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TECHNICAL NOTE

CRIMINALISTICS

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Development of a Fast, Simple Profiling Method for Sample Screening Using High Resolution Melting (HRM) of STRs*

ABSTRACT: A screening assay has been developed to provide preliminary individualization of crime scene samples thus eliminating expensive, time-consuming short tandem repeat (STR) profiling of nonprobative samples. High resolution melting performed in a real-time PCR instrument is used to detect the slight melting differences between the length and sequence variations of 22 forensic STRs. Three STRs (vWA, D18S51, THO1) were chosen to develop an assay which was optimized for Mg++ concentration, annealing/extension time/temperature, assay volume, and bovine serum albumin addition. The assay was tested for reproducibility, uniformity for genotype, melting profile consistency, effects of inhibitors, and mixture effects. The assay could be used to determine DNA concentration when a standard curve is run simultaneously. Calculations of costs show that the assay can save significant time and money for a crime with many samples or suspects.

KEYWORDS: forensic science, high resolution melting, STR, screening, real-time PCR, DNA quantitation

The forensic laboratory must continually meet the challenge of increased casework and the criminal justice community's demand for timely analysis of evidence. Short tandem repeat (STR) profiling using conventional typing techniques, that is, gel-based or capillary electrophoresis methods, is routinely performed for sample individualization. However, it is impractical to use such costly and labor-intensive methods to analyze every sample found at a crime scene. A simple analytical method, which could allow the analyst to select only those probative samples for further analysis, would save time and resources. This assay need not yield complete profile information about the donor of a particular stain, but reveal sufficient data to allow the examiner to differentiate between those individuals present at the crime scene. Only stains which yielded information that indicated a need for further examination, would be tested using conventional methods thereby eliminating many stains which would provide no useful purpose if subjected to lengthy testing. A method to quickly type only a few STR loci, while certainly not by any means a definitive test of identity, will usually allow determination of whether a blood sample came from a particular individual. That information is sufficient for a quick screen of crime scene stains to determine the most probative samples.

Analysis of melt curves is often used in real-time PCR to determine if amplification generated a specific product. This approach assesses the PCR products by monitoring the fluorescence of an intercalating dye (or dye probe/primer combination) associated with double-stranded DNA. After PCR amplification but before melting commences, fluorescence is high because the dye is intercalating into the many copies of the double-stranded amplicon. As the temperature rises, fluorescence decreases slightly until the temperature at which the two strands begin to separate is reached. Once complete denaturation is accomplished, the fluorescence decreases to background as intercalation of the probe to dsDNA is required to observe fluorescence. The data is plotted as the change in fluorescence with respect to the change in temperature (−df/dt) which results in a maximum at the melting temperature where the change in fluorescence is greatest. It is this melting profile that may be used to assess the specificity of the generated PCR product or detect differences between products.

High resolution melting (HRM) (1,2) goes beyond the power of classical melting curve analysis by allowing study of the thermal denaturation of a double-stranded DNA in much more detail and with more information. PCR products can be differentiated based on length, sequence or complementarity; single base changes (single-nucleotide polymorphisms) can be genotyped (3–5) or single base mutations can be detected (6–8). The HRM method requires the two amplification primers, a PCR mastermix containing a saturating, intercalating dye that binds specifically to dsDNA, but negligibly to ssDNA (such as Eva Green™; Biotium, Hayward, CA) and a proper instrument (such as the Qiagen Rotorgene Q; Qiagen, Valencia, CA) utilizing specific melting parameters and dedicated software to interpret the results. Eva Green™ is often used for these studies because it has excitation and emission spectra very close to those of fluorescein (FAM), so it is compatible with real-time instruments. It is stable, nonmutagenic, noncytotoxic, and nonfluorescent when not bound to dsDNA and it generates much less PCR inhibition than SYBR® Green at saturating concentrations (9). SYBR® Green also performs "dye jumping," where dye from a melted duplex may get reincorporated into regions of dsDNA

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which had not yet melted causing inaccuracies in the resulting melt curves (1,10).

This article describes the development of a three STR HRM assay which can be used as a screening assay for forensic evidence. Twenty-two STRs were surveyed for their ability to distinguish individuals based on HRM melting patterns. Three STRs that allowed the largest number of patterns to be discerned were chosen for an assay which was optimized and validated. The assay was tested with inhibitors, degraded DNA, on two different real-time instruments, and on samples containing mixtures of DNA.

Materials and Methods

Table 1 lists the primers used for amplification of the 22 STRs tested. Primers were purchased from Integrated DNA Technologies (IDT) (Coralville, IA). Amplification for HRM analysis was performed using SensiMix HRM™ mastermix (Quantace, Inc., Norwood, MA) which contains the dye Eva Green™. The DNA samples used were from laboratory personnel, convicted offenders, 9947A (Applied Biosystems, Foster City, CA), or samples obtained from the National Institute of Standards and Technology. Samples were purified by a modified sucrose-based extraction (11), a Maxwell 16 using the manufacturer's protocol (Promega, Madison, WI), and phenol/chloroform (12,13). Degraded DNAs were created by treating nine high concentration DNAs with DNaseI (Promega) for 0–128 min according to manufacturer's directions except using a 1:10 dilution of enzyme. Aliquots were taken at 11 intervals (0, 15, 30 sec, 1, 2, 4, 8, 16, 32, 64, and 128 min) to create DNAs with various levels of degradation.

All chemicals used for the inhibitor studies were purchased from Sigma (St. Louis, MO) and prepared as follows: hematin (100 mM in 0.1 M NaOH), indigo (100 mM in 2% Triton X), humic acid (1 mg/mL in DIH_2O), tannic acid (1 mg/mL in DIH_2O), calcium hydrogen phosphate (100 mM in 0.5 M HCl), melanin (1 mg/mL in 0.5 M ammonium hydroxide), and collagen (1 mg/mL in 0.1 M acetic acid). Bovine serum albumin (BSA) (nonacetylated) was also purchased from Sigma.

Initially, the PCR conditions in the Quantace SensiMix HRM™ manual were followed using a 56°C anneal temperature and a 20 μL reaction volume (instead of the recommended 25 μL).

TABLE 1—*Primers for STR high resolution melting (14,15).*

Locus	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product Size
CSF1PO	ACAGTAACTGCCTTCATAGATAG	GTGTCAGACCCTGTTCTAAGTA	276–320
D1S1677	TTCTGTTGGTATAGAGCAGTGTTT	TGACAGGAAGGACGGAATG	81–117
D2S1338	TGGAAACAGAAATGGCTTGG	GATTGCAGGAGGGAAGGAAG	289–341
D3S1358	CAGAGCAAGACCCTGTCTCAT	TCAACAGAGGCTTGCATGTAT	97–149
D4S2364	CTAGGAGATCATGTGGGTATGATT	GCAGTGAATAAATGAACGAATGG	67–83
D5S818	GGGTGATTTTCCTCTTTGGT	AACATTTGTATCTTTATCTGTATCCTTATTTAT	134–178
D7S820	GAACACTTGTCATAGTTTAGAACGAAC	TCATTGACAGAATTGCACCA	253–297
D8S1179	TTTGTATTTCATGTGTACATTCGTATC	ACCTATCCTGTAGATTATTTTCACTGTG	123–175
D10S1248	TTAATGAATTGAACAAATGAGTGAG	CAACTCTGGTTGTATTGTCTTCAT	79–123
D13S317	TCTGACCCATCTAACGCCTA	CAGACAGAAAGATAGATAGATGATTGA	193–237
D14S1434	TGTAATAACTCTACGACTGTCTGTCTG	AATAGGAGGTGGATGGATGG	70–98
D16S539	ATACAGACAGACAGACAGGTG	GCATGTATCTATCATCCATCTCT	233–277
D18S51	TGAGTGACAAATTGAGACCTT	GTCTTACAATAACAGTTGCTACTATT	264–394
D19S433	GCACCCATTACCCGAATAAA	CCTGGGCAACAGAATAAGATT	106–140
D21S11	ATTCCCCAAGTGAATTGC	GGTAGATAGACTGGATAGATAGACGA	138–256
D22S1045	ATTTTCCCCGATGATAGTAGTCT	GCGAATGTATGATTGGCAATATTTTT	82–115
FGA	AAATAAAATTAGGCATATTTACAAGC	GCTGAGTGATTTGTCTGTAATTG	196–348
Penta D	GAGCAAGACACCATCTCAAGAA	GAAATTTTACATTTATGTTTATGATTCTCT	76–449
Penta E	GGCGACTGAGCAAGACTC	GGTTATTAATTGAGAAAACTCCTTACA	79–474
THO1	CCTGTTCCTCCCTTATTTCCC	GGGAACACAGACTCCATGGTG	160–204
TPOX	CTTAGGGAACCCTCACTGAATG	GTCCTTGTCAGCGTTTATTTGC	209–257
VWA	AATAATCAGTATGTGACTTGGATTGA	ATAGGATGGATGGATAGATGGA	52–212

TABLE 2—*Effects of altering Mg++ concentration, anneal time, extension time, and extension temperature and reaction volume.*

PCR Amplification Conditions					C_t				
Anneal Conditions	Extension Conditions	Mg++ (mM)	Primer (nM)	Reaction Volume (μL)	THO1 C_t	D7S820 C_t	Penta E C_t	D22S1045 C_t	FGA C_t
56°C 10 sec	72°C 10 sec	3.0	200	20	29.33	>45	>45	34.15	34.59
56°C 10 sec	72°C 10 sec	4.5	200	20	30.06	>45	>45	>45	34.44
56°C 10 sec	72°C 10 sec	6.0	200	20	30.31	>45	>45	>45	34.60
56°C 20 sec	65°C 30 sec	3.0	200	20	28.50	26.91	29.31	27.68	27.33
56°C 20 sec	65°C 30 sec	3.0	200	20	29.71	31.27	38.96	29.51	29.52
56°C 10 sec	65°C 30 sec	3.0	200	20	29.05	30.26	36.89	32.15	31.98
56°C 10 sec	72°C 10 sec	3.0	400	20	28.12	29.61	34.48	29.93	29.33
56°C 10 sec	65°C 30 sec	3.0	400	20	28.29	28.44	33.00	29.46	28.52
56°C 10 sec	65°C 30 sec	3.0	400	20	26.96	28.32	32.03	30.08	28.54
56°C 10 sec	72°C 10 sec	3.0	400	20	28.56	31.15	34.24	31.17	29.35
56°C 20 sec	65°C 30 sec	3.0	400	20	27.39	26.70	29.23	27.60	26.40
56°C 20 sec	65°C 30 sec	3.0	400	20	27.37	ND	29.50	ND	26.62
56°C 20 sec	65°C 30 sec	3.0	400	15	27.47	ND	30.17	ND	26.76
56°C 20 sec	65°C 30 sec	3.0	400	10	28.03	ND	31.29	ND	27.21

ND, not determined.

TABLE 3—*The approximate number of genotypes that could be easily differentiated for the 22 STRs studied.*

STR	Number of Samples Tested	Number of Genotypes Present in Samples	Number of Distinct Melting Patterns
vWA	48	21	10
THO1	45	14	8
D18S51	30	22	8
TPOX	44	10	8
Penta D	29	19	7
D8S1179	30	21	7
D16S539	45	15	6
D3S1358	30	14	6
D19S433	21	16	4
D13S317	30	15	4
D21S11	30	14	3
D4S2364	21	3	3
FGA	30	20	3
Penta E	29	25	3
D5S818	30	11	3
D22S1045	21	4	2
D14S1434	21	8	2
D10S1248	20	11	2
CSF1PO	30	10	1
D7S820	30	16	1
D2S1338	21	19	1
D1S1677	21	14	(Noise only)

However, optimizations were performed for anneal time, extension temperature and time, and reaction volume. The final PCR reactions were performed in a 15 μL volume including 4.8 μL of DIH_2O, 7.5 μL of mastermix, 0.6 μL of Eva Green™ dye solution, 0.3 μL of each 20 μM primer, and 1.5 μL of sample DNA. Amplification and HRM was performed in a two color + HRM Corbett Rotorgene RG6000 (now Qiagen Rotorgene Q; Qiagen). Amplification was performed using the following cycling parameters: 95°C for 10 min, 45 cycles of 95°C for 5 sec, 56°C for 20 sec, 65°C for 30 sec, then 72°C for 2 min, 95°C for 20 sec, 55°C for 20 sec, and lastly 56°C for 2 min; the HRM melting was subsequently performed from *c.* 3°C below the first melting peak to *c.* 3°C above the highest melting peak (from 66 to 86°C for the combination of vWA, D18S51, and THO1) with 0.1°C/step and a 90 sec wait before the first step and 2 sec thereafter.

Some data was initially interpreted using the HRM software of the Rotorgene. One example of each genotype was used as a standard and the software was allowed to call the genotypes for the rest of the samples. This software method had difficulties, in that many samples were not called; therefore, future sample assessments were performed by visual inspection.

Results

Twenty-two STRs used in the forensic field were chosen for the initial analysis (Table 1). For each locus, primers were chosen to generate small PCR products (14,15). Each STR was initially tested with several DNA samples to check that the primers worked by performing real-time PCR and HRM melting in a Rotorgene RG6000. Initial studies showed a great deal of variation in the amplification efficiency of some of the STRs (data not shown). To remedy this, a number of amplification parameters were adjusted (Table 2). This included Mg++ concentration, anneal time, primer concentration, and cycle number. Increasing the Mg++ concentration gave various results with different loci, but did not improve overall amplification (Table 2). A number of experiments testing the other parameters were performed. It was determined that a combination of increasing the anneal time to 20 sec (from 10 sec), increasing the extension time to 30 sec (from 10 sec), decreasing the extension temperature to 65°C (from 72°C), and doubling the primer concentrations (Table 2) gave the best amplification and these conditions were used for all subsequent experiments. PCR reaction volumes of 10, 15, and 20 μL (Table 2) were also investigated. Equivalent results were obtained for 15 and 20 μL reactions

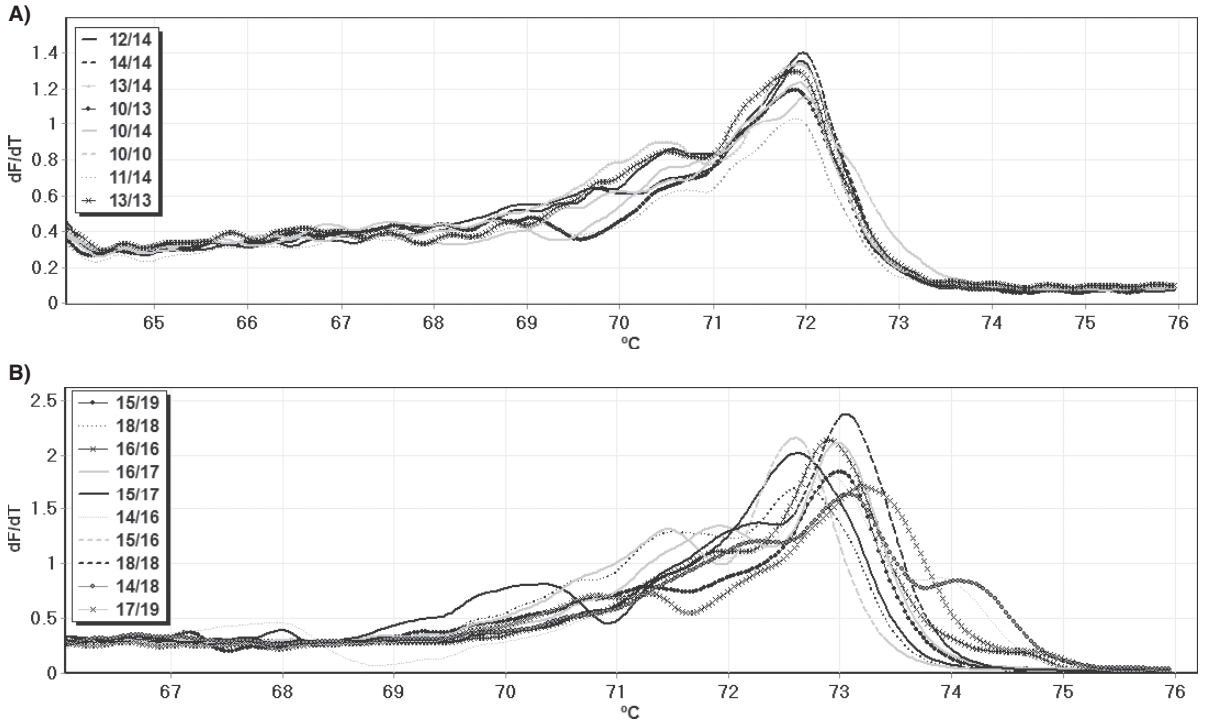


FIG. 1—*High resolution melting profiles for two STR loci. (A) Eight genotypes for an STR (D14S1434) with little ability to distinguish genotypes. (B) Ten different genotypes for vWA showing 10 different melting patterns. The inset legend lists the genotypes of the samples.*

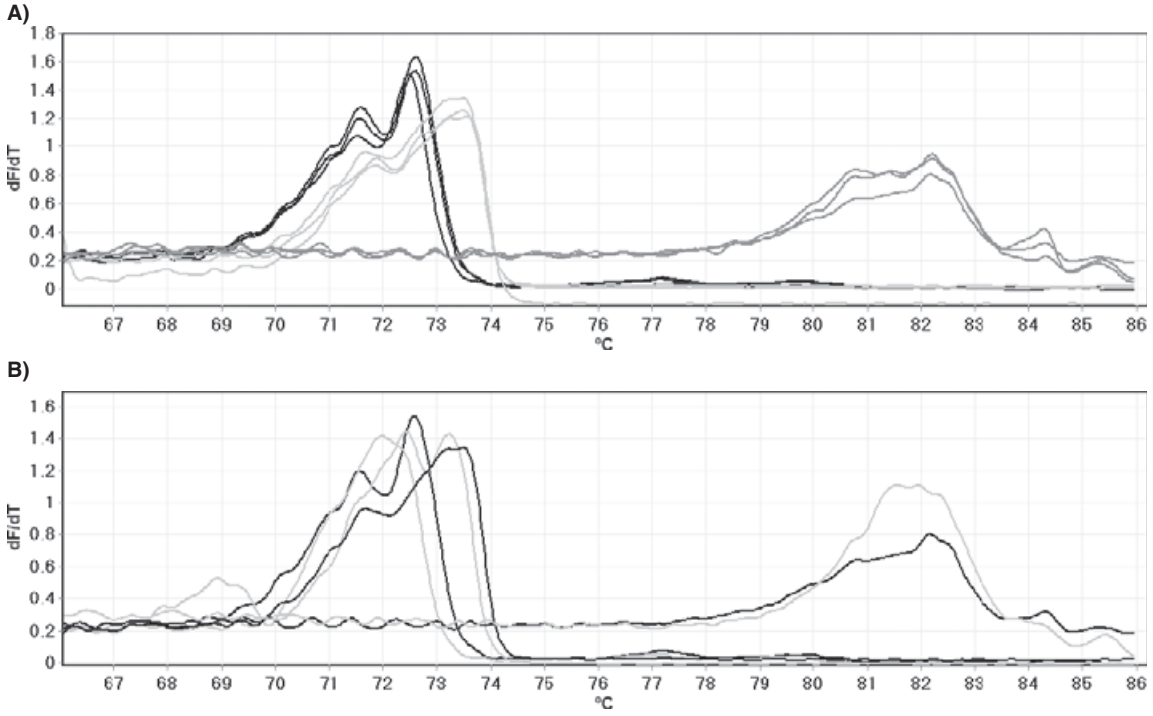


FIG. 2—*Triplex STR high resolution melting assay. (A) Results showing triplicate results of all three loci on the same graph (DNA 9947 [vWA—black, D18S51—light gray, THO1—dark gray]). (B) Two different DNA samples showing that the profiles are quite different (9947—black, DNA2—gray).*

TABLE 4—*Reproducibility of Tm's of high resolution melting peaks from four individuals run three times.*

Sample	Melting Peak	Day 1	Day 2	Day 3	Average	Stdev	%Stdev
vWA							
Individual A	1	72.80	72.75	72.43	72.66	0.201	0.28
Individual B	1	72.40	72.23	71.95	72.19	0.227	0.31
Individual C	1	71.58	71.63	71.50	71.57	0.066	0.09
Individual C	2	72.67	72.70	72.40	72.59	0.165	0.23
9947	1	71.60	71.70	71.48	71.59	0.110	0.15
9947	2	72.68	72.72	72.37	72.59	0.192	0.26
D18S51							
Individual A	1	72.57	72.55	72.40	72.51	0.093	0.13
Individual A	2	73.40	73.35	73.25	73.33	0.076	0.10
Individual B	1	72.48	72.52	72.35	72.45	0.089	0.12
Individual B	2	73.28	73.30	73.20	73.26	0.053	0.07
Individual C	1	72.45	72.40	72.30	72.38	0.076	0.11
Individual C	2	73.32	73.28	73.17	73.26	0.078	0.11
9947	1	71.60	71.75	71.57	71.64	0.096	0.13
9947	2	73.40	73.30	73.27	73.32	0.068	0.09
THO1							
Individual A	1	82.15	82.05	81.97	82.06	0.090	0.11
Individual B	1	82.08	81.95	81.97	82.00	0.070	0.09
Individual C	1	82.03	81.95	81.93	81.97	0.053	0.06
9947	1	82.05	82.00	82.05	82.03	0.029	0.04

(10 μL gave slighter higher C_t values); thus, 15 μL was chosen to save on reagent costs.

Each STR primer set was then tested with 21–45 sample DNAs with an array of known genotypes to determine the ability of HRM of the STR product to differentiate individuals by differences in their melting profile. Table 3 summarizes the results of these tests, giving the approximate numbers of genotypes that could be resolved by differences in melting profile. The results varied from very good (many to most of the genotypes could be resolved) to very poor (none could be resolved). There was no correlation of number of distinguishable patterns with STR length or amplicon length (data not shown). Figure 1*A* shows the result for the STR D14S1434 which yielded HRM patterns that only distinguished a few genotypes, whereas Fig. 1*B* shows results for an STR (vWA) which gave a variety of different melting patterns for different genotypes.

The three STRs which allowed the most genotypes to be distinguished by their melting patterns (vWA, D18S51, THO1) were chosen to generate a triplex assay. Figure 2 shows how the data for all three loci can be displayed simultaneously so that comparisons of two samples can be made easily. Figure 2*A* displays all three loci for 9947 DNA (triplicates shown) whereas Fig. 2*B* demonstrates how two different samples can be differentiated.

To show the reproducibility of the assay, Tables 4 and 5 give information of the Tm's for peaks at all three loci for four individuals over 3 days (Table 4) or for individuals with the same

TABLE 5—*Reproducibility of Tm's of high resolution melting peaks from individuals with the same genotype.*

Genotype	Melting Peak	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Average	Stdev	% Stdev
vWA 17/18	1	71.58	71.60	71.77			71.65	0.104	0.146
	2	72.67	72.68	72.80			72.72	0.072	0.099
vWA 16/17	1	71.68	71.72	71.62	71.65		71.67	0.043	0.060
	2	72.82	72.82	72.75	72.82		72.80	0.035	0.048
D18S51 12/14	1	72.57	72.58	72.55			72.57	0.015	0.021
	2	73.32	73.28	73.20			73.27	0.061	0.083
D18S51 14/14	1	72.60	72.50				72.55	0.071	0.097
	2	73.40	73.30				73.35	0.071	0.096
D18S51 12/17	1	71.27	71.30				71.29	0.021	0.030
	2	73.27	73.28				73.28	0.007	0.010
THO1 6/9.3	1	82.03	82.12	82.07	82.07	82.08	82.07	0.032	0.039
THO1 9.3/9.3	1	80.35	80.48	80.35	80.30		80.37	0.077	0.096
	2	82.15	82.17	82.08	82.10		82.13	0.042	0.051
THO1 7/9.3	1	81.17	81.20	81.20	81.15		81.18	0.024	0.030
	2	82.15	82.12	82.15	82.10		82.13	0.024	0.030

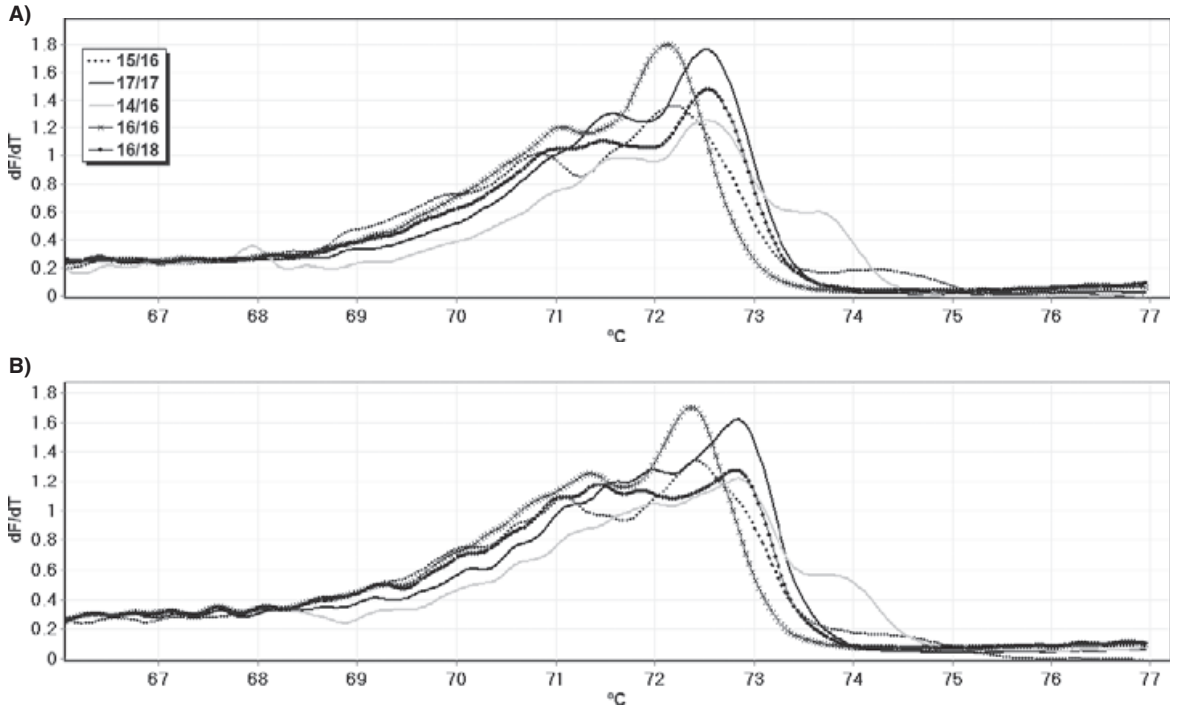


FIG. 3—*Five different samples were amplified with vWA primers and then high resolution melting (HRM) was performed twice, 6 days apart. The amplified samples sat undisturbed in the instrument between the HRMs. The results from the comparisons (A, day 1; B, day 6), show the stability of the amplicons and the similar melt profiles over time. The inset legend lists the vWA genotypes of the samples.*

genotype in the same run (Table 5). Percent standard deviations (standard deviation divided by the mean) are all <1% (most were <0.1%).

This assay was then tested for reproducibility, uniformity for a known genotype, and melting profile consistency over dilution. The HRM results are very consistent and stable. Melting profiles are essentially unchanged after the tubes have remained for 6 days at room temperature in the instrument (Fig. 3). Figure 4 shows results for four individuals performed on three different days for one STR. The assay also gives consistent melting profiles across dilutions of a sample from neat to 1/512 (Fig. 5). This relates to a C_t of <35 in the amplification and a concentration of *c.* 20 pg (expected for a single copy locus).

As BSA is often included in forensic PCR mixes to ameliorate the effects of inhibitors, five BSA concentrations (0, 166, 250, 333, 417 ng/μL) were tested to see their effects on amplification and melting. There was little, if any, effect (data not shown). A concentration of 250 ng/μL was chosen for future experiments as this concentration is used in other assays performed in our laboratory.

Whether or not DNA degradation would affect the assay was tested. Figure 6 demonstrates that degradation did not affect the melting profiles. Figure 6*A* shows the degraded DNA (DNaseI treated) and Fig. 6*B* shows the melting of the same DNA samples. Also tested was whether different DNA extraction methods would affect the HRM profiles. Two different DNAs extracted in duplicate with the Maxwell 16, the usual organic method or a simple sucrose extraction, were tested. No difference was seen in melting profiles between DNAs isolated by the three different methods (data not shown).

The assay was tested with seven different inhibitors (hematin, indigo, melanin, collagen, calcium phosphate, tannic acid, and humic acid). Although many of the inhibitors do affect the

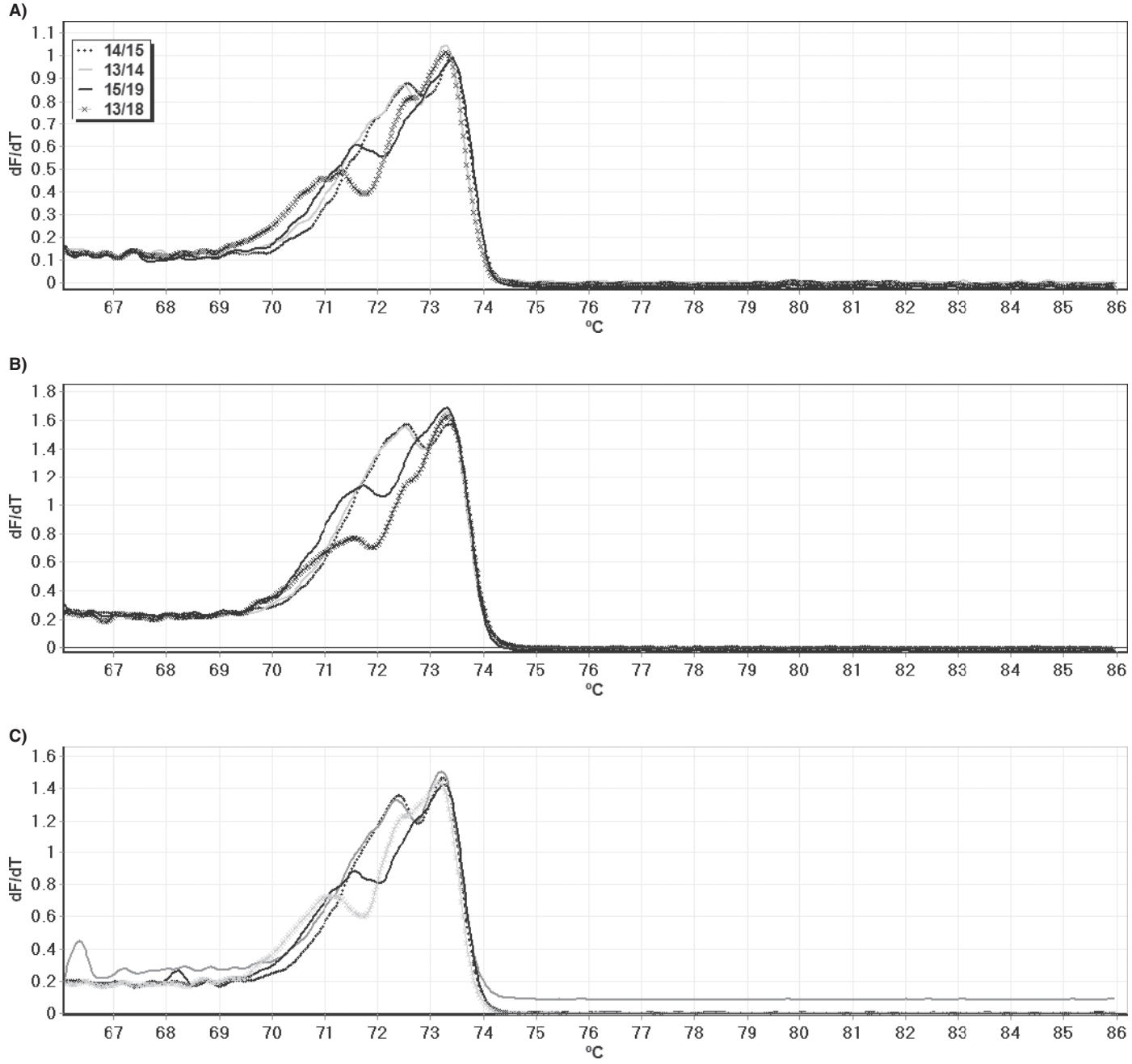


FIG. 4—*Results for four individuals performed on three different days (A–C) for D18S51. Each DNA is shown in a different shade of gray. The inset legend lists the genotypes of the samples.*

amplification at high concentrations, most do not affect the melting characteristics (data not shown). Collagen and calcium phosphate do shift the profile to slightly higher melting temperatures; however, this appears to be due to the buffers rather than the inhibitor (data not shown). Also, very high concentrations of indigo appear to give 100% amplification, but this is due to the dye causing spurious fluorescence measurement (data not shown).

The assay cannot definitively identify all genotypes for each locus (e.g., the melt curve of a 17/17 vWA genotype can look the same as an 18/18 or a 19/19 genotype) (Fig. 7). Table 6 shows the grouping of the genotypes by HRM patterns and which common genotypes were not distinguished by visual inspection. However, it should be noted that as a screening assay, it need not distinguish all genotypes. In addition, in some cases, HRM can be used to distinguish between individuals with the same STR genotypes. Figure 8 shows that some individuals with the same genotype (vWA 16/17) had different melting profiles. These differences were reproducible and probably due to the fact that some alleles, although the same length (thus, have the same allele designation based on fragment analysis) have different sequences and, thus, different melting characteristics. The Short Tandem Repeat DNA Internet DataBase (STRBase, http://www.cstl.nist.gov/strbase/) lists a number of known sequence differences for vWA alleles including

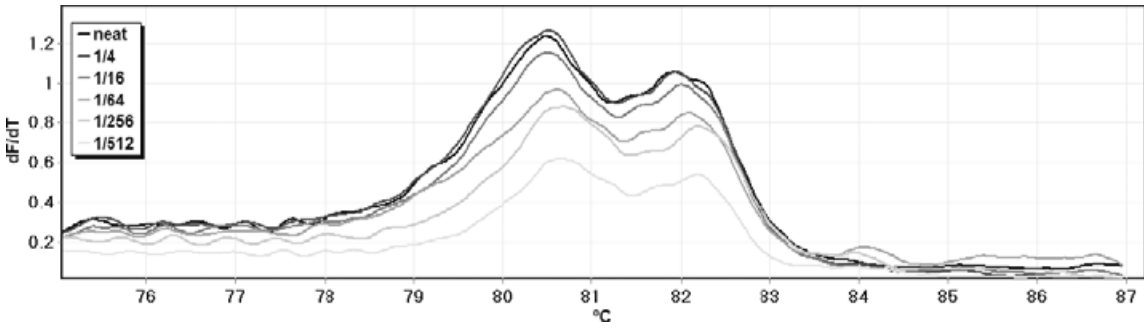
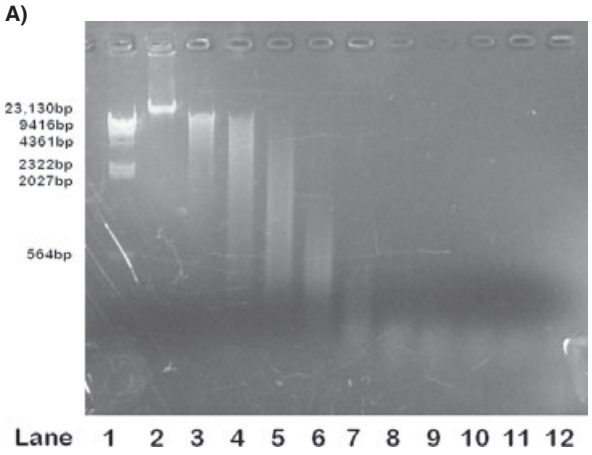


FIG. 5—*Melting patterns for THO1 are constant even when a sample is diluted 1/512. The inset lists the dilutions of the sample.*



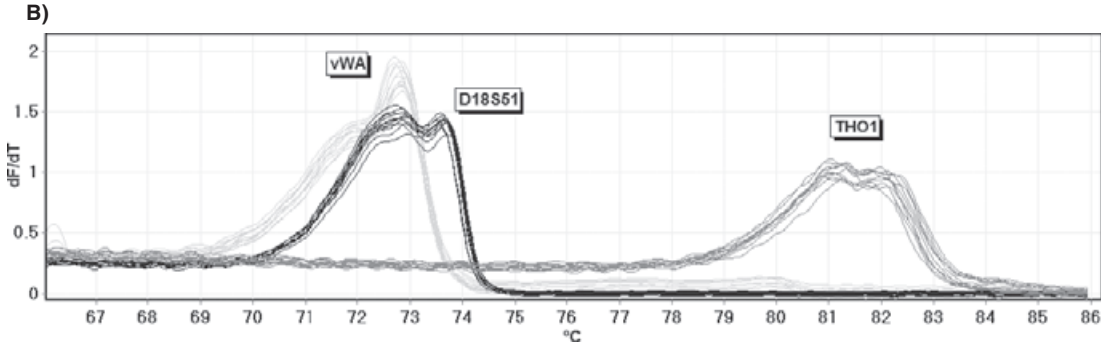


FIG. 6—*DNA degradation does not affect the melting profiles. Panel (A) shows the degraded DNA (DNaseI treated) samples run on an agarose gel and (B) shows the high resolution melting results for the same DNA samples.*

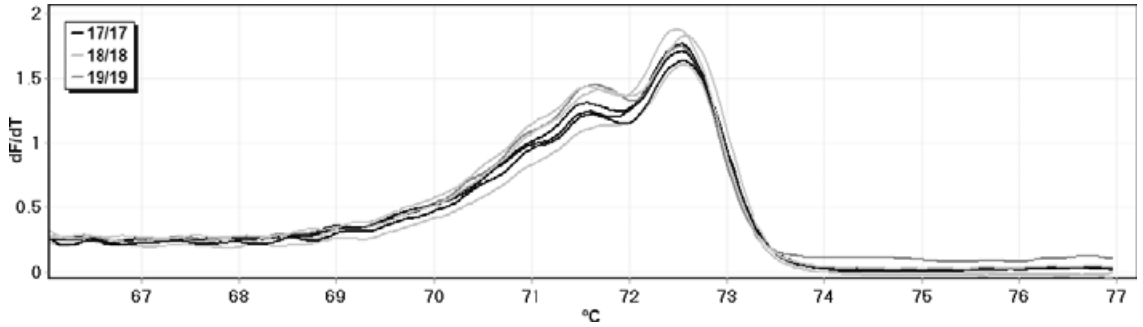


FIG. 7—*Some genotypes cannot be differentiated by high resolution melting. vWA genotypes 17/17, 18/18, and 19/19 cannot be differentiated. The inset legend lists the genotypes of the samples.*

TABLE 6—*The common genotypes in each group that could (different groups) or could not (same group) be distinguished.*

Group	vWA	D18S51	THO1
A	16/18, 17/19, 16/19	12/13, 12/14, 14/15, 15/16	6/9.3
B	14/18 (some 17/18)	17/17	7/9.3, 8/9.3
C	17/18, 16/17	11/16, 12/17	6/6
D	16/16, 17/17	11/19, 12/19	9/9, 9.3/9.3
E	18/18	14/14	9/9.3
F	15/17, 17/19	13/16, 14/17, 15/18, 15/19	5/9.3
G	14/16	12/16	6/7, 6/8, 6/9
H		12/14, 13/15, 14/16	7/7
I		17/20	
J		15/18	

alleles 14, 15, 16, and 18 (Table 7); no sequence differences are listed for allele 17 or 19.

To determine if the melting differences were due to sequence differences, DNA sequencing was performed on five pairs of samples that had the same vWA genotype by capillary electrophoresis, but different HRM patterns (17/18, 14/18, 16/18, 16/17, 17/19). For the 17/18 pair, it appeared that one sample was 17/18 and the other 17/18′. For the 14/18 pair, interpretation was difficult as the variant sequences in STRBase were incomplete (short); however, one sample appeared to be a 14′/18′ because of heterozygosities in the third, fifth, sixth, and 11th repeat. The second sample appeared to be a variant reported in Lins et al. (16), but not in STRBase ((TCTA)[TCTG]$_3$[TCTA]$_{10}$) paired with 18′ because of the heterozygosity in the fifth repeat. For the 16/18 pair, the two samples were 16/18 and 16′/18. For the 16/17 pair, the samples were 16/17 and 16′/17. For the 17/19 pair, STRBase did not report any sequence variants; however, the sequences showed a clear difference between the two samples, either the 17 was a 17′ of (TCTA)[TCTG]$_3$[TCTA]$_{13}$(TCCA)(TCTA) or the 19 was a 19′ of (TCTA)[TCTG]$_3$[TCTA]$_{15}$(TCCA)(TCTA).

As the amplification part of the assay is quite robust, it should be possible to use it to quantitate the DNA in the sample when a standard curve is simultaneously run. An assay run with a standard curve (64–0.0156 ng/μL) gave an R^2 of 0.993 and an efficiency of 1.05 (Fig. 9).

A test of 20 DNA samples with replicates (0–6 replicates for a total of 72 tubes assayed) to ascertain the accuracy of determining whether two samples were alike or different was made. This experiment was designed to mimic what might be found at a crime scene where different numbers of samples from different individuals might be found and in addition, the analyst who reviewed the profiles (blind to the results) would not know how many replicates of each sample to expect. Results for one DNA sample (five duplicates/tubes) were excluded because of poor amplification. For the remaining 19 DNA samples/67 tubes, 53 tubes were correctly identified with their duplicates. Two sets of two samples were found, that is, the two samples and their duplicates, which could not be differentiated from each other, but were correctly called as a group (a total of 12 wells). There were four incorrect calls where the duplicate was put into the wrong set, and one had only one tube (no replicate).

A second experiment involved three separate runs of the instrument. Each run tested for THO1, vWA, and D18S51 and had four samples in common (DNA1, DNA2, DNA3, and 9947) and 20 unique (different samples). The experiment was performed in this manner so that in a single amplification all three loci for the four test samples could be compared with the 20 DNA samples (to make for easier analysis.) For all of the 240 comparisons (4 × 60), only one melting profile match for the three loci was found. When this was checked against the actual genotypes, the two individuals had the exact same genotypes for the three loci. The assay performed very well in this test, showing an excellent ability to distinguish individuals and a perfect detection of the one true genotype match. The difficulties encountered in the test described in the previous paragraph were possibly due to the complexity of comparing the large amount of data spread over three amplifications. In reality, when this test would be performed in the laboratory on casework, the data would be comparing several melting profiles for all three loci in the same run.

For the mixture experiments, four combinations of different DNAs were made at five mixture combinations (100% DNA1, 75% DNA1 and 25% DNA2, 50% DNA1 and 50% DNA2, 25% DNA1 and 75% DNA2, 100% DNA2). Melting profiles of the mixtures were often not the combination of the two melt profiles of the individual DNAs. This is not surprising as having two new DNAs in the amplification/melt allows for unique hybrid molecules to be formed and melt. For example, a 7/7 homozygote typically has only the 7-7 molecule to melt with whereas a 9/9.3 heterozygote has 9-9, 9.3-9.3, and 9-9.3 molecules. However, a mixture of the two includes all of those plus 7-9 and 7-9.3 which could give an entirely new dimension to the melt curve (data not shown). It can get even more complex if both DNAs are from heterozygotes. In general, the 75/25 mixes were reasonably close to the 100/0 patterns, but the 50/50 mixes were often quite different. However, there were exceptions. If both patterns were initially similar then the mixture was usually similar as well.

Because the melting temperature of THO1 is so much higher than either vWA or D18S51, it was thought that it might be possible to amplify either THO1 with vWA or THO1 with D18S51 as a

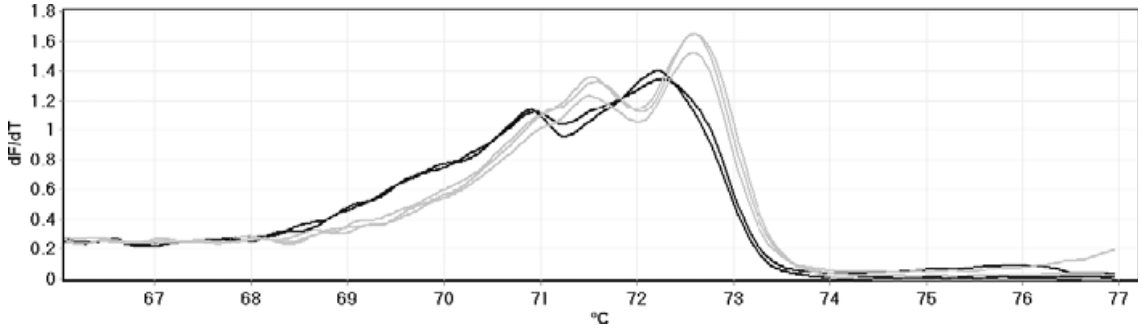


FIG. 8—*Some samples with the same genotype have different melting profiles. Five DNA samples with genotype 16/17 for vWA. Two show one melting pattern (black) while the other three show a clearly different pattern (grey).*

TABLE 7—*vWA sequence variants (data taken from STRBase).*

Allele	Sequence Variants
14	14 (TCTA)[TCTG]$_4$[TCTA]$_{11}$
	14′ (TCTA)(TCTG)(TCTA)[TCTG]$_4$[TCTA]$_3$(TCCA)[TCTA]$_3$
	14″ (TCTA)[TCTG]$_5$[TCTA]$_3$(TCCA)[TCTA]$_3$
15	15 (TCTA)[TCTG]$_4$[TCTA]$_{10}$(TCCA)(TCTA)
	15′ (TCTA)[TCTG]$_3$[TCTA]$_{11}$(TCCA)(TCTA)
16	16 (TCTA)[TCTG]$_4$[TCTA]$_{11}$(TCCA)(TCTA)
	16′ (TCTA)[TCTG]$_3$[TCTA]$_{12}$(TCCA)(TCTA)
17	17 (TCTA)[TCTG]$_4$[TCTA]$_{12}$(TCCA)(TCTA)
18	18 (TCTA)[TCTG]$_4$[TCTA]$_{13}$(TCCA)(TCTA)
	18′ (TCTA)[TCTG]$_5$[TCTA]$_{12}$(TCCA)(TCTA)
19	19 (TCTA)[TCTG]$_4$[TCTA]$_{14}$(TCCA)(TCTA)

duplex. As a proof of concept, amplifications of THO1 together with vWA were performed on 24 DNA samples, both with separate amplifications/melts and a duplex amplification/melt. The results for the duplex were surprisingly good, perhaps even better than for the separate amplifications and melts. Figure 10 shows the results for these of the 24 samples. In Fig. 10*A*, the separate melts are displayed on the same graph whereas Fig. 10*B* shows the duplex. The results for most samples look quite similar. The peaks for THO1 are also higher in the duplex which helps with differentiation of the profiles. This is an area for further research as duplexing THO1 and vWA can lend itself to the possibility of multiplexing a second high melting STR with D18S51, creating a much more powerful assay using only two wells.

Most forensic laboratories do not have access to an HRM instrument and it was important to determine if a non-HRM instrument could be used for these types of experiments. To assess this, three samples were amplified and melted with the vWA, D18S51, and THO1 primers using a six-color, non-HRM RG6000 real-time instrument using the standard melt software set to parameters similar to those of an HRM melt (0.1°C/step, 2 sec wait/step). The results showed that similar melt curves could be obtained between the instruments (Fig. 11). For example, sample 2 (vWA 14/18, THO1 6/7) amplified with vWA primers shows the same broad peak at 72.4°C and small peak at 69.5°C in both the HRM and non-HRM instruments and sample 3 (vWA 17/18, THO1 8/9.3) amplified with D18S51 primers has the same dip at 72.2°C in both instruments. A greater number of comparisons would need to be performed to understand if the non-HRM instrument gave sufficiently discriminating data for a forensic assay or if other brands of real-time instruments could be utilized.

Discussion

With the choice of the proper STRs, HRM can be a very useful technique in distinguishing between genotypes. While HRM cannot distinguish all genotypes, an assay consisting of three STRs gives sufficient power to make a useful screening assay. If all genotypes could be distinguished, then an assay based on THO1, D18S51, and vWA would only give a match at about 1/5400 for these three loci. As the assay cannot distinguish all genotypes, the match probability would be less than this (*c.* 1/2000). This number is very conservative, but was difficult to calculate because of the many genotype combinations for D18S51, many of which, because of their rarity, were not tested in the experiments described here. This 1/2000 is without the added ability of the assay to distinguish alleles of the same length, but different sequence [important for such alleles as vWA 18 ((TCTA)[TCTG]$_4$[TCTA]$_{13}$(TCCA)(TCTA)) and 18′ ((TCTA)[TCTG]$_5$[TCTA]$_{12}$(TCCA)(TCTA))]. The assay is relatively impervious to inhibitors and degraded DNA.

As evidentiary samples can often be composed of more than one donor profile (i.e., mixtures), it is imperative that the forensic scientist understand that melting patterns may change with these samples. Furthermore, the complexity of melting patterns in single source samples would prohibit the identification of the contributors in a mixture. However, the usefulness of this HRM assay remains. Intended as a screening procedure, the melting pattern analysis

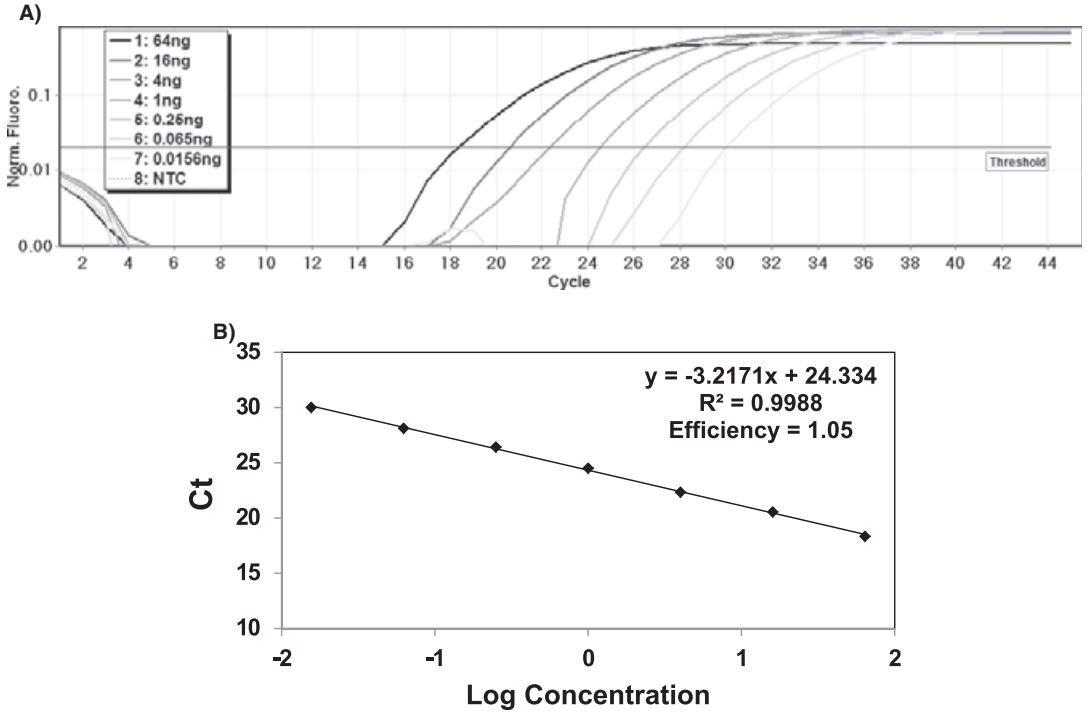


FIG. 9—*Amplification curve (A) and standard curve (B) for a dilution series of DNA. The inset shows the amount of DNA added to the amplifications.*

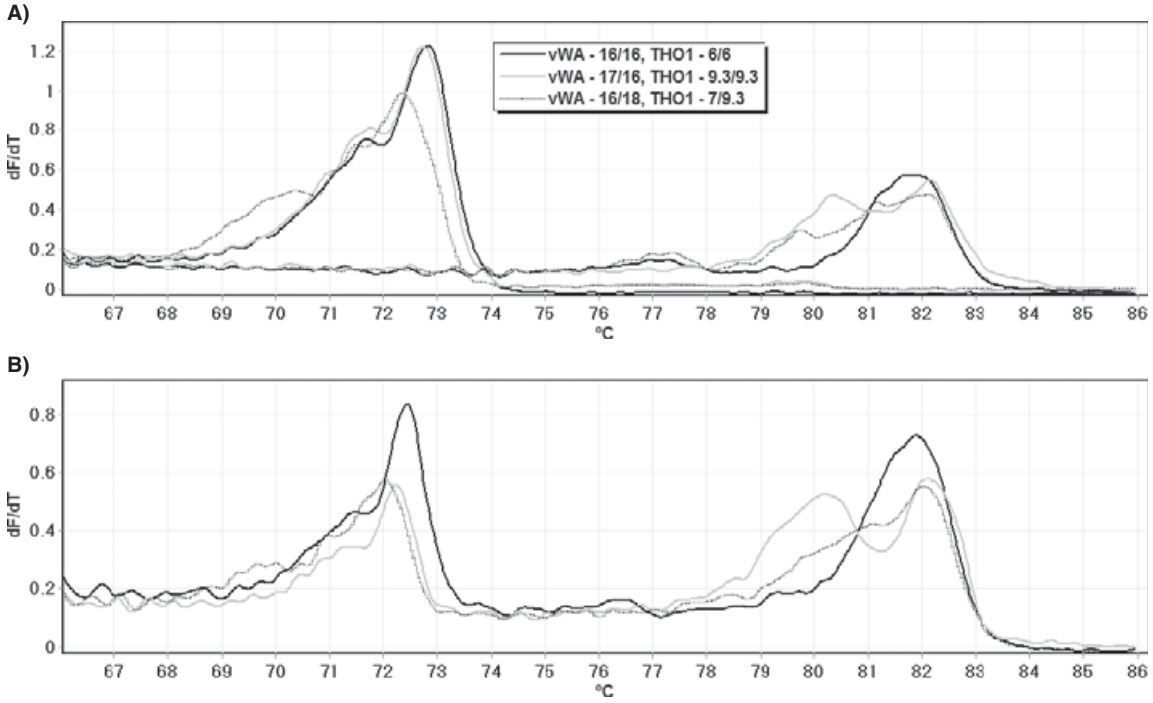


FIG. 10—*High resolution melting results for DNA samples from three individuals using THO1 and vWA primers. (A) Amplified and melted in separate wells. (B) Amplified and melted together in the same well. The inset legend lists the genotypes of the samples.*

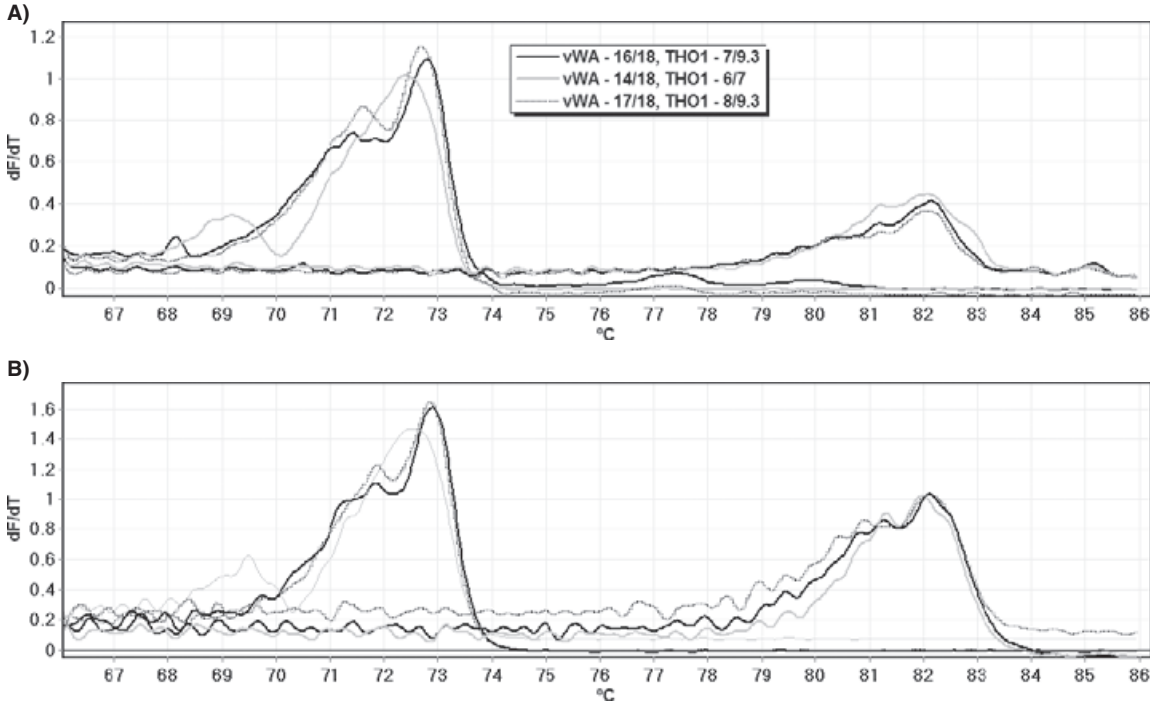


FIG. 11—*Comparison of melting results for the same three samples in a high resolution melting (HRM) Rotorgene real-time instrument (A) versus in a non-HRM Rotorgene real-time instrument (B). The inset legend lists the genotypes of the three samples.*

would allow the sorting of samples into groups and therefore, reduce the number of samples deemed worthy of STR analysis. For instance, evidentiary samples matching the victim's melting profile could be eliminated, whereas samples showing different melting patterns (i.e., possible suspect(s), mixtures, etc.) would be further analyzed. Alternatively, if an evidentiary sample is determined to originate from the suspect and is a single-source sample, it would be more efficient and cost-effective to screen putative suspect samples via melting patterns before performing STR analysis on every sample.

This assay is designed as a screening tool to allow the examiner the flexibility to sort stains found at crime scenes. Many labs do

this already when they sort stains donated by male and female individuals. This test is the logical extension of that analysis. The assay may not work in every circumstance, but when it does, it will give the examiner the ability to take a large number of stains and select a handful for STR analysis. The ability to quickly and efficiently group stains having a common HRM pattern would be a powerful procedure in the forensic examiner's tool box.

The cost savings of using this assay would be considerable. If use of the screening assay on 20 samples from a crime scene (at <$1.00 each in supplies and a couple hours of time to setup and run the assay), determined that 18 were from the same individual, then only three samples would need to have STR analysis performed. This would save the cost of STR analysis on 17 samples which is >$400 (*c.* 17 × $25 = $425, plus a day of analyst time).

In addition, the assay could be used to simultaneously quantitate DNA samples for full 13 locus STR analysis. Quantitating DNA separately costs *c.* $2.50/sample using a commercial kit plus eight standards ($20) whereas this assay would do it for the $8.00 total needed to run eight standards.

It may even be possible to perform "HRM" in a regular real-time instrument with the melting ramp set to finer resolution or to duplex several STRs with very different melting profiles in the same tube. Future work needs to be performed to see if these approaches are viable.

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