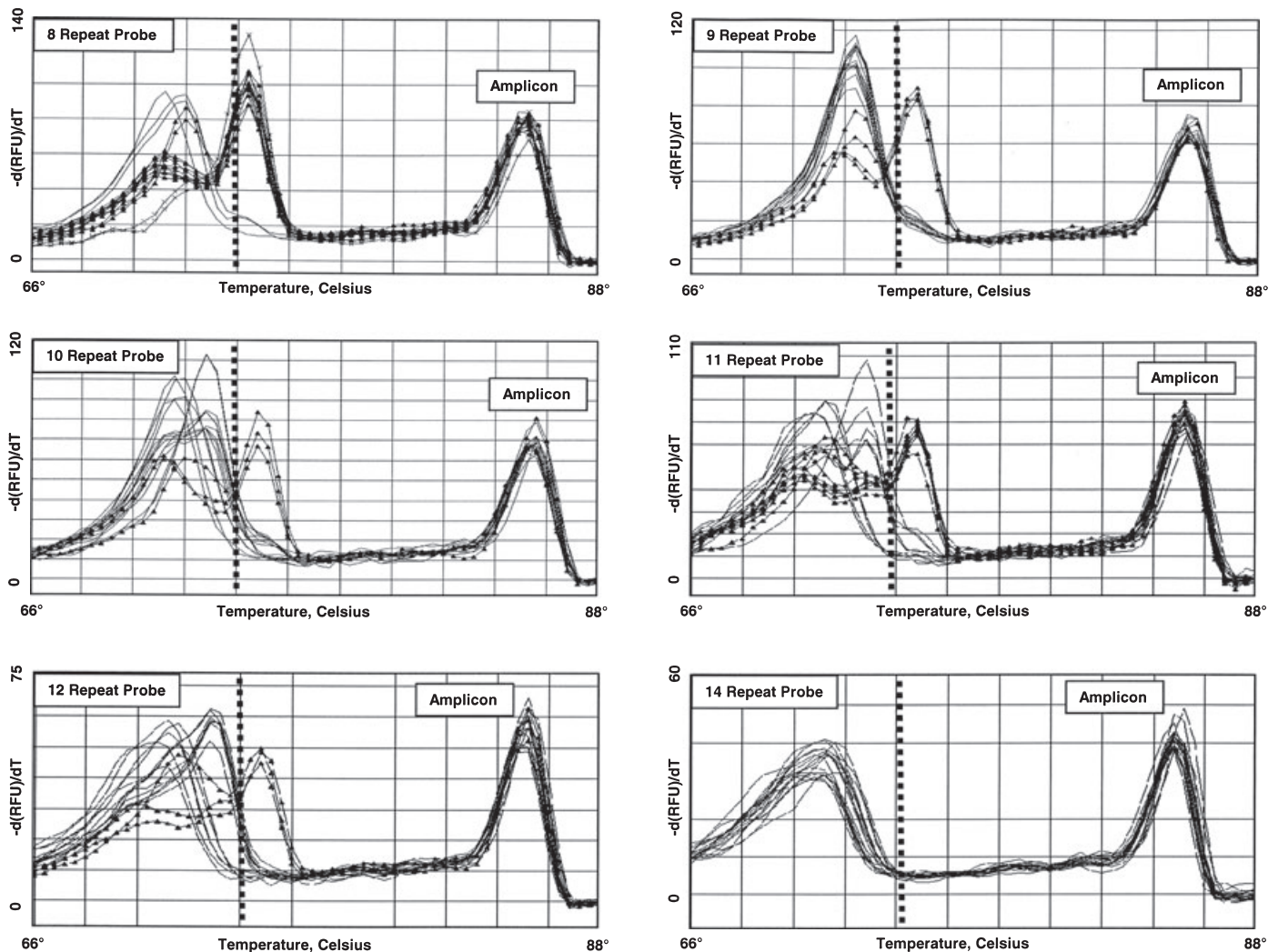

FIG. 3—*TPOX analysis by dpFRET. dpFRET was performed using repeat allelic probes designed to uniquely recognize the 8, 9, 10, 11, 12, and 14 alleles. Matched and mismatched alleles are separated by a dashed line with amplicon peaks included to illustrate specific amplification. Homozygotes (cross), heterozygotes (triangle), and samples lacking the allele (smooth) are indicated.*

TABLE 1—*TPOX CE genotypes for 16 samples tested using dpFRET.*

Sample	Allele 1	Allele 2
1	8	8
2	8	8
3	8	9
4	8	9
5	8	10
6	8	10
7	8	11
8	8	11
9	8	11
10	8	11
11	8	11
12	8	12
13	8	12
14	9	11
15	9	11
16	10	12

CE, capillary electrophoresis.

STR Assay Sensitivity and Allelic Dropout

Preliminary evaluation of the dynamic range and limits of detection using dpFRET for STR analysis was carried out using the TPOX locus. Correct genotyping results were obtained using 5.0 ng, 0.5 ng, and 50 pg of input DNA for both homozygote and heterozygote samples (Fig. 5). Interestingly, the fluorescent signal showed no marked decrease for less concentrated samples, and no allelic dropout was observed for the heterozygote.

Mixed Sample Testing

Laboratory-Prepared Mixture—Artificial mixtures of homozygote and heterozygote samples tested with a TPOX 8 repeat allelic probe resulted in fluorescent match and mismatch signal intensity changes indicative of the concentration of allele within the sample

(Fig. 6). The first mix composed of a homozygote and heterozygote (left panel) contained *c.* three times the amount of target allele (eight repeats) compared to nontarget allele (10 repeats) and resulted in a higher match peak signal intensity by qualitative analysis. It should be noted that the match and mismatch peak fluorescent intensities did not correlate quantitatively with sample allelic content (match ~ 175 RFU, mismatch ~ 80 RFU) in the expected 3:1 ratio. The second mix (middle panel) contained an equal proportion of target and nontarget allele and resulted in *c.* equivalent fluorescent intensities for the match (~ 110 RFU) and mismatch (~ 90 RFU) peaks. The third mix (right panel) was composed of three times the amount of nontarget allele and resulted in higher mismatch peak signal intensity. Again the peak height intensity did not correlate quantitatively with sample allelic content (match ~ 90 RFU, mismatch ~ 130 RFU) at the expected 1:3 ratio.

Clinical Mixture—dpFRET analysis using the TPOX locus for samples from two bone marrow transplant cases produced results similar to those obtained by CE analysis (Fig. 7). Case 1 (left panels) resulted in all cellular fractions displaying donor genotype for both alleles tested. This was in agreement with results generated by CE that detected 90–95% donor for all fractions. dpFRET testing for case 2 (right panels) resulted in successful donor genotyping for all cellular fractions except granulocytes, which showed a mix of both donor and recipient at *c.* a 1:1 ratio. This result was in agreement with prior CE testing that showed a 50% contingent of donor genotype within this sample. Two additional cases were tested (data not shown) and showed similar results to case 1 with concordance between CE and dpFRET genotyping, and no significant difference from donor genotype.

Discussion

STRs are composed of repetitive sequences, and the greatest advantage of these markers compared to SNPs is their multi-allelic
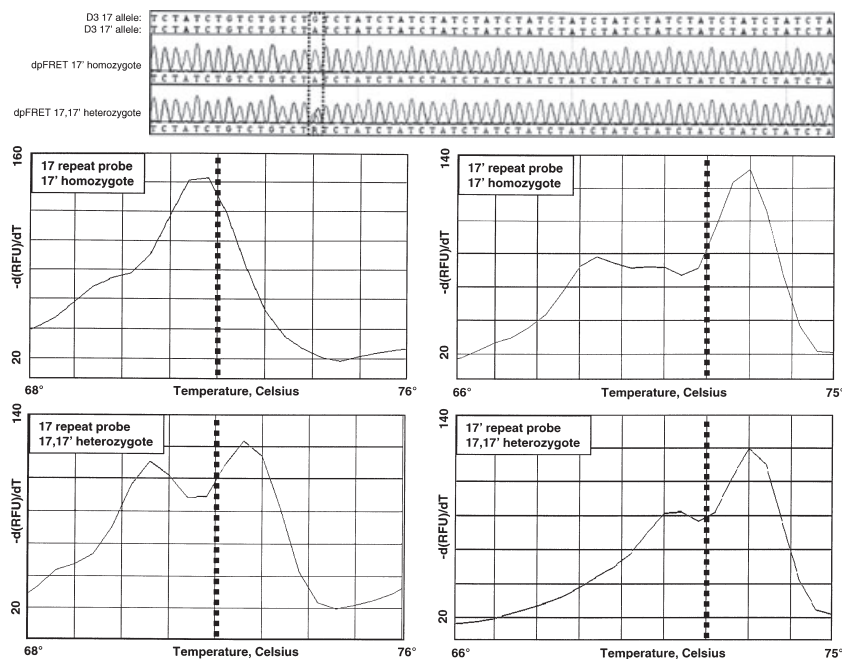

FIG. 4—*D3S1358 analysis by dpFRET. Complex short tandem repeats (D3S1358) dpFRET testing for two samples typed as homozygote 17 by capillary electrophoresis are shown that resulted in a homozygote 17′ (top panels) and a heterozygote 17, 17′ (bottom panels) when tested using allele-specific 17 and 17′ probes that differ by a single base change. Sequence for both samples and probes are indicated at the top of the figure with the expected mixed base position highlighted (R = A or G).*
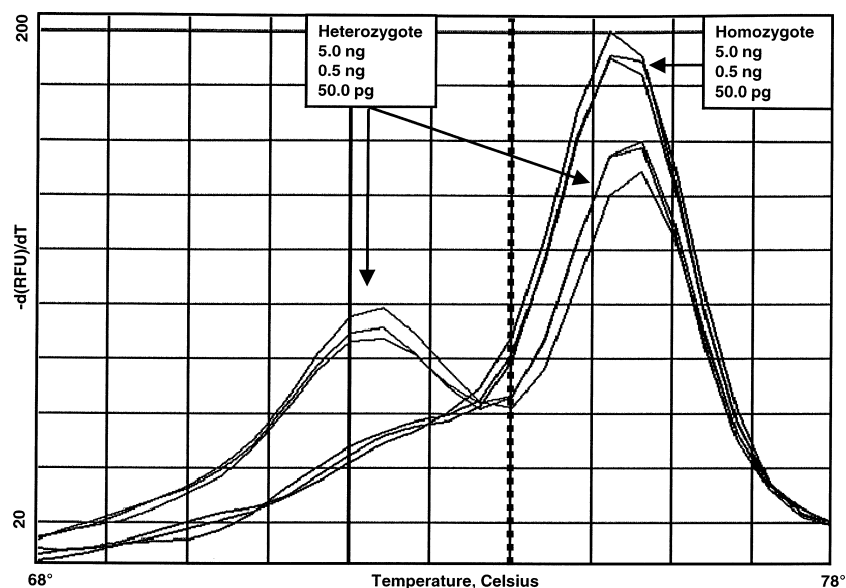
FIG. 5—*dpFRET short tandem repeats Sensitivity and Lack of Allelic Dropout. A TPOX 8 repeat probe was used to test two serially diluted genomic samples. Both a homozygote (8,8) and a heterozygote (8,10) were diluted to final concentrations of 5.0 ng, 0.5 ng, and 50 pg.*
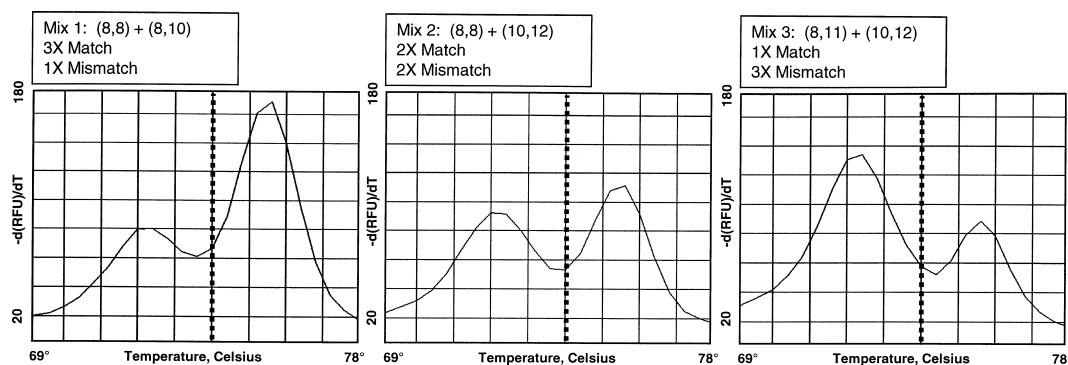

FIG. 6—*dpFRET TPOX short tandem repeats laboratory-prepared mixed sample analysis. Mix 1 (left panel) contained equal amounts of both a homozygote (8,8) and a heterozygote (8,10) sample. Mix 2 (middle panel) was composed of a homozygote (8,8) and a sample lacking the eight repeat allele (10,12), and Mix 3 (right panel) was composed of a heterozygote (8,11) and a sample lacking the eight repeat allele (10,12). All mixes were tested using a TPOX eight repeat allelic probe.*

variation that provides superior discrimination at each locus. Genotypes are generated by amplification followed by sizing of the alleles by CE. This approach is subject to a number of artifacts and requires specialized equipment and extensive analyst training to generate and interpret genotypes.

In contrast to CE, hybridization-based genotyping of DNA variants depends on oligonucleotide melting temperature (Tm). The Tm of duplex DNA is defined as the temperature where one-half of the nucleotides are paired and one-half are unpaired (21). Tm can be predicted using a variety of formulas with the most accurate being the thermodynamic nearest neighbor model (22). The nearest neighbor model is based on the assumption that probe hybridization energy can be calculated from enthalpy and entropy of all nearest neighbor pairs, including a contribution from each dangling end (23). Dangling ends (also known as "end effects" or "end-fraying") account for the effects seen when a shorter probe is bound to a target with flanking sequence (24,25). Various interactions contribute to probe/template stability, but it has been demonstrated that

melting of the complex is initiated at the ends of the duplex (26). The results of the present work suggest that this dangling end effect provides dpFRET with a higher level of resolution when compared to an intercalating dye.

Initial assay development for STR dpFRET genotyping consisted of both a simple (TPOX) and a complex (D3S1358) STR loci. Following brief optimization for 80 cycle amplifications (data not shown), initial probes were designed using the previously established strategy for the most common alleles of the TPOX locus (8–12 repeats). Blinded samples previously genotyped using standard forensic CE protocols were provided by the JCCL and analyzed by dpFRET. dpFRET produced the same genotypes for TPOX when compared to the current CE approach, but in less time (2.5 h) than required for the current CE approach (4 h). Following assay development for a locus with a simple repeat structure, similar design strategies and testing were used for the complex locus D3S1358. Results were equally successful and may even have the potential to provide higher allelic resolution than current

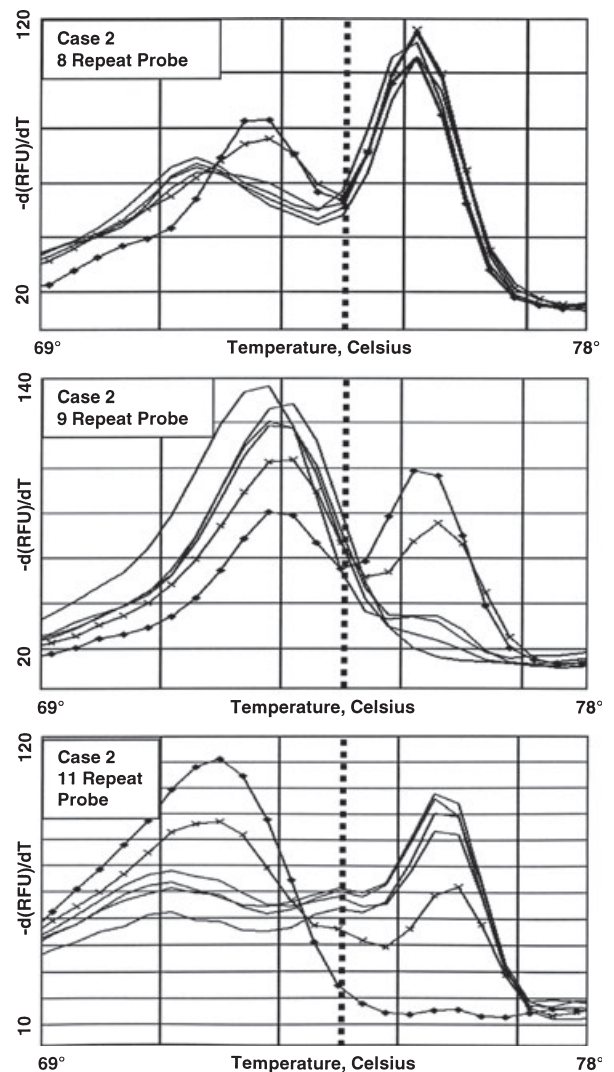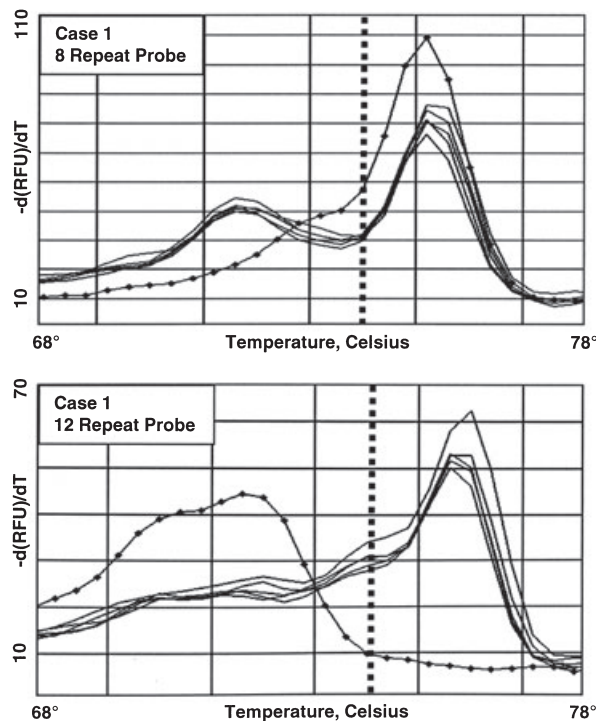| Cell fraction	Case 1		Case 2	
	% Donor (8,12)	% Recipient (8,8)	% Donor (8,11)	% Recipient (8,9)
Peripheral blood	90	10	80	20
Monocytes	95	5	90	10
Granulocytes	95	5	50	50
Bone Marrow	95	5	90	10

FIG. 7—*dpFRET TPOX short tandem repeats clinical mixed sample analysis: bone marrow transplant testing. Case 1 (left panels) was typed by both capillary electrophoresis (CE) and dpFRET for a donor (8,12), a recipient (8,8), and four cellular fractions. All fractions for case 1 showed majority donor genotype (smooth) by both methods with undetectable amounts of recipient (diamond). Case 2 (right panels) was similarly typed for a donor (8,11), a recipient (8,9), and four cellular fractions. Granulocytes (cross) showed a reduced proportion of donor genotype (smooth) with increased proportion of recipient genotype (diamonds) for both dpFRET and CE testing. Results generated by CE for both cases are listed.*

approaches. This preliminary work demonstrated that dpFRET analysis can be accomplished using existing primer designs and amplification strategies for both simple and complex loci.

Results for D3S1358 locus testing suggest that dpFRET has the potential to provide higher resolution of complex STR markers than possible with CE-generated profiles. A complex locus with more than one core repeat has the potential to generate the same size product with different alleles. For example, D3S1358 17 and 17′ are different alleles but cannot be differentiated by size. Results generated using 17 and 17′ specific dpFRET probes were able to differentiate between these two genotypes because of differential probe hybridization. Additional support is required to prove this hypothesis with future development for complex loci necessitating the cloning of amplification products followed by sequenced verification of sample allelic content.

No matter the approach for STR genotyping, allelic dropout is an important consideration for forensic analysis of low template DNA samples (27,28). This phenomenon is because of preferential amplification of one allele owing to stochastic effects (29). Because

of the potential for allelic dropout, it is important to quantify starting material prior to CE-based testing. Results of preliminary sensitivity testing using dpFRET showed no allelic dropout for a heterozygote sample. Tenfold diluted concentrations of starting material were tested, and preliminary results demonstrated no marked change in final fluorescent signal for diluted samples. Current forensic protocols incorporating CE-based genotyping typically utilize 28–32 cycles for PCR amplification as opposed to dpFRET protocols that utilize a total of 50–80 cycles of amplification. The increased number of cycles for dpFRET potentially alleviates sampling error that can be seen with fewer cycle amplification approaches. Thus, unlike CE where detector saturation effects complicate interpretation, additional amplification opportunity (cycles) in dpFRET is provided to produce an equivalent signal for both alleles. It is realized that 50–80 cycles posits concerns of increased contamination artifacts and future testing, and evaluation studies will address this concern in great detail.

Both allelic dropout and prequantification are also important considerations with mixed sample testing. Results for both
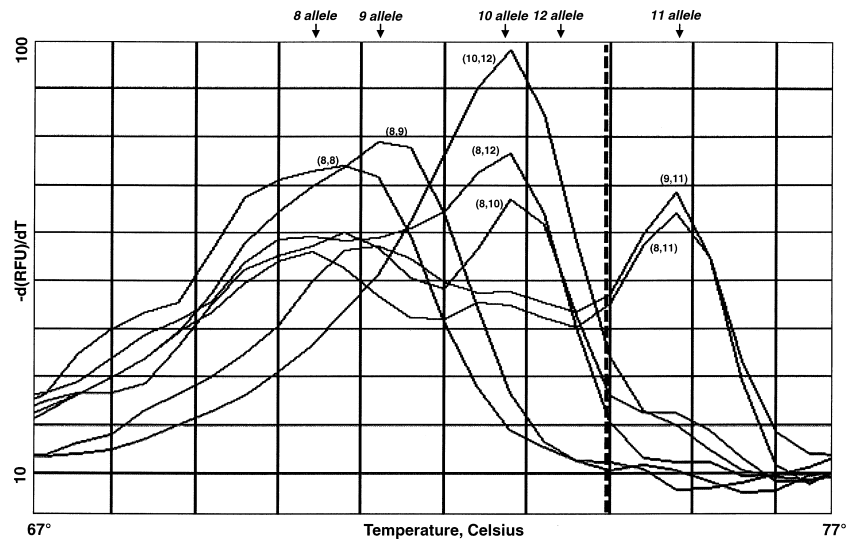
FIG. 8—*dpFRET TPOX 11 repeat probe differential mismatch peak profiles. Samples containing genotypes (8,8), (8,9), (8,10), (8,12), (10,12), (8,11), and (9,11) were tested with a TPOX 11 repeat probe. Melt curves generated in the mismatched region of the graph appear to be indicative of allelic content. Generalized regions for mismatch melting of each allele are indicated above the graph.*

laboratory-generated and clinical sample admixtures demonstrated dpFRET's potential to detect samples containing more than one genotype. Success with producing equivalent results to size-based testing for the percent donor contribution to cell fractions for bone marrow transplant provided evidence that clinical application is possible, albeit with further development. It was noticed that results were somewhat variable for correlation of peak height intensity with true allelic content for mixed samples. A potential explanation for this result is sampling error because of the amplification approach. The protocol that was used for amplification of both laboratory and clinical samples was 50 cycles of double-stranded amplification followed by introduction of a small portion of this reaction into another reaction consisting of 30 cycles of single-stranded amplification containing only one primer. Because of the need to test multiple probes per sample, this method was used in an effort to minimize the amount of sample used for testing. Following the 80 cycles of amplification, allele-specific probes are supplemented in each reaction. This minimizes the amount of sample required but also provides potential for introduction of sampling error that could result in peak height variability. Protocols were also tested based on closed tube 80-cycle amplification protocols (data not shown) that demonstrated less variability and better correlation with signal intensity. Unfortunately, this would require multiple aliquots of sample for testing of multiple alleles at each locus. Although limited success for dpFRET mixed sample testing was demonstrated, there is good potential for obtaining better correlation through additional protocol development with particular attention to sampling methodology.

Current strategies for dpFRET STR analysis are based on standard melt curve analysis of each potential allele. Although proven successful, this approach requires multiple reactions per locus, additional time for analysis, and acquisition of relatively large data sets. These limitations prompted exploration of alternative methods to either reduce the number of reactions required per locus and/or further simplify the melt curve analysis required to differentiate the presence/absence of an allele. A reduction in the number of reactions required to genotype an individual at a locus would necessitate the ability to genotype with a reduced number of probes. This approach would require moving from a match/mismatch-based
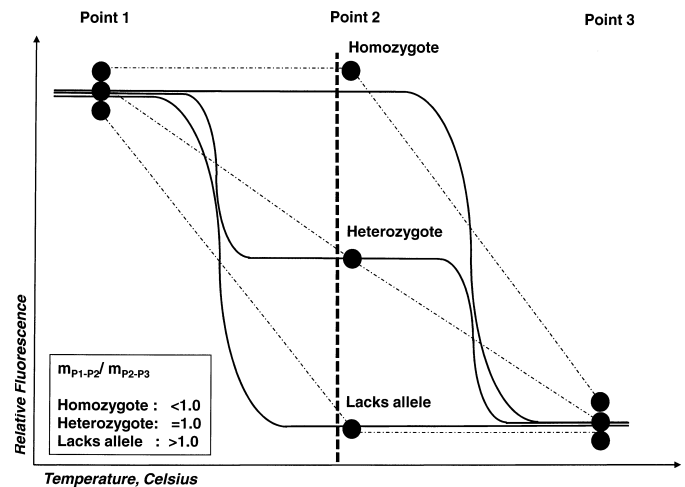

FIG. 9—*dpFRET short tandem repeats slope ratio analysis. As opposed to standard melt curve analysis that requires a large number of fluorescent readings, minimal data measurements are taken at three temperatures represented by points 1–3. Hypothetical examples for a homozygote, heterozygote, and a sample lacking the allele are shown. The ratio of the two slopes (mP1-P2 and mP2-P3) generated for the three points defines the genotype.*

analysis to a more classical melt-based analysis similar to genotyping SNP mutations. Early results demonstrated variation in the mismatch peak melt curve that appeared potentially correlative with the mismatched allele present in the sample. Thus, it appeared that a higher level of discrimination was possible beyond a basic presence/absence type analysis. Similar results were generated with other repeat probes, and it was determined that higher repeat number probes resulted in better resolution of mismatched melt peaks. For example, testing with an 11 repeat probe (Fig. 8) demonstrated potential to differentiate the full allelic complement of a sample beyond a simple match/mismatch-based analysis. The potential to generate an STR genotype for a sample using dpFRET and a minimal number of probes appears likely but will require the

use and development of higher resolution approaches and curve fitting analysis.

Classical melt curve-based analysis requires time and additional resources to generate a multitude of data points for every temperature point along the curve. Current dpFRET STR analysis produces distinct match/mismatch melt peaks separated by *c.* 3–4°C. To reduce the time and complexity of analysis, a minimal number of fluorescent data points can be taken at three temperature points: (1) prior to probe/template denaturation, (2) a point midway between melting of a matched and mismatched hybrid complex, and (3) following complete probe denaturation. By comparing the slope ratios between these points (points 1–2 and points 2–3), a more rapid quantitative method for STR genotyping is possible that requires only three temperature measurements. This method of analysis is depicted in Fig. 9 and is capable of genotyping both homozygotes and heterozygotes. With careful design, this same analysis can potentially be applied for all probes at multiple loci further simplifying analysis. This would necessitate careful control of probe melting temperatures based on reporter and anchor flank sequence design. Further development of this approach could improve the speed and reduce the complexity of dpFRET STR testing when compared to current classical melt curve analysis.

Although displaying great potential, there is currently a limit to the loci that can be tested using dpFRET STR genotyping. For example, not all of the CODIS loci used for forensic applications are capable of being interrogated using this approach because of a limit of 100 base pairs imposed by commercial suppliers on the size of fluorescently labeled probes. Approximately 20 base pairs of flanking sequence is required for probe design, which leaves <80 base pairs for the repeat section of the probe, and therefore the dpFRET approach is only presently compatible with tetranucleotide repeat loci with a maximum repeat number of 19 (or 76 base pairs). Based on these limitations, limited alleles at loci VWA, D18S51, D21S11, and FGA are currently amenable to dpFRET genotyping because their maximum repeat sizes for larger alleles are >80 base pairs. Technology for oligonucleotide synthesis is continually improving, and this limitation should be overcome in the near future. However, there are many other STR loci not included in the CODIS core set that have been characterized for forensic applications (30) and are presently adaptable for dpFRET genotyping.

In summary, dpFRET requires only an acceptor probe and can be designed against any appropriate length repetitive sequence. Most importantly, this strategy provides an objective allele match/mismatch-based analysis that is automatable, capable of multiplexing, and can be analyzed using standard technology presently established for DNA melt curve analysis. The extraction and amplification portion of the protocol for dpFRET genotyping of an STR locus is similar to the current approach used in forensic laboratories. No changes to either primer sequences or thermal protocol are required. The only deviations from current protocols are an increased concentration of the hybridization template generating primer for asymmetric PCR and the addition of an intercalating dye to the reaction. Both these approaches (SYBR Green I and asymmetric PCR) are common methodologies employed in molecular biology. The benefits to using dpFRET for STR screening are numerous. It is less costly than many other approaches because of the use of an intercalating dye, a probe with a single fluorophore, and minimized equipment requirements. Probe design is fairly flexible, and initial results suggest it to be somewhat sequence independent. Equipment requirements are minimal needing nothing more than is required for basic real-time PCR. Application of this new approach has the potential to alleviate many of the shortcomings of current CE-based genotyping approaches and could be applied to other fields that require genotyping of repetitive sequences.

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