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PAPER

CRIMINALISTICS

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STR Melting Curve Analysis as a Genetic Screening Tool for Crime Scene Samples*

ABSTRACT: In this proof-of-concept study, high-resolution melt curve (HRMC) analysis was investigated as a postquantification screening tool to discriminate human CSF1PO and THO1 genotypes amplified with mini-STR primers in the presence of SYBR Green or LCGreen Plus dyes. A total of 12 CSF1PO and 11 HUMTHO1 genotypes were analyzed on the LightScanner HR96 and LS-32 systems and were correctly differentiated based upon their respective melt profiles. Short STR amplicon melt curves were affected by repeat number, and single-source and mixed DNA samples were additionally differentiated by the formation of heteroduplexes. Melting curves were shown to be unique and reproducible from DNA quantities ranging from 20 to 0.4 ng and distinguished identical from nonidentical genotypes from DNA derived from different biological fluids and compromised samples. Thus, a method is described which can assess both the quantity and the possible probative value of samples without full genotyping.

KEYWORDS: forensic science, criminalistics, forensic biology, high-resolution melting curve analysis, short tandem repeat typing, genetic screening

Forensic laboratories have been greatly impacted by the expanded use of DNA analysis to solve major crimes and will be further burdened by its application in the investigation of property crimes. Forensic casework can include tens or even hundreds of biological items of evidence and can consist of varying types including blood, semen and saliva stains, hairs, and touch DNA samples. Because resources are limited, laboratories typically restrict the number of samples to be tested in any one case. Unfortunately, the sample selection process is often based on case context or presumptive testing, neither of which provide direct information as to the donor. Thus, any effort to locate the few suspect-associated stains in the vast background of victim-associated stains typically requires a tour d'force approach where resources are wasted on over-sampling or over-testing. If a genetic screening technique was available that could rapidly detect samples foreign to the victim or suspect, or determine the number of donors present, it could be used to include or exclude subjects. Such a tool could reduce the wasteful use of full genetic profiling simply to search for probative evidence.

The evolution of methods to establish human identity has uncovered an important trade-off between specificity and the optimal utilization of the evidence at hand. Early serological tests based upon antigenic (i.e., ABO typing) and protein markers (i.e., PGM) could establish source attribution quickly and cheaply on a large numbers of samples but were not highly discriminating. Conversely, restriction fragment length polymorphism and short tandem repeat (STR) markers, though highly discriminating, are expensive and time-consuming to perform limiting the number of stains that are economically feasible to analyze. What is desirable is a screening method that will allow genetic comparisons to be made while simultaneously permitting more evidence to be processed from the crime scene without departing from the standard protocol of DNA extraction, quantification, and STR amplification and typing.

Numerous genotypic testing methods have been proposed and used to establish the inclusion or exclusion of suspects, or to elucidate the possible number of unique stains. single nucleotide polymorphism (SNP)-based methods (e.g., nuclear HLA typing [1] and mitochondrial HVI/HVII [2] screening) are useful for establishing donor attribution but are typically biallelic and possess a low power of discrimination. Conversely, STR genotyping, which utilizes electrophoresis to measure size differences in alleles possessing different numbers of full or partial repeats, is highly discriminating. Electrophoretic mobility, however, is not the only characteristic that varies with fragment size, but the dissociation temperature or melting temperature (T_m) of a double-strand DNA (dsDNA) molecule also varies as a function of its nucleotide sequence, its length, and the degree to which its strands are perfectly complementary. This difference in the melting behavior of STR alleles can also be exploited to genotype dsDNA molecules by a process known as melt curve analysis.

The T_m, also the midpoint of the melting curve, is defined as the temperature at which the equilibrium constant for the following [dsDNA] $\rightarrow$ [ssDNA] $\times$ [ssDNA] equals one, that is, $[ssDNA]^2/[dsDNA] = 1$. This condition can be satisfied by two populations

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of molecules—one double-stranded and the other single-stranded, or where a portion (e.g., the low-melting domain) of each dsDNA molecule is single-stranded, or some combination of these two extremes.

In practice, the melting behavior of a dsDNA molecule that encompasses an allelic region of interest is correlated with the genotype(s) present. For homozygous samples, the melting behavior is typically sharp, symmetrical, and dependent on product length and sequence composition. Thus, homozygotes that differ in length or sequence exhibit small differences in their T_ms. On the other hand, melt curves derived from heterozygous alleles reflect the melt behavior of the linear combination of two or more alleles that differ by one or more bases or repeats. In heterozygotes, homoduplexes will form for each of the two alleles present, but a small number of heteroduplexes will form as well between mismatched alleles, or the probes that bind to them. In fact, the relative percentage of heteroduplexes can be enriched by introducing a rapid cooling step following polymerase chain reaction (PCR) but prior to melting the sample.

High-resolution melt curve (HRMC) analysis relies on the property of DNA intercalating cyanine dyes, such as TOTO, YO-PRO-1, and SYBR Green (Invitrogen, Carlsbad, CA; Molecular Probes, Eugene, OR), and DNA minor groove-binding dyes, such as LCGreen (3,4), SYTO 9 (5), BEBO (6), among others, to undergo a dramatic decrease in dye fluorescence because of solvent quenching upon the melting of duplex DNA. However, because the latter dyes do not inhibit PCR at saturating levels, and capture the melting behavior of mixed PCR products more accurately, with higher sensitivity, and with greater reproducibility, many HRMC applications designed to use the intercalating dyes have evolved to use the minor groove-binding dyes.

An analysis by Schütz and von Ahsen (7) examined both fundamental thermodynamic and operational parameters that effect the melting of nucleic acids and discovered several variables that are key to obtaining successful genotyping by HRMC. Necessarily, the melting of the amplicon must approximate a two-state system (i.e., duplex vs. the melted state), and the domain of interest (i.e., the polymorphism) must melt in the region of detection. Operational parameters also contribute to success such as the rate of melting, the ability of the saturating dye to raise the enthalpy (i.e., the stability) of the amplicon without inhibiting PCR, and the temperature resolution of the instrument. Because other variables, such as the concentration of DNA undergoing melting, and the particular solvent and solutes that are present during melting, can affect the melting of DNA, these factors must be considered and their effects mitigated when designing an HRMC experiment.

Recently, a high-sensitivity master mix (LightScanner® Master Mix; Idaho Technology, Salt Lake City, UT) has become available that can adjust for these effects. This mastermix contains the saturating dye LCGreen Plus and 0.05 μM of a high ($T_m \sim 92°C$) and a low ($T_m \sim 62°C$) melting double-strand (ds) oligonucleotide. These 3′-blocked, short oligonucleotide duplexes serve as internal temperature calibrators to compensate for positional differences in optical detection and well temperature, and adjust for volume and compositional differences in PCRs. Following melting, the first derivative peak signatures for these calibrators serve to normalize sample-to-sample differences, resulting in less dispersion, and greater accuracy and reproducibility (e.g., standard deviations in T_m are reduced from ~0.17°C to between 0.03 and 0.10°C), in grouping genotypes by difference curve and T_m comparisons (8).

Accurate genotyping of two or more alleles using melting curve analysis can use either probes that hybridize to the polymorphic region, or PCR amplicons. Probe-based methods have proven quite promising versus amplicon melting in HRMC applications because melting temperature differences are more pronounced (~2–8°C) for detecting dissimilarities in probes between 15 and 35 nts in size that are complementary to one allele (9). However, fluorescently labeled probe-based methods are expensive, and sequence detection using unlabeled, nonextendable, oligonucleotides is difficult to design. Mutation scanning, on the other hand, uses short amplicons, saturating dsDNA dyes, and is both cost effective for genotyping and easy to design and optimize. These studies typically use an intercalating dye, such as SYBR Green I (10,11), or a dsDNA-binding dye such as LCGreen (12), SYTO 9 (5), and Eva Green (13) during PCR and melting. Because the magnitude of the T_m shift resulting from a mismatched base-pair is inversely correlated with amplicon length, smaller PCR amplicons that encompass the polymorphism are typically easier to genotype (14,15).

The use of low-resolution melt curve analysis as a forensic DNA screening method has been developed for sex determination (16), the detection of heterozygozity at the HUMTHO1 locus (17), and SNPs (18) on quantitative PCR (qPCR) thermal cyclers using SYBR Green dye. Similarly, HRMC analysis has been performed on instruments that are optimized to detect 0.1–0.05°C differences in melting temperature, and developed for forensic screening applications for the detection of SNPs in HLA types (19,20), discriminating SNPs (21), limited STR analysis by probe hybridization (22), and a plethora of other mutation scanning and genotyping analysis applications in genetics and medical diagnostics (23).

Numerous instruments with HRMC analysis capabilities were available when this investigation was initiated in 2006. The Light Scanner®-96 (Idaho Technology Inc.) was chosen because of its superior well-to-well temperature uniformity (near ±0.15°C), its data sampling rate of 10–12 points/°C (each point consists of 7–12 optical and temperature acquisitions that are averaged), and its excellent signal-to-noise ratio (1800), paired with its data analysis software that compensates for differences in fluorescent intensity and other subtle sample effects (4,24). The LS-96 analysis software also has the ability to utilize the T_m information provided by the internal temperature-calibrating oligonucleotides. Based upon our theoretical calculations of the T_m differences between the different alleles (approximately 0.1 and 0.2°C per repeat unit for CSF1PO and HUMTHO1), comparisons would have been impossible on less sensitive instruments, which are not as well controlled for positional and thermal irregularities, and slight compositional differences in PCRs.

Here, in this proof-of-concept study, we describe the use of the LightScanner-96 to detect differences in the melting profiles of mini-STR amplicons, produced from the CSF1PO and HUMTHO1 STR loci, and quantified during qPCR, in the presence of the intercalating dye SYBR Green or dsDNA-binding dye LCGreen Plus. The utility of this approach was demonstrated on single-source samples, two-person mixtures, and forensically challenging samples typifying those that might be encountered in crime scene investigations. The sensitivity, reproducibility, and discriminatory ability of HRMC analysis of STR loci is also discussed in the context of other techniques for evidence screening, as well as the techniques' sufficiency to screen biological material, to gather intelligence, and to make the most efficient use of the evidence at hand.

Material and Methods

DNA Samples

Typed blood lines were provided by the Los Angeles Police Department Scientific Investigative Division Serology Unit and

extracted with a QIAGEN EZ1 BioRobot using the QIAGEN EZ1 DNA Tissue kit (Qiagen, Valencia, CA). A total of eight CSF1PO genotypes [(9,11), (10,10), (10,11), (10,12), (10,13), (11,11), (11,12), and (12,13)] and eight HUMTHO1 genotypes [(6,7), (6,9.3), (7,7), (7,9), (8,9.3), (9,9), (9.3,9.3), and (9.3,10)] were used. In each instance, one donor was used per genotype with the exception of THO1 genotypes (6,7) and (6,9.3) in which two donors were used (i.e., 10 donors were used for the THO1 genotyping experiment). For the substrate study, DNA from blood and non-blood sources was extracted using the organic method (phenol, chloroform, and isoamyl alcohol mixture in a 50:49:1 ratio) followed by washing three times on a Centricon YM-100 spin column (Millipore, Bedford, MA).

The eight DNA standards used for qPCR were prepared from human placental DNA purchased from Sigma Aldrich Chemical Corp. (St. Louis, MO) and encompassed a concentration range of 50–0.023 ng/μL.

HRMC experiments performed at Idaho Technology utilized staff DNA samples extracted from 16 individuals with the Gentra Autopure LS robotic workstation (Gentra Systems Inc., Minneapolis, MN) and quantified with the Nanodrop UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Altogether, nine CSF1PO genotypes [(8,11), (9,12), (10,10), (10,11), (10,12), (11,11), (11,12), (11,13), and (12,12)] and eight THO1 genotypes [(6,6), (6,7), (6,9), (6,9.3), (7,9.3), (8,8), (8,9.3), and (9,9)] were represented with multiple donors possessing two CSF1PO types [(10,11) and (10,12)] and five THO1 types [(6,6), (6,7), (6,9.3), (7,9.3), and (8,9.3)].

Quantitative PCR

DNA extracts were quantified on an ABI 7000 Sequence Detection System using either a TaqMan, total human DNA assay (the Canadian Forensic Science HUMTHO1 assay or CFS-HUMTHO1 assay) as described by Richard et al. (25) or a dye intercalation assay using either SYBR Green I Master Mix™ (with ROX) or LightScanner Master Mix with the LCGreen Plus dye (without ROX), and the same CSF1PO primers used to generate short amplicons for melting analysis. The CFS-HUMTHO1 assay amplifies a 62-bp amplicon that is 31-bp downstream of the polymorphic repeat region of the HUMTHO1 locus (Accession D00269). Five microliters of DNA extract in a total reaction volume of 25 μL are quantified by comparison with an eight-point DNA standard curve (50–0.023 ng/μL). PCR cycle conditions for both assays enlist a hot-start step (95°C) followed by 40 cycles of two-temperature PCR.

Amplification

Specific SYBR Green amplification of the CSF1PO locus and THO1 locus was achieved by real-time PCR on the ABI 7000 using reactions performed in a standard ABI 96-optical-well plate covered with optical adhesive film. Unless otherwise stated, between 1 and 6 ng of DNA was used in all genotype experiments.

For experiments utilizing SYBR Green, a 25 μL reaction volume was used with the ABI SYBR Green Master Mix™ (Applied Bio-Systems Inc., Carlsbad, CA) and 0.3 μM of each primer. The cycling parameters for qPCR of the CSF1PO locus included an initial "hot-start" step at 95°C for 10 min, followed by 40 cycles of two-temperature PCR with the following temperatures and hold times: denaturation at 95°C for 15 sec and an annealing/extension at 60°C for 1 min. Amplification of the THO1 locus was identical except that 65°C was used as the annealing/extension temperature.

LCGreen reactions utilized 0.25 μM of each primer, 4 μL of DNA extract, and 4 μL of 2.5× LightScanner Master Mix in a total reaction volume of 10 μL. The cycling parameters for PCR included an initial "hot-start" step at 95°C for 2 min, followed by 45 cycles of two-temperature PCR with the following temperatures and hold times: denaturation at 95°C for 30 sec and annealing/extension at 68°C for 30 sec. PCR-amplified products were subjected to a final 30 sec denaturation at 95°C followed by a rapid drop to 28°C to favor the formation of heteroduplexes in heterozygous samples.

Primers

Mini-STR primer sequences (Fig. 1), taken directly, or adapted, from the paper by Butler et al. (26), were used to amplify short DNA fragments, which encompassed the polymorphic region of the CSF1PO and THO1 loci. These primers were synthesized by Sigma Genosys (The Woodlands, TX).

Melting Curve Analysis

Following PCR, the SYBR Green samples were transferred, in duplicate, from an ABI 96-optical-well reaction plate to an ABgene (AB-0800/W) 96-white-well plate (a step that was not required for the LCGreen reactions) and were overlaid with mineral oil. The samples were melted by heating from 55 to 95°C at a ramp rate of 0.1°C/sec using the LightScanner-96. Data were analyzed using the LightScanner Analysis software vers. 1.5.0.972 (vers. 2.0.0.1331

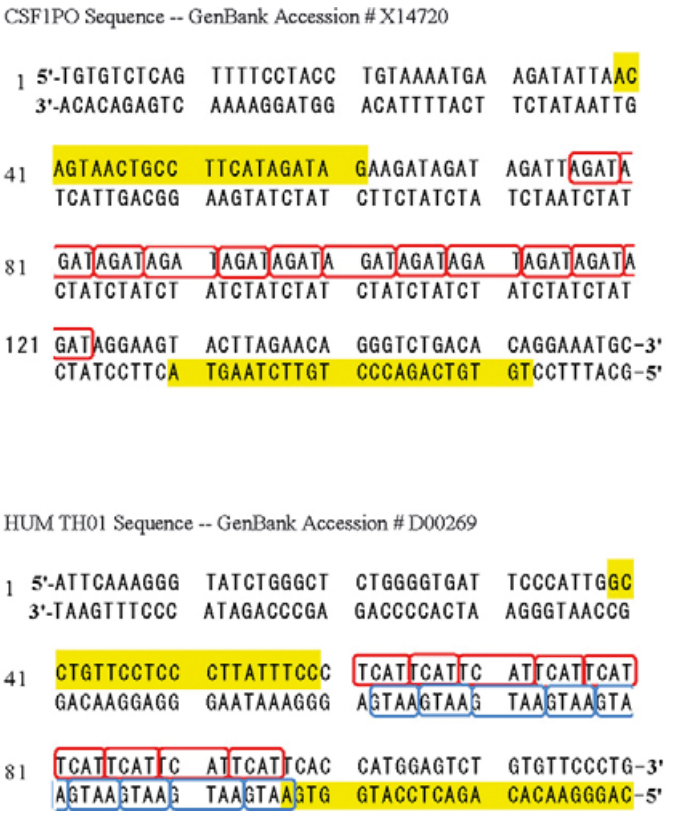

FIG. 1—*Nucleotide sequences depicting the tetranucleotide STR repeat region for the CSF1PO and HUMTHO1 loci. The mini-STR primer sequences (highlighted in yellow) flank the repeat regions (red outlined boxes) of the CSF1PO and HUMTHO1 loci. The blue outlined boxes in the HUMTHO1 sequence depict the four-nucleotide repeat (5′-AATG-3′) on the opposite strand for the HUMTHO1 locus which is referred to in some literature.*

was used for LCGreen reactions). Raw melt curve data were first normalized by selecting a temperature range over which the premelt fluorescence is set to 100%, and the postmelt fluorescence is set to 0%, for all samples. The SYBR Green temperatures used for the premelt and postmelt normalization were 66.1–68.1°C and 74.3–75.3°C for the CSF1PO melt curves, respectively, and 65.0–66.0°C and 75.7–76.7°C for the HUMTHO1 melt curves, respectively. The LCGreen premelt and postmelt normalization temperatures were 71 and 80°C, respectively, for the CSF1PO melt curves and 73.5 and 84°C, respectively, for the HUMTHO1 melt curves. For all the melt curves, a temperature normalization was also carried out at 5% of the normalized fluorescence in an attempt to mitigate small position-dependent differences in temperature across the wells of the 96-well plate. The LightScanner software then creates a difference plot of fluorescence versus temperature by comparing a reference sample's melt curve, selected from among all of the melt curves by either the user or the software, to all other melt curves. It then attempts to group morphologically similar melt curves together using an unbiased hierarchical clustering scheme. Because the reproducibility of melt curves is dependent on instrumental, optical, and enzymatic (i.e., PCR-dependent) factors, the stringency with which two curves are assigned to the same or separate genotypes can be set by the user, that is, by adjusting the sensitivity setting. Specifically, both the pre- and postmelt normalization temperature ranges were optimized by choosing temperatures that allowed the maximum change in the sensitivity setting while maintaining the proper grouping of melting curves by genotype. This preserves the correct grouping of melting curves according to genotype while setting the greatest tolerance to individual variation in melting curve morphology because of differences in the quantity, purity, and/or integrity of DNA template used for PCR. For precisely this reason, the sensitivity setting was adjusted between experiments, thus allowing for more or less dispersion in the melt curves of identical genotypes while avoiding the false inclusion of melt curves arising from a different genotype.

Results

Melt Curve Analysis

When melting curve analysis is performed with SYBR Green I (an intercalating dye), the dsDNA is fluorescent until a temperature is reached near the T_m. At this point, the fluorescence decreases rapidly because of strand dissociation (hence dye elimination) and then plateaus at some residual fluorescent value. This is owing to the dye properties for SYBR Green I whose fluorescence decreases several hundredfold when it is not intercalated into dsDNA (27). Because the dissociation of strands can begin internally, both in lower melting domains and mismatched regions, STR amplicons that differ by integral numbers of four base-pair repeats can be discriminated from one another.

Software Analysis

Raw melt curve data show individual variation between reaction samples because of differences in starting template concentrations, in the optical detection efficiency of fluorescence between samples, slight positional temperature differences between wells, and solution components such as the solvent used and the salt/solute concentration (Fig. 2*A*). Following PCR, the genotype-calling software on the LightScanner-96 allows the user to normalize well-to-well differences in both the fluorescent intensity (Fig. 2*B*) and the temperature (Fig. 2*C*), allowing melting curves to be accurately compared from different PCRs. This aids in grouping similar genotypes together. The display of difference curves (Fig. 2*D*) helps to visually distinguish different genotypes from one another by setting one genotype as the reference profile or baseline for comparison. The extent to which other melting curves differ from this reference is then plotted as a positive or negative displacement or change in fluorescence (*y*-axis) as a function of temperature (*x*-axis).

Genotype Discrimination

Either eight or 10 DNA extracts from typed blood lines were primed with either CSF1PO or HUMTHO1 mini-STR primers, respectively, and amplified, in duplicate, in mastermix containing SYBR Green I dye. Following PCR, fluorescence melt data were collected and processed on a LightScanner-96 high-resolution melt instrument to generate and compare the difference curves arising from each genotype.

All of the eight duplicate CSF1PO (Fig. 2*A–D*) and 10 duplicate HUMTHO1 (Fig. 3) samples produced difference curves that could be recognized and correctly grouped by the software into eight possible unique genotypes. Despite the fact that the CSF1PO (10,11) and (12,13) melt curves in red and green, respectively (Fig. 2*D*), and the HUMTHO1 (7,7) and (9.3,9.3) melt curves in pink and blue, respectively (Fig. 3), were nearly identical to one another, it was possible to calibrate the sensitivity of software through the visual inspection of data to correctly group samples possessing the same genotype while simultaneously discriminating between different genotypes. Additionally, the THO1 (6,7) and (6,9.3) genotypes from two different donors were grouped together with their isotypic counterpart, demonstrating that melt curve morphology is associated with the donor's STR genotype rather than subtle differences in robotic sample preparation or other donor-specific differences.

In a blind study conducted at Idaho Technology Inc., 16 donor samples comprising nine CSF1PO and eight THO1 genotypes were analyzed by HRMC on a LightScanner-96 using the high-sensitivity LCGreen mastermix (Figs 4 and 5). Genotyping by normalized −d(fluorescence)/dT analysis and T_m comparisons were performed on duplicate PCRs and resulted in the recognition and discrimination of all genotypes, demonstrating the utility of the internal temperature calibrators for STR melt curve comparisons. The slight temperature differences that separate the inflection points in the difference curves of the homozygous genotypes are also displayed, and correlate with the T_m differences of approximately 0.08°C (Fig. 4) and 0.06°C (Fig. 5) for CSF1PO and HUMTHO1 alleles that differ by one repeat, respectively.

Sensitivity Studies

Serial dilutions of DNA from individuals possessing CSF1PO genotypes (9,11) and (10,10) and THO1 genotypes (7,9) and (8,9.3) were prepared and used for qPCRs in amounts ranging from 20 to 0.1 ng of DNA per reaction. Melt curves for both CSF1PO genotypes were distinct and reproducible down to 0.4 ng per PCR, while THO1 melt curves were reproducible down to 0.2 ng per PCR (Fig. 6*A,B*). With the exception of a single duplicate reaction in the HUMTHO1 (7,9) and CSF1PO (10,10) dilution series, all other amplification reactions throughout the concentration series produced melt curves that grouped with their genotypic counterparts.

Quantitative PCR

qPCR was performed on 24 donor DNA samples, in duplicate, using LCGreen Plus dye and CSF1PO primers, and the

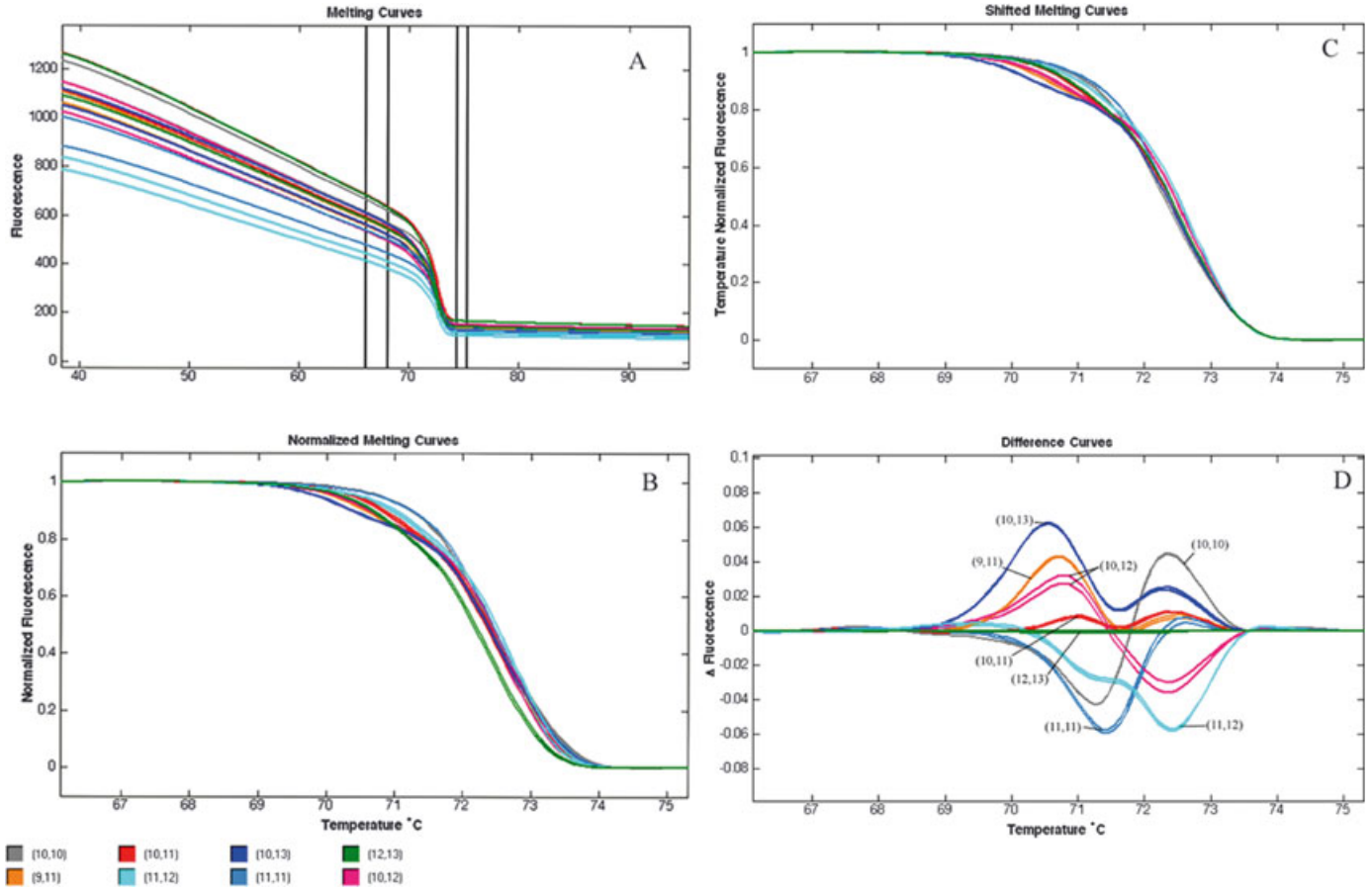

FIG. 2—*Melt curve profiles and difference curves of eight CSF1PO genotypes: (9,11), (10,10), (10,11), (10,12), (10,13), (11,11), (11,12), and (12,13). (A) Raw melt curve data. (B) Normalization of raw melt curve before and after major melt transition. (C) Temperature axis shift to correct for well-to-well temperature and fluorescence/optical data collection variations. (D) Difference curves to easily distinguish different genotypes. The CSF1PO (12,13) genotype was selected by the software as the reference to which other samples were compared (shown as the baseline across the temperature range). Melt curves were assigned to eight different groups by the software using a high-sensitivity setting (+3.2).*

concentrations obtained were compared to the same samples quantified using the TaqMan CFS-HUMTHO1 assay (Table 1). Following PCR, average DNA concentrations were calculated for all 24 DNA samples by comparison with a standard curve constructed from eight standards encompassing a range of 50 and 0.023 ng/μL. Although the plot of the standard curve showed good linearity ($R^2 = 0.993$), and the slope and *y*-intercept of −3.63 and 29.47, respectively, are within validated tolerances, the DNA concentrations measured by the dsDNA dye method were on average 38% less than the same samples quantified using the TaqMan CFS-HUMTHO1 assay with a definite negative bias, that is, the concentrations measured with the dye-binding assay underestimated the DNA concentration versus the TaqMan assay.

Mixture Studies

Two DNA mixtures composed of DNA from two donors with different CSF1PO and HUMTHO1 genotypes were examined by HRMC analysis over a range of mixture ratios. PCRs contained a total of 5 ng of DNA per reaction and were comprised of either CSF1PO genotypes (10,13) and (11,11) or THO1 genotypes (6,7) and (8,9.3) divided according to the following mixture ratios: 1:0, 15:1, 10:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5, 1:10, 1:15, and 0:1. Difference curves generated from any of the DNA mixtures at either the CSF1PO or the HUMTHO1 were correctly differentiated from the single-source samples used to prepare the mixtures as required of a screening technique used to identify stains with dissimilar genotypes. Unique melt curves were also produced for many of the CSF1PO (10,13) and (11,11) mixtures with the exception of the CSF1PO melt curves for the 2.5:1, 5:1, 10:1, and 15:1 mixture ratios which formed a tight cluster (Fig. 7). Complex melt curves were also generated for various DNA mixtures of a (6,7) and a (8,9.3) genotype at the THO1 locus and were unique for all but the 15:1, 10:1, and 5:1 mixture ratios which appeared to overlap (Fig. 8).

Substrate Studies

To assess the effect of conditions and inhibitors that might challenge the reproducibility of CSF1PO and THO1 STR melt curves, DNA was extracted from four different substrates that emulate the nature and quality of those recovered in field investigations—whole blood collected from a garage area covered in used motor oil, degraded DNA, a reference buccal swab, and blood containing hematin. DNA extraction from blood contaminated with old automobile oil was performed on a Qiagen EZ1 BioRobot using Qiagen's EZ1 Tissue kit, while DNA from the remaining substrates was obtained by organic extraction (phenol, chloroform, and isoamyl alcohol 50:40:1 mixture) followed by washing three times with TE on Centricon YM-100 spin columns. The biological material

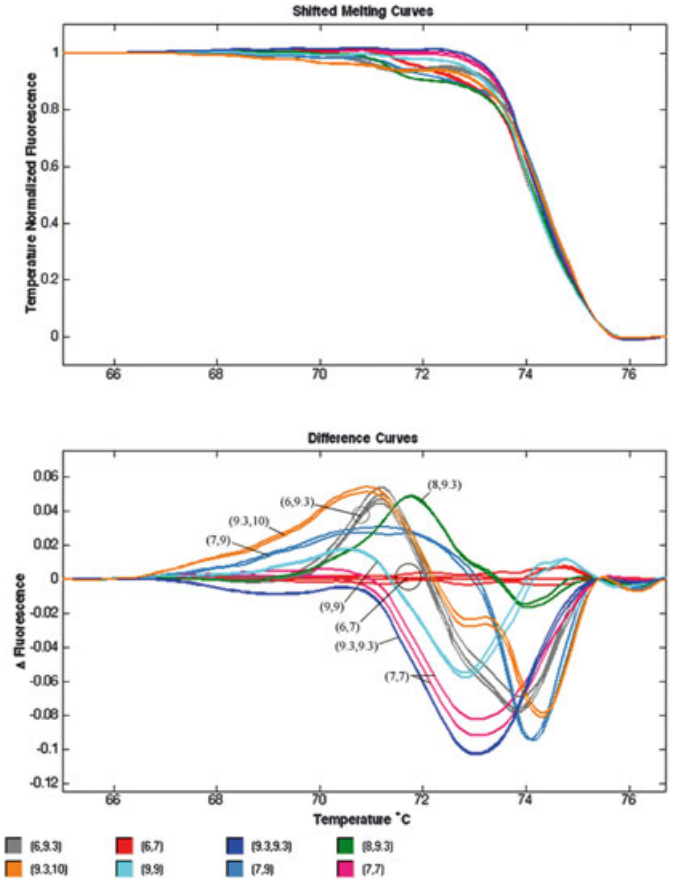

FIG. 3—*Melt curve profiles and difference curves of eight HUMTHO1 genotypes: (6,7), (6,9.3), (7,7), (7,9), (8,9.3), (9,9), (9.3,9.3), and (9.3,10). Two different donors for genotype (6,7) and (6,9.3) were used to demonstrate reproducibility in melt curve morphology among donors sharing the same THO1 genotype. Melt curves were assigned to eight different groups by the software using a high-sensitivity setting (+2.1).*

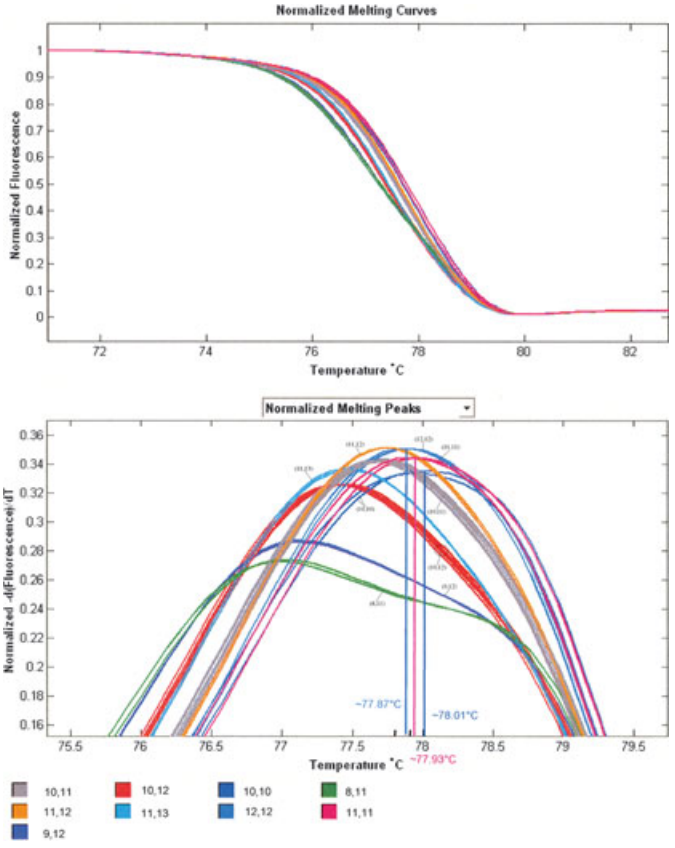

FIG. 4—*Melt curve profiles and difference curves produced from 16 donors comprising nine CSF1PO genotypes: (8,11), (9,12), (10,10), (10,11), (10,12), (11,11), (11,12), (11,13), and (12,12). The CSF1PO amplicons were amplified on an LS32 LightScanner system using the high-sensitivity LCGreen master mix followed by high-resolution melting and difference curve analysis on a LightScanner-96 HRM instrument with genotype calling software (v. 2.0.0.1331). All nine genotypes curves are resolvable from one another. The melting curves derived from all six individuals who share a (10,11) and the three persons who possess a (10,12) genotype grouped together according to their genotype. Genotypes were grouped blindly, that is, without reference to the number of different CSF1PO genotypes represented or the genotypic identity of each sample. The genotype-grouping algorithm was set to a high sensitivity (+4.5). The vertical lines labeled with the temperatures ~77.87, ~77.93, and ~78.01°C refer to the maximum value of the first derivative (–d(fluorescence)/dT or normalized fluorescence intensity change as a function of the temperature change) for homozygous genotypes (10,10), (11,11), and (12,12), respectively.*

deposited on all four substrates originated from one donor with the following genotypes: CSF1PO (10,12) and HUMTHO1 (9,9.3). Following PCR using approximately 2 ng of purified DNA, three of the four samples produced identical melt curves at the CSF1PO and HUMTHO1 loci. The one exception was blood containing hematin; here the melt curve was similar in shape but shifted to a higher temperature when compared to the other three melt curves (Fig. 9*B*).

Purification Studies

CSF1PO and THO1 melt curves, produced from DNA extracted by either organic extraction (PCI) or robotic extraction (EZ1), were compared. Four donors were selected with the following CSF1PO:THO1 genotypes: Person#1: (11,12):(8,9), Person#2 (12,12):(9,9.3), Person#3: (10,12):(9,9.3), and Person#4: (11,11):(6,7). CSF1PO (11,12) and (10,12) melt curves from both extraction methods were grouped correctly. Regardless of the extraction method used, nearly identical CSF1PO melt curves were obtained for the CSF1PO (12,12) and (11,11) genotype donors, although discrimination was possible in all but one of the duplicate samples (Fig. 10) purified robotically. The tripartite grouping of HUMTHO1 melt curves was correctly performed with the exception of the (6,7) genotype (Fig. 11). Here, the curve morphology differed significantly between the EZ1 and organically extracted samples causing their melt curves to be exclusive from one another.

Discussion

Techniques for Screening Evidence

Techniques to screen forensic evidence for donor attribution using STRs or SNPs have been previously described, and two commercial products are available. Promega Corporation (Madison, WI) produces the PowerPlex S5, a five-locus mini-STR system that includes D8S1179, D18S51, FGA, THO1, and Amelogenin, for low-copy and degraded samples with a power of discrimination of 1.9×10^{-5} (Caucasian population). Reverse dot blot kits are also available through Roche Applied Science (Indianapolis, IN) for an HVI/HVII mtDNA typing assay (Linear Array™ mtDNA HVI/HVII Region-Sequence Typing Kit) (28). Some labs utilize the CTT STR triplex for screening (personal communication) while the use of the single highly polymorphic locus, HUMACTBP2 (SE33), has also been reported (29).

Methods utilizing HRMC analysis to detect SNPs have been published and include forensic applications. Panels of autosomal- and Y-SNPs, and DIPs (deletion/insertion polymorphisms) have been

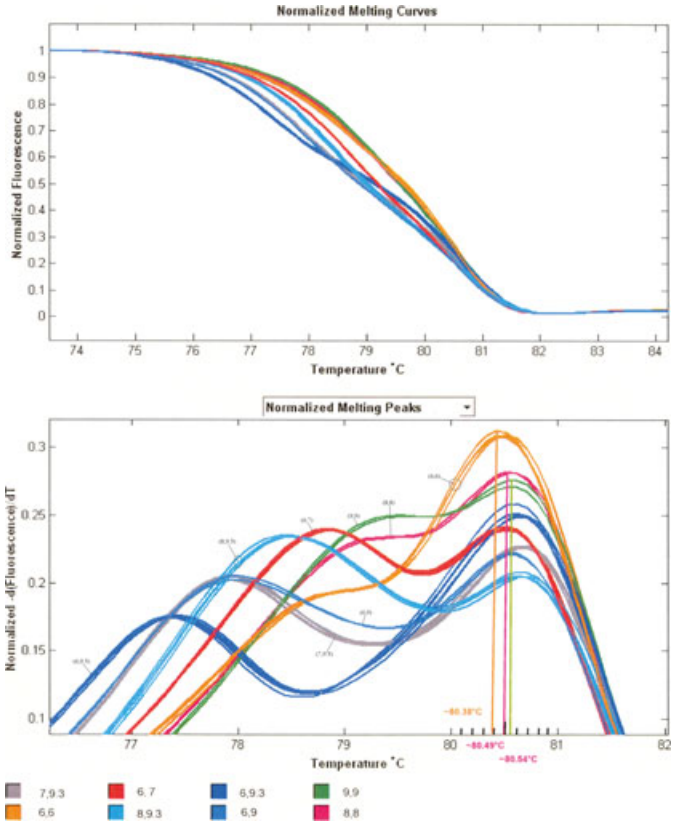

FIG. 5—*Melt curve profiles and difference curves produced from 16 donors comprising eight HUMTHO1 genotypes: (6,6), (6,7), (6,9), (6,9.3), (7,9.3), (8,8), (8,9.3), and (9,9). The HUMTHO1 amplicons were amplified on an LS32 LightScanner system using the high-sensitivity LCGreen mastermix followed by high-resolution melting and difference curve analysis with genotype calling software (v. 2.0.0.1331). All eight genotypes curves are easily resolved from one another. Additionally, genotypes (6,6) and (8,9.3) shared by two donors, and (6,7), (6,9.3), and (7,9.3) shared by three donors, were used to demonstrate melting curve reproducibility among different donors who possess the same genotype. Genotypes were grouped blindly, that is, without reference to the number of different HUMTHO1 genotypes represented or the genotypic identity of each sample. The genotype-grouping algorithm was set to a high sensitivity (+4.0). The vertical lines labeled with the temperatures ~80.38, ~80.49, and ~80.54°C refer to the maximum value of the first derivative (–d(fluorescence)/dT or normalized fluorescence intensity change as a function of the temperature change) for homozygous genotypes (6,6), (8,8), and (9,9), respectively.*

analyzed by low-resolution melting curve analysis (18), and SNPs in the HLA-DQ region have also been described (19,20). Recently, an evidence screening technique has been reported that uses a multiplexed, FRET-based, fluorescent probe hybridization assay to genotype two panels of six and one panel of seven highly polymorphic SNPs, and two of these possess gender discrimination (21). The SNP assay has superior sensitivity (down to 18 pg), the ability to perform DNA quantitation with adequate precision and accuracy, and sufficient power of discrimination to exclude one in 2500 unrelated individuals (using eight SNPs) in just a one- or two-tube assay. Despite these advantages, the assay may not be able to adequately address mixtures (other than in a 1:1 ratio), or severely degraded samples given the relatively large amplicons selected (141–245 bp).

The general problem with most SNP-based assays is their power of discrimination—it is limited to two to the power of the number of SNPs examined. This results in a relatively low power of discrimination across all ethnicities—0.83 to 0.94 for HLA-DQα (1), and only 95–99% of different individuals according to the Nicklas

TABLE 1—*qPCR conducted on 24 donor DNA samples using LC Green Plus mastermix and the CSF1PO primers yielded the following DNA concentrations (ng/μL). The table displays the average DNA concentration measured in this assay versus that measured by the CFS-HUMTHO1 TaqMan DNA assay. On average, the LC Green Plus assay underestimated the DNA concentrations by approximately 42% versus the CFS-HUMTHO1 TaqMan human DNA assay.*

Samples #	#9	#12	#14	#19	#20	#22	#23	#25	#26	#30	#31	#38	#42	#61	#62	#67	#69	#70	#71	#72	#76	#79	#81	#82
CFS-HUMTHO1 assay	0.23	0.2	0.082	0.035	0.27	0.3	0.2	0.27	0.44	0.092	0.24	0.19	0.18	0.66	0.167	0.124	0.22	1.07	0.35	0.2	0.066	0.08	0.19	1.17
LC Green Plus assay	0.12	0.108	0.015	0.04	0.09	0.15	0.11	0.18	0.24	0.059	0.09	0.076	0.12	0.32	0.077	0.062	0.18	0.51	0.17	0.09	0.09	0.064	0.08	0.7
% difference	–48	–46	–82	14	–67	–50	–45	–33	–45	–36	–63	–60	–33	–52	–54	–50	–18	–52	–51	–55	36	–20	–58	–40

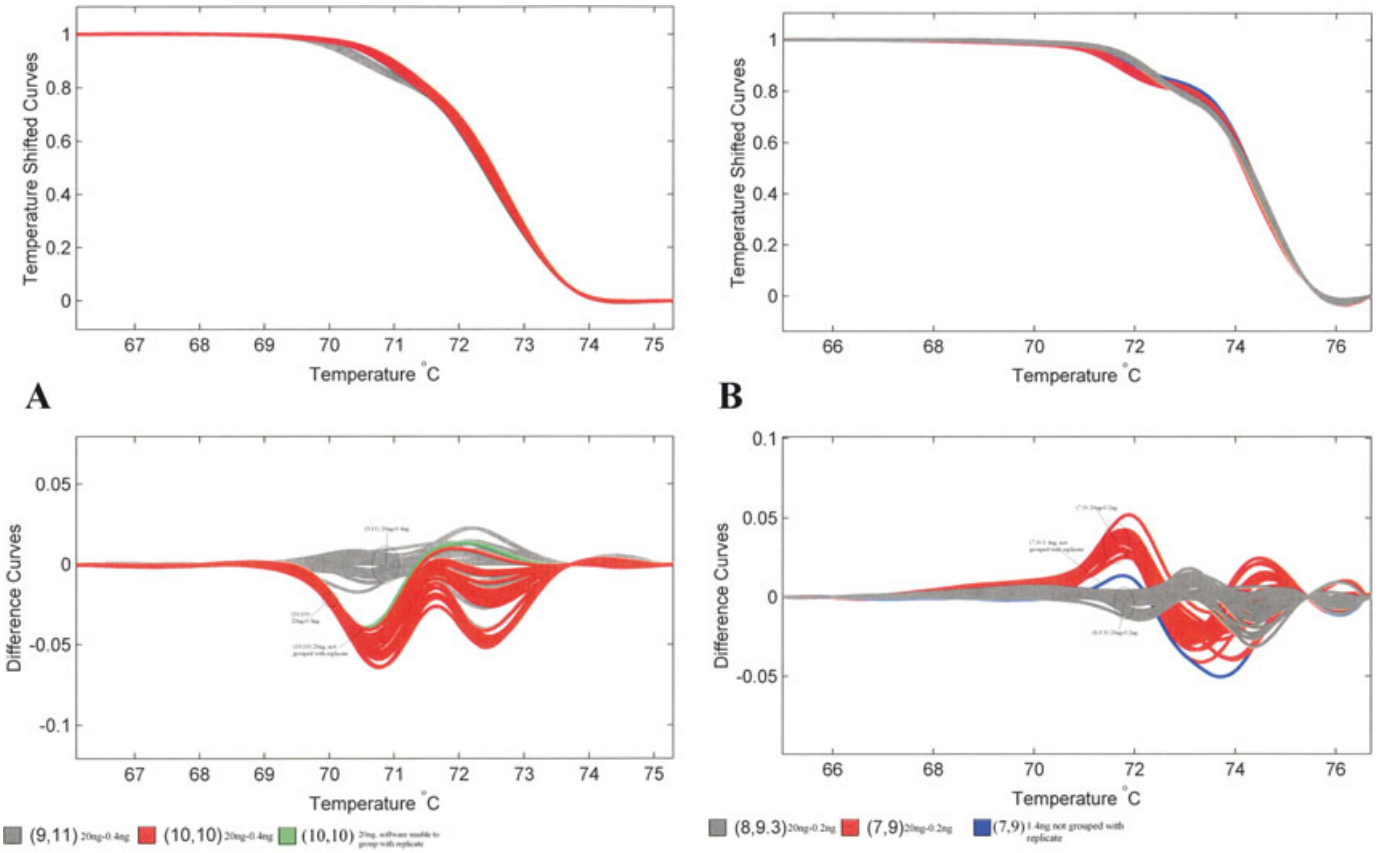

FIG. 6—*Sensitivity study using different quantities of DNA from donors with CSF1PO (9,11) and (10,10), and HUMTHO1 (7,9) and (8,9.3), genotypes as input for PCRs followed by melt curve analysis. The DNA concentrations tested in duplicate were as follows: 20, 10, 5, 2.5, 2, 1.8, 1.6, 1.4, 1.2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 ng (not shown). The genotype-grouping algorithm was set to a high sensitivity of −1.6 and +1.0 for CSF1PO and HUMTHO1 melt curve analysis, respectively. (A) With the exception of one replicate sample (20 ng DNA input [10,10]), melt curves were grouped according to CSF1PO genotype down to 0.4 ng DNA input. (B) With the exception of one replicate sample (1.4 ng DNA input [7,9]), melt curves were grouped according to HUMTHO1 genotype down to 0.2 ng DNA input.*

and Buel assay (21). SNP-based methods also lack the capacity to address complex mixtures.

HRMC analysis has also been described for the detection of both short and long repeat polymorphisms, and to identify sequence variants. Using HRMC analysis, variants have been detected in strains of *Bacillus anthracis* (30), internal tandem repeats have been identified in cancer cells (31), and it has been used in BAC clone screening for repeats (32). Most recently, it has been used to obtain genotypes for samples for three CODIS-13 STR loci (22).

Using a two-probe system consisting of an anchor, a blocking probe, and a HyBeacon fluorescent probe, French et al. (22) were able to genotype the D18S51, THO1, and D8S1179 loci with allelic T_m values that were separated by 2–3 standard deviations, and a power of discrimination of ~1 in 10,266. Unfortunately, the need to perform asymmetric PCR to prepare single-stranded DNA to hybridize to the probe precludes its use to simultaneously assess donor attribution and quantify DNA, meaning that it is mainly a screening assay. Another drawback is that it requires 11 separate PCRs making it impractical to use for the analysis of forensic unknowns of limited quantity for each analysis.

Unlike a method to genotype STRs, a comparison-based method to screen evidence for donor attribution must correctly assess when the genotype of an unknown sample or stain differs from that of the reference(s) to which it is being compared. Because this technique is not required to genotype the unknown, but rather to highlight genotypic distinctions between the melt curves of a reference and one or more unknowns, the application must possess greater stringency in not calling two different genotypes as equivalent (a false inclusion) versus the opposite error of misinterpreting identical genotypes as different (a false exclusion).

The melting of PCR amplicons containing tetranucleotide repeat polymorphisms entails different assay design considerations than those used for SNPs. Whereas the influence of a single mismatched base on an amplicon's melting behavior can be modeled by enthalpy and entropy estimates, and nearest neighbor effects, the melting of internal, low-melting domains has been studied to a lesser extent (33). An AT-rich domain interior to a DNA fragment that is clamped by GC-rich barrier domains will melt more sharply, and as a closed loop with a higher T_m than if the same domain was positioned at the ends of the fragment. This is because the T_m of internal repetitive sequences is a linear function of the domains' sequence composition (slope ≈1.2°C for every 0.1 part change in the mole fraction of AA), and is inversely related to the length of the domain. After passing a threshold of 20 nts, the domain's melting becomes cooperative meaning that unzipping will occur rapidly (34,35). The T_m also increases with salt concentration where sodium ions serve to neutralize the polyanionic backbone and stabilize the helical state. Therefore, increasing the salt concentration will compress the melt transition temperature range, increase the slope of the melt curve, and make melting more specific to base-stacking interactions.

The above criteria were taken into account for selecting STR loci that would yield informative melt curves. Primers that amplify

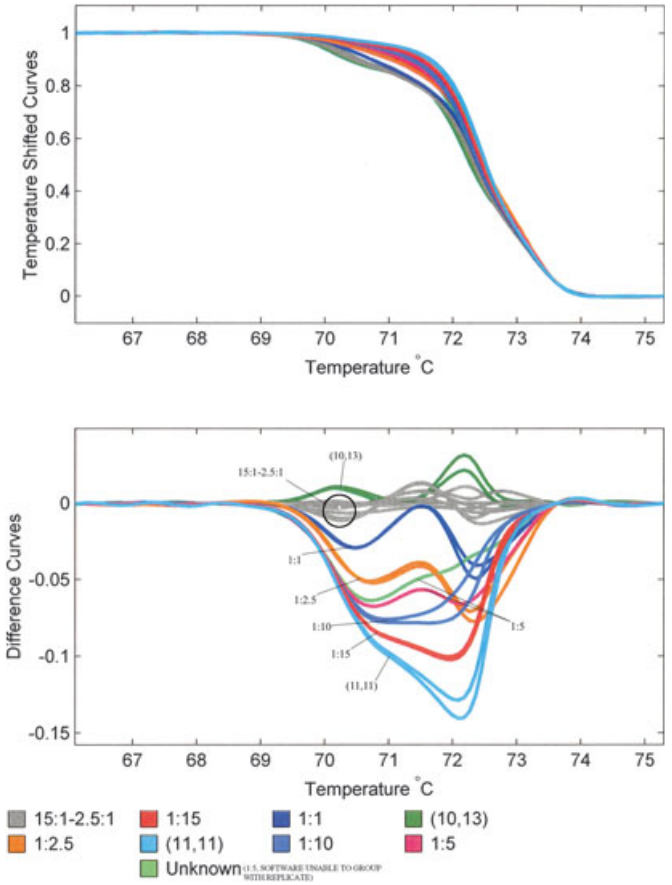

FIG. 7—*CSF1PO mixture melt curves analysis. DNA from two contributors with CSF1PO genotypes (10,13) and (11,11) was used to prepare duplicate PCRs containing various mixtures of DNA as shown for each colored curve. The total amount of DNA used in each PCR was 5 ng. The melt curves, from top to bottom, represent the following (10,13):(11,11) mixture ratio: 0:1, 1:15, 1:10, 1:5, 1:2.5, 1:1, 2.5:1, 5:1, 10:1, 15:1, and 1:0. Melt curves for ratios 2.5:1, 5:1, 10:1, and 15:1 appear together as a cluster. The genotype-grouping algorithm was set to a high sensitivity (+2.0).*

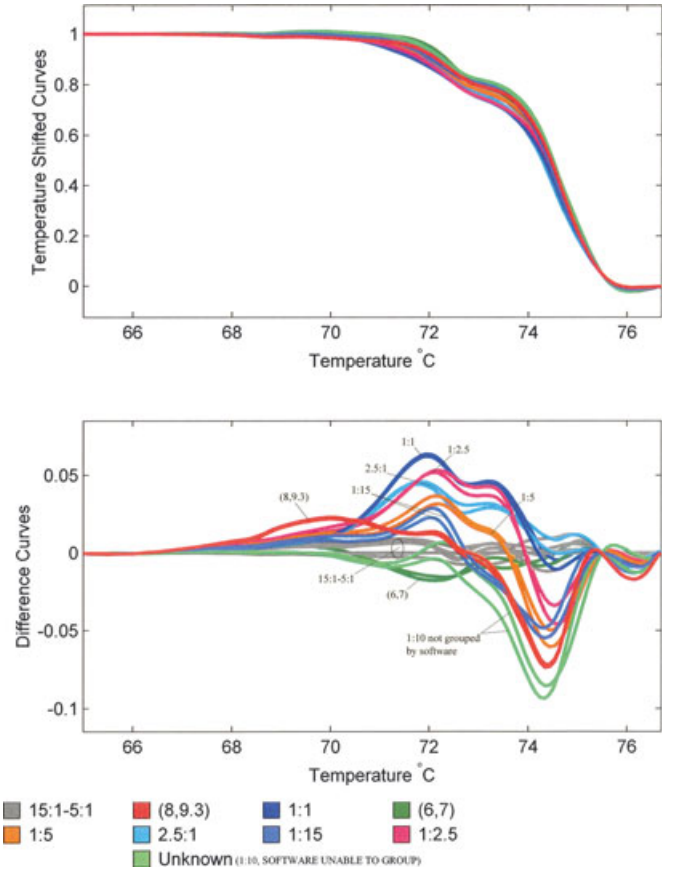

FIG. 8—*HUMTHO1 mixture melt curve analysis. DNA from two contributors with HUMTHO1 genotypes (6,7) and (8,9.3) was used to prepare duplicate PCRs containing various mixtures of DNA as shown for each colored curve. The total amount of DNA used in each PCR was 5 ng. The relative ratio of (6,7):(8,9.3) reaction template was 1:0, 15:1, 10:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5, 1:10, 1:15, and 0:1. Unlike Fig. 7, there is no discernable trend in melt curve transition between the genotypes. The difference curves generated display unique curves at almost every mixture ratio. The exceptions are the 15:1, 10:1, and 5:1 ratios. The genotype-grouping algorithm was set to a high sensitivity (+2.2).*

mini-STRs were selected for both THO1 and CSF1PO loci creating short amplicons of 55–98 and 89–129 bps, respectively, that span the possible allelic range of 3–14 repeats and 6–16 repeats, respectively, for each locus. Furthermore, both loci possess polymorphic regions with AT-rich repeats (mole fraction 25%) bracketed at each end by GC-rich sequences (42% and 55% for THO1 and CSF1PO, respectively). This results in T_m differences for each additional tetranucleotide repeat of approximately 0.08°C (Fig. 4) and 0.06°C (Fig. 5) for CSF1PO and HUMTHO1 melt curves, respectively, derived from homozygous genotypes.

SYBR Green I and LCGreen—Evidence Screening Using STRs

Genotype-specific melt curves were generated from mini-STR amplicons amplified from the CSF1PO and THO1 loci in the presence of SYBR Green I. Biological samples obtained from different sources (blood, semen, and saliva), collected on different substrates, and purified by different extraction methods were compared. HRMC analysis correctly identified all eight of the CSF1PO and HUMTHO1 genotypes tested. Although resolvable, two homozygous THO1 genotypes (THO1: [7,7] and [9.3,9.3]), and one pair of CSF1PO heterozygotes (CSF1PO: [10,11] and [12,13]) whose alleles differed by a single repeat, produced very similar melt curves.

STRs Yield Better Discrimination Using Internal Temperature Calibrators

Good precision and accuracy in assessing the T_m of the amplicons is necessary, and this can be aided by the inclusion of internal calibrating oligonucleotides at the low (62°C) and high (92°C) melting range (8,9,36). Using these calibrators, imprecision in the melting of amplicons in different PCRs caused by temperature and solute differences (i.e., because of positional temperature effects and the extraction method, respectively) can be mitigated, improving the accuracy of T_m determination and the assignment of genotype by decreasing the standard deviations in T_m measurements by 47–82% (8).

When genotyping was performed with the LCGreen Plus high-sensitivity mastermix, the T_m differences for the homozygotes, and melting curve fine structure for the heterozygotes, were more easily discernable and grouped correctly by the software. The reproducibility and thermal separation in melt curves improved dramatically for both the HUMTHO1 and the CSF1PO amplicons. The ability to discriminate eight HUMTHO1 genotypes and nine CSF1PO genotypes in a blind study substantially supports the notion that high-resolution melting analysis can reliably perform as a genetic screening tool for donor attribution.

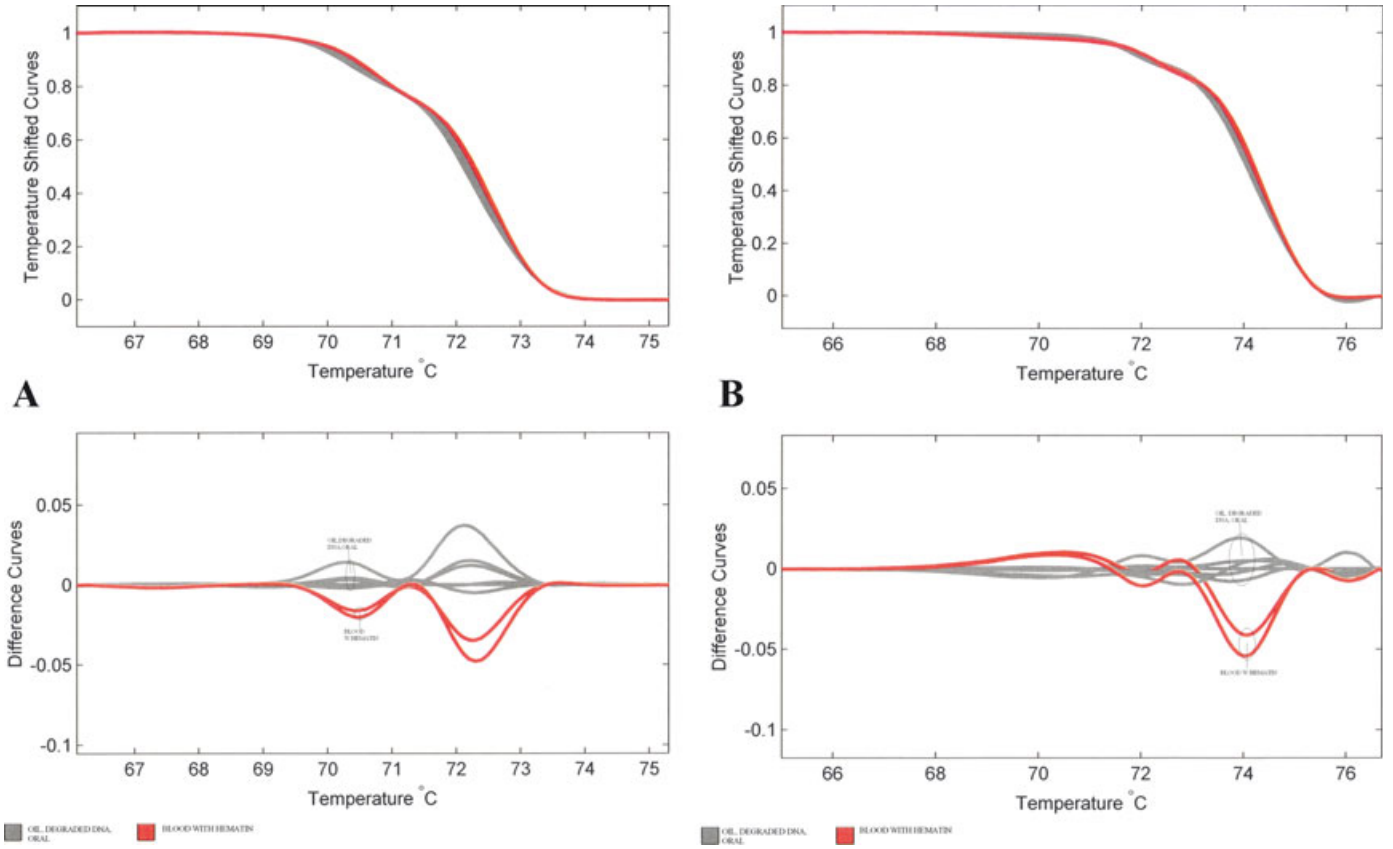

FIG. 9—*The effect of forensically challenging sample issues on (A) CSF1PO and (B) HUMTHO1 melt curves. The effects of degradation and two different PCR inhibitors on an individual's CSF1PO (10,12) and HUMTHO1 (9,9.3) melt curves were investigated by comparing melt curves generated from DNA recovered from whole blood contaminated with used automobile oil, degraded DNA, and blood containing hematin to duplicate melt curves produced from DNA obtained from an oral reference swab belonging to the same donor. Extraction of DNA from used automobile oil was performed with an EZ1, while organic extraction was performed on the remaining substrates. With the exception of the blood sample containing hematin, the CSF1PO and HUMTHO1 samples were indistinguishable from one another using a high-sensitivity setting of +0.7 and +2.2 for CSF1PO and HUMTHO1 melt curve analysis, respectively.*

Sensitivity of the SYBR Green I Assay for Evidence Screening Using the CSF1PO and HUMTHO1 Amplicons

In the sensitivity study, melt curves were shown to be unique and reproducible over a wide range of input DNA quantities from 20 ng down to 0.4 ng and 0.2 ng for CSF1PO and THO1 loci, respectively. In the 13 and 14 sets of duplicate samples evaluated in the CSF1PO and HUMTHO1 concentration series, only two sample replicates deviated from grouping with their genotyping counterparts—the 20 ng replicate from the CSF1PO series and the 1.4 ng replicate from the HUMTHO1 series. Although T_m is affected by amplicon concentration, the effect is small (only a 0.05°C/0.1 μM change in amplicon concentration over a 0.2–1.0 μM range) and is only detectable in instruments with superior temperature resolution (27). If greater sensitivity is desired, the number of PCR cycles could be increased from 40 to 45 thus amplifying well into the plateau phase of PCR, where differences in template concentration should be equalized (13).

DNA Quantitation Using the LCGreen Assay

DNA quantitation using LCGreen dye and either the HUMTHO1 and CSF1PO locus is desirable so that DNA concentrations may be obtained for subsequent DNA profiling while simultaneously providing information on the number of unique genotypes present by HRMC analysis. While the DNA concentrations measured by dsDNA dye binding to CSF1PO amplicons were approximately 40% of those obtained with our TaqMan assay, the TaqMan assay generates a 62-bp amplicon versus an amplicon size of between 89 and 129 bp for the CSF1PO alleles. Because the elongation of larger amplicons is less efficient in qPCR, it is quite possible that this effect contributes to underestimating the amount of DNA present when compared to the TaqMan assay. In fact, DNA concentrations measured using our CFS-HUMTHO1 qPCR assay are approximately 50% greater than those reported using the DuoQuant kit (ABI) whose amplicons are on the order of 130 bp. Whereas ABI includes ROX in all of its qPCR mastermixes as a passive internal reference to normalize and correct for fluorescence differences caused by nonhomogeneities in PCR volumes and optical detection efficiencies across the thermal block, the LCGreen Plus reactions do not, so some of the variability in C_t values between duplicate reactions may be due to the absence of ROX in the LCGreen Plus dye reactions.

HRMC analysis of mixtures of two donors at various mixture ratios was performed on amplicons produced from both the CSF1PO and the THO1 loci. Six unique groups were identified at the CSF1PO (Fig. 8) and HUMTHO1 (Fig. 9) loci, from among the nine mixture ratios tested. One exception was the CSF1PO sample replicate for the 1:5 (10,13):(11,11) mixture that did not group with its isotype. In the CSF1PO mixture series, the melt curves followed an ordered progression (from top to bottom) as the mixture ratio transitioned from one donor to the other (Fig. 8). This was not the case for the HUMTHO1 mixture series, suggesting that differentiation between different mixture ratios may depend on the donor genotypes. In both mixtures, however, the donor's sample

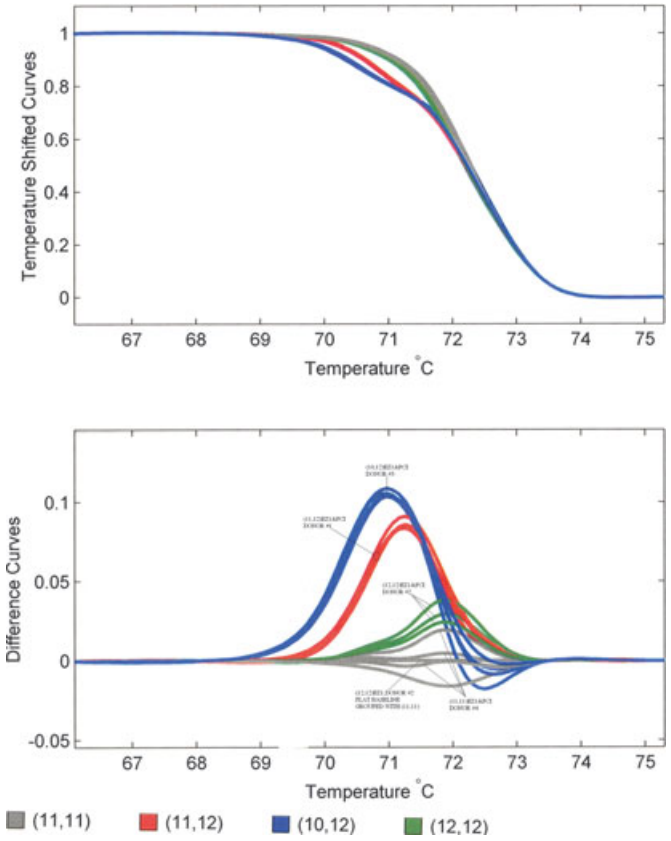

FIG. 10—*The effect of purification method (PCI vs. EZ1) on CSF1PO melt curves. Four donors with the following genotypes were used: #1 (11,12), #2 (12,12), #3 (10,12), and #4 (11,11). DNA extraction from blood was performed for donors #1–3, while an oral swab was the source of DNA extracted from donor #4. With the exception of one sample replicate for donor #2, all genotypes grouped together with their isotypic counterparts but separately from melt curves generated from other donor genotypes. The genotype-grouping algorithm was set to a high sensitivity of +1.6.*

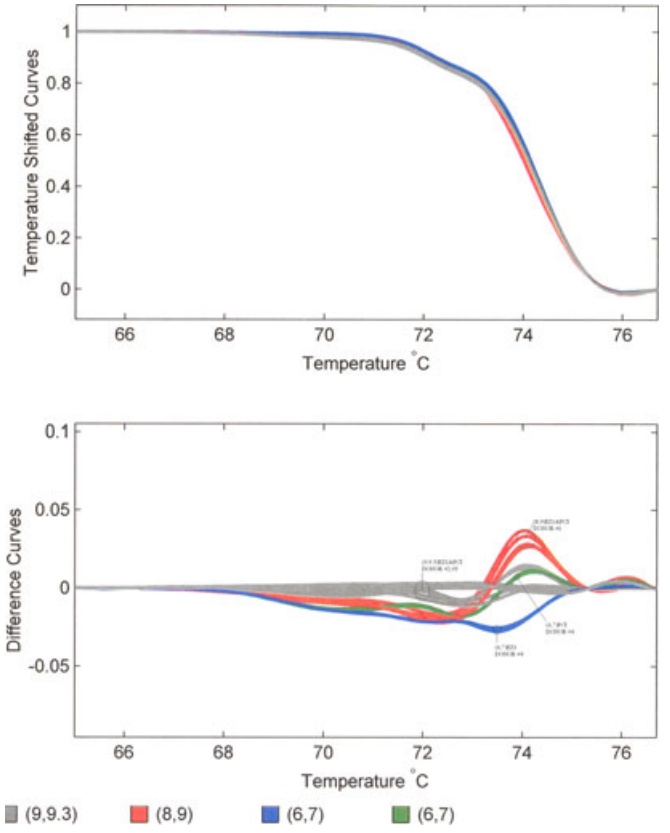

FIG. 11—*The influence of purification method upon HUMTHO1 melt curve reproducibility. Four donors with the following genotypes were used: #1 (8,9), #2 (9,9.3), #3 (9,9.3), and #4 (6,7). DNA extraction from blood was performed for donors #1–3, while an oral swab was the source of DNA extracted from donor #4. Donor samples were grouped according to genotype with the exception of donor #4 whose duplicate melt curves grouped differently for the EZ1 and PCI purified templates. The genotype-grouping algorithm was set to a high sensitivity of +2.2.*

could be clearly differentiated from the mixed samples, demonstrating the utility of HRMC analysis in revealing samples whose genotype(s) differs from the reference, whether in part or in total.

How Sample-Specific Challenges and Purification Method Impact HRMC Analysis

Sample-specific challenges often associated with forensic field samples were also examined by HRMC analysis, as was the influence of the DNA extraction method. CSF1PO and HUMTHO1 STR melting curves produced from DNA extracted either robotically or organically from the blood of three donors, and the oral swab of a fourth donor, were grouped correctly by the software for all four donors for the CSF1PO samples (Fig. 10) and three of the four donors for the HUMTHO1 samples (Fig. 11). The exceptions were the sample replicate for donor #2 (CSF1PO [12,12]) purified by robot and the oral swab sample from donor #4 (HUMTHO1 [6,7]). Failure to group sample replicates has been seen in other experiments and can be resolved by repeating the analysis. However, the reproducible difference in melt curve morphology between a donor sample purified by two different methods reinforces the notion that solute differences can interfere with reproducibility in melting analysis when different extraction methods are used.

To investigate the extent to which HRMC analysis is impacted by various forensically challenging sample issues, melt curves specific for the CSF1PO and HUMTHO1 loci were generated from DNA obtained from one donor that was compromised in either its purity or integrity. In this study, DNA purified by robot from a blood stain mixed with used motor oil, or by organic extraction from degraded DNA and DNA containing hematin (a PCR inhibitor), was compared to DNA organically extracted from an oral swab, by HRMC analysis. Melt curves were identical for all but the hematin-containing sample at both the CSF1PO and HUMTHO1 loci. As anticipated, the melting of mini-STR amplicons for CSF1PO and HUMTHO1 performed well even for moderately degraded DNA. However, to avoid the detrimental effects of PCR inhibitors and sample solute differences on melt curve analysis, it is recommended that all samples submitted for HRMC analysis be purified robotically.

Power of Discrimination

We have shown that HRMC can be used to differentiate between a total of 12 different CSF1PO and HUMTHO1 genotypes, and using DNA isolated by two different methods from a variety of biological sources over a wide range of DNA concentrations, and from a number of samples which possess forensically challenging issues. The observation that uniform pre- and postmelt fluorescence normalization parameters and a single temperature normalization value can be determined for the HRMC analysis of CSF1PO and

HUMTHO1 mini-STR amplicons underscores the definable nature of the melting behavior of STR polymorphisms in short amplicons. These parameters also resulted in a low number of false exclusions, and in only one false inclusion (a single replicate in the purification method study), which supports the reliability and objectivity of this tool for screening evidence for unique stains. Notwithstanding, some adjustments were necessarily made to the sensitivity (or tolerance) used to determine whether the melt curves from two unknowns were grouped together or separately, and these decisions were made with foreknowledge of the sample genotypes. These adjustments uncovered limitations in the assay.

Over the course of this enquiry, it became obvious that the major limitation in melting curve reproducibility is sample-to-sample consistency in the PCR and the quality of the DNA template. It was also realized that laboratories that utilize real-time chemistries that are not amenable to HRMC analysis will have to consume additional samples to obtain melt curve genotypic data. It is therefore recommended that all samples submitted for this assay be extracted by the same method, that such method should generate DNA of high purity, that real-time PCR be performed using a mastermix that contains a saturating dsDNA-binding dye with internal temperature calibrators, and that post quantitation the real-time plate be submitted for HRMC analysis to obtain additional genotypic discrimination data without the need to consume additional samples. Replicate samples encompassing a wide range of common CSF1PO and HUMTHO1 genotypes and as well as a wide range of concentrations performed using these recommendations should then inform the validating laboratory of what software settings may be applied uniformly for obtaining discriminating results.

Future efforts will address shortcomings of this assay. For example, using only the THO1 and CSF1PO loci, the power of discrimination is only about 0.009 (i.e., one in 100); therefore, additional loci must be added to increase the technique's value for evidence screening. Additionally, loci for gender which include a Y- and X-STR locus will render it more useful for screening evidence in which one or more suspects or victims may be related. Finally, the need to melt the amplicons from each locus in a separate tube is cumbersome; therefore, a strategy to multiplex each locus by color and/or temperature will be devised so that the melting of 4–5 loci may be performed in a single tube.

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