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Janice A. Nicklas,[1] *Ph.D. and Eric Buel,*[1] *Ph.D.*

A Real-Time Multiplex SNP Melting Assay to Discriminate Individuals

ABSTRACT: A method that quickly and inexpensively differentiates crime scene samples from multiple donors would expedite casework analysis by allowing the selection of probative items requiring comprehensive testing. This new method need not be perfectly definitive nor give a complete 13 locus short tandem repeat (STR) profile; it simply must be able to differentiate between most victim and suspect samples. We describe the development of multiplex, single nucleotide polymorphism (SNP), fluorescence resonance energy transfer-based real-time polymerase chain reaction (PCR) assays to fulfill this need. Dual probes, one fluorescently labeled and the other labeled with a quencher, are monitored during a melt analysis to reveal an increase in fluorescence, which allows the assessment of the two SNP alleles. Two alternate 6-plex assays (with and without gender determination) have been developed for the six-color RG6000 real-time instrument (Corbett Robotics, Inc.) and one seven SNP plus gender assay (performed as two 4-plex assays, one with gender the other without) have been developed for use in four/five color real-time instruments. This technique can discriminate between 95% and 99% of samples from different individuals. This assay is fast (~2 h), much less expensive than STR analysis, and uses a real-time PCR instrument which is found in most forensic and molecular biology labs.

KEYWORDS: forensic science, fluorescence resonance energy transfer, polymerase chain reaction, single nucleotide polymorphism, real-time polymerase chain reaction, human discrimination, multiplex

Short tandem repeat (STR) profiling using conventional typing techniques, i.e., 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 each and 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.

Single nucleotide polymorphisms (SNPs) are commonly used for identification purposes, for example, determination of family heritage, disease states, and linkage studies. Reviews of SNP applications in forensics (1–4) reveal advantages and disadvantages to using SNPs. The smaller polymerase chain reaction (PCR) product size allows typing of degraded samples and SNP analysis is amenable to rapid, high throughput technologies yielding possibly lower analysis costs. Disadvantages include the lack of published databases, problematic mixture interpretation, and limited discrimination compared to STRs. Two recent reviews summarize technologies for typing SNPs (5,6). The main technologies available for SNP interrogation include: single nucleotide extension (Applied Biosystem's SNaPshot kit, Foster City, CA; Orchid's SNPstream, Princeton, NJ), allele-specific hybridization (e.g., DQ alpha PolyMarker kits, Perkin Elmer Corporation, Norwalk, CT), fluorescence resonance energy transfer (FRET) hybridization probes, TaqMan™ (Applied Biosystems), molecular beacons, oligonucleotide ligation followed by hybridization (Illumina, San Diego, CA), invasive cleavage (Invader®; Third Wave Technologies, Madison, WI), minisequencing (Pyrosequencing, Charlottesville, VA), genechip arrays, mini-sequencing followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, CA), fluorescence polarization, and allele-specific PCR. Many of these technologies require an expensive piece of specialized equipment (MALDI-TOF and Pyrosequencer) or cannot perform more than a duplex SNP assay in a four-color instrument or triplex assay in a six-color instrument (TaqMan™).

There are a number of reports describing the detection of SNPs or disease-related mutations using FRET hybridization probes in the LightCycler® (Roche Applied Science, Indianapolis, IN) or the LightTyper (Roche Applied Science) (7–30) and at least one report using the iCycler (Bio-Rad Laboratories, Hercules, CA) (31). Most of these assays detect only a single SNP although several duplex assays (15,21), a triplex assay (18), and a 4-plex (32) are reported.

As a number of assays are available to detect SNPs, one could envision using SNPs to differentiate stains found at a crime scene. The chance of two random individuals having the same result (same genotype), using an assay with four SNPs where both alleles are represented in the general population at about 50% ($p = q = 0.5$) is only 2%, for six SNPs it is 0.3%, while for eight SNPs it is only 1 in 2500 (Appendix A). To determine if two crime scene samples are from the same individual, a four-locus screening test would suffice, but for more complex scenes, a six- or eight-locus SNP screening test would offer a greater degree of discrimination. Many forensic labs are now acquiring real-time PCR instruments for human DNA quantitation. Rapid SNP profiling methods developed on such an instrument could be easily performed and would require no additional investment in equipment.

Herein, we describe the development of several multiplex SNP assays using FRET chemistry, quenchers, and melting curve analysis for discriminating crime scene samples originating from different individuals. Two alternate 6-plex assays (with and without gender determination) have been developed for the six-color RG6000 real-time instrument (Corbett Robotics, Inc., San Francisco, CA) and one seven SNP plus gender assay (performed as two 4-plex assays, one with gender the other without) has been developed for use in four/five color real-time instruments. The data presented also suggests that by running appropriate quantitation standards, this SNP assay could double as a means to quantitate the DNA in samples.

[1]Vermont Forensic Laboratory, Department of Public Safety, 103 S. Main St., Waterbury, VT 05671.

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Methods and Materials

DNA Samples

The DNA samples utilized in this study were mostly in-house controls used for STR testing in the laboratory (lab personnel) or DNA samples from convicted offenders. These were isolated using an organic extraction method (33) as modified in Akane et al. (34). The GM9947A DNA control DNA from AmpFlSTR® COfiler™ and Profiler Plus™ PCR Amplification Kits (Applied Biosytems) was also utilized as a DNA sample. Human Genomic DNA:Male (Catalog #G1471; Promega, Madison, WI) was used for the standard curves.

FRET Hybridization Probe Quenching Melting Assay to Detect SNPs

An assay detecting melting differences of a probe with the two different alleles of a SNP was utilized in the studies reported here. Melting differences were detected using a FRET hybridization quenching assay. In this assay, PCR is performed using two primers flanking the SNP. Two probes are also present: one 5′ situated probe with a 3′ dye label which covers the SNP, referred to as the reference or sensor probe, and a second, 3′ situated probe called the anchor probe, labeled with a 5′ quencher and a 3′ phosphate to prevent elongation. The reference/sensor probe is designed as a perfect match to one allele but has one mismatch with the other allele. As PCR proceeds, additional fluorescence is quenched because when both probes bind to the PCR product, the energy from the excited 3′ dye is transferred to the quencher and there is no emission of light (see Fig. 1). In the melting phase, fluorescence is gained because as the temperature increases, the probes melt off the PCR product and FRET between the dye and the quencher is lost in solution. Determination of which allele(s) is present depends on the melting temperature where the fluorescence is regained. This method uses only one reporter dye per SNP; thus, theoretically in a four-color real-time instrument, four SNPs can be multiplexed making a simple, one tube profiling assay feasible. Currently, several five-color and one six-color real-time instruments (the Corbett RG6000 used here) are commercially available allowing for even greater multiplexing.

SNP Selection and Primer Design

Review of the data provided from the ALFRED database (http://alfred.med.yale.edu/alfred/) permitted the selection of SNPs such that the variations in allele frequencies between populations were low (low Fst). This makes the SNP useful in any population. Additional criteria were that the SNPs were: noncoding, not medically relevant, allele frequencies (p and q) close to 0.5, located on different chromosomes, and not patented. In order for the assay to determine the gender of the individual from whom a sample derived, detection of a sequence difference (SD) between the ZFX and ZFY genes was incorporated. While this is not technically a SNP, it is still a nucleotide difference and it can be treated as if it

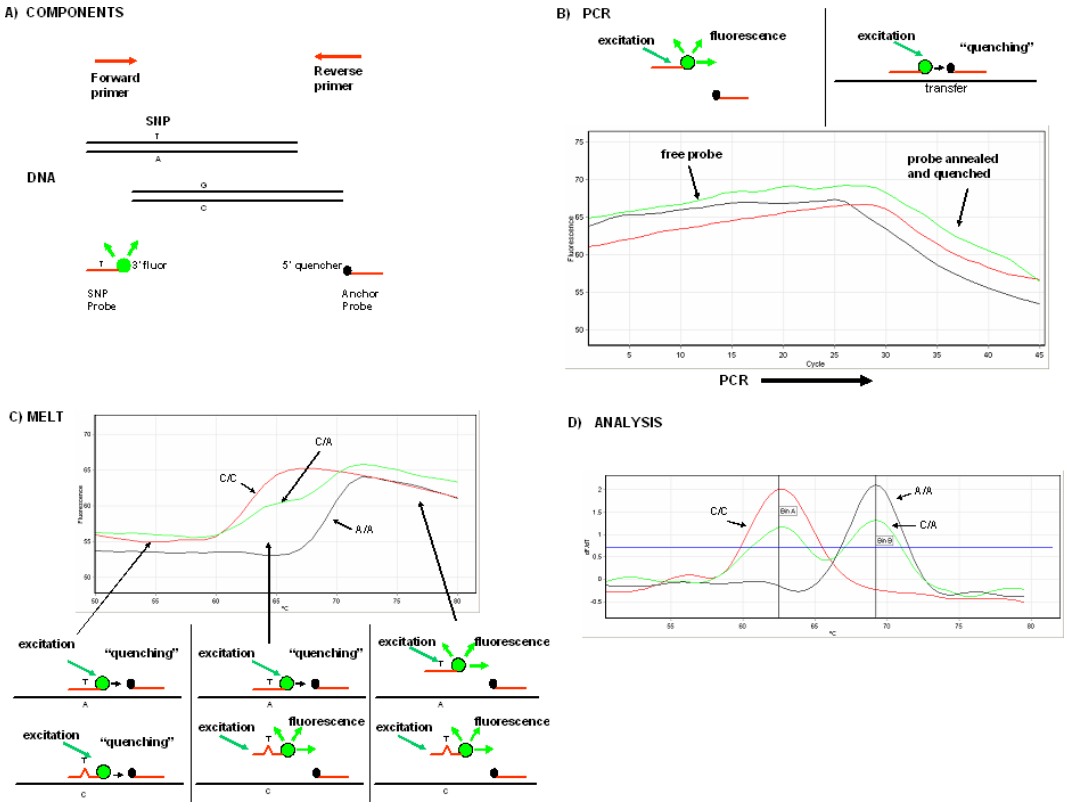

FIG. 1—*The SNP assay. (A) Lists the components used in the assay. i.e., two unlabeled PCR primers, a fluorescently tagged SNP probe complementary to allele A with a 1 bp mismatch to C at the SNP position and an anchor probe which has a 5′ quencher. (B) Demonstrates that as PCR proceeds, additional probes anneal and thus, fluorescence is quenched. (C) Presents the melting phase that takes place from 50°C to 80°C after 45 rounds of PCR are completed. The mismatched SNP probe will melt off first; thus, the DNA from an individual with this allele will regain fluorescence at a lower temperature. The perfect match probe melts off later; thus, this allele will regain fluorescence at a higher temperature. A heterozygote will gain half the fluorescence at the lower temperature and half at the higher temperature. (D) Shows the plot generated by the Corbett software with the change in fluorescence over the change in temperature (dF/dT) plotted versus the temperature. This demonstrates the low (mismatch) and high (perfect match) peaks.*

were a SNP in assay design. The chosen probe contains two differences between the X and Y sequences which leads to greater discrimination in melting temperature (T_m) between the selected sequences.

Primers and probes for the assays were designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA) which has a module for designing FRET primers and probes (see primer/probe sequences in Table 1). For the first several SNPs, the default design parameters were utilized; however, later the probe lengths were shortened from the 35 bp default to 30 bp in an attempt to increase the T_m difference between probes. Also, Beacon Designer 4.0 was often not successful in finding a primer/probe set (~60% of the SNPs yielded sets). In these cases, several parameters individually or in combination were adjusted as follows: the T_m difference between probe and primer was decreased to 6°C (from the recommended 8°C), the primers were made longer (up to 25 bp), or the PCR product size was increased (up to 250 bp).

After making probes to a number of SNPs, it appeared that the SNPs assays worked best with the match of C:G and a mismatch of C:T, C:C, or C:A although some probes with an A:T match and of A:C were used. The C:G SNPs were chosen when possible for the SNP assays.

Primers were purchased from Applied Biosystems and 200 μM stocks in TE buffer (10 mM Tris and 0.1 mm EDTA, pH 7.5) were created. The primers were initially amplified alone and in multiplex to assure that the correct size products were generated. Probes were purchased from Biosearch Technologies (Novato, CA) and kept as 200 μM stocks in TE. Seven different dyes and associated quenchers (in parentheses) were utilized on the probes: FAM (BHQ-1), Biosearch Blue (BHQ-1), CAL Fluor Orange 560 (BHQ-1), CAL Fluor Red 610 (BHQ-2), Quasar 607 (BHQ-2), Quasar 705 (BHQ-2), and CAL Fluor Gold 540 (BHQ-1). Each SNP assay was amplified separately with its probes in the Corbett RG3000 or RG6000 real-time instrument and amplification and melting monitored.

Real-Time Multiplex SNP Assay

Twenty microliter PCR reactions were used in these experiments. These consisted of 10 μL of 2x commercial master mix (see below), the final concentration of primers and probes listed in Table 1, 2 μL of the sample DNA and deionized H_2O to make up the 20 μL. The sample DNAs were not quantitated before use to parallel the generally unknown concentrations experienced with evidentiary samples but generally were in the range of 0.1–10 ng/μL. Individual SNP assays up to 4-plex experiments were performed initially in a Corbett RG3000 (Corbett Robotics)—until a RG6000 (Corbett Robotics) was purchased to perform the 6-plex experiments. Some 4-plex experiments were also performed in the Stratagene MX3005P (La Jolla, CA) to develop a seven SNP plus gender assay as two 4-plexes.

Six commercial master mixes were initially evaluated using a 4-plex assay to determine compatibility with multiplexing (ABgene Absolute QPCR Mix, Rochester, NY; Qiagen QuantiTect Multiplex PCR NoROXaster Mix, Valencia, CA; Sigma JumpStart Taq Ready Mix, St. Louis, MO; Stratagene Brilliant Multiplex QPCR Master Mix; Eurogentec qPCR Mastermix NoROX, San Diego, CA; Roche Fast Start TaqMan™ Master Mix). The Qiagen QuantiTect master mix performed the best, generating sharp peaks in the melting results (data not shown); thus, it was chosen for all future experiments.

Experiments run in the RG3000 were analyzed using the Corbett Robotics Rotorgene software (currently the Rotorgene 6 version 6.0, Build 38 software). Experiments run in the RG6000 were

TABLE 1—*Primers/probes used in this study.*

Locus (chromosome)	Ref SNP ID# (alleles)	Primers (For/Rev is relative to the probes)	Final Concentrations of Primers (nM)	Product Size (bp)	3′ Probe with SNP (has 3′ Fluor) 5′ Probe (has 5′ BHQ)	Final Concentrations of Probes (nM)
FLJ43720 (chr 5)	rs315791 (C/A)	For: TTGTACCAGGGGTGTTTCC	20	141	ACTAATGCATAGGC**C**AGTTTCATCCTTAT	200
		Rev: GGCTAGTGGCTACCAAATTG	200		TGGCAGACAGAAATTAACAAAGGAGCAAATAAAGA	200
A2BP1 (chr 16)	rs7205345 (C/G)	For: CCTTGGGTCATCTCTATCATAG	40	156	TCTGTGTCTGCC**T**CTCACACTAGA	200
		Rev: GAACCAGGAACTCTTCTCACATC	200		TCCACATCCTTAGTGCAGGTGCC	400
THSD2 (chr 6)	rs2503107 (A/C)	For: AATCCAAAAGGAGTGTTGTATC	50	175	TGTTCATATTTTGCTATCTATACCTAACTTTCTCA	200
		Rev: AGTCAGTGTTTCTCATTTGTTC	400		TGCCTAAATGGTTGTGGAGACCTAGCC	400
PALLD (chr 4)	rs6811238 (C/A)	For: ACTGTTCAGGTCCTCAAAGC	40	176	TACTATCATAAC**C**CTTTAAACAAACCTGGC	200
		Rev: ATCCCAGGGAAGGAATAAGTAC	200		AAACACACAGTCTTCTCCTCTCAGTGACT	400
ATP13A4 (chr 3)	rs6444724 (C/T)	For: AAAGGTTAGGGATTGGAATGG	20	180	GAACACTGGTTAC**C**GTGCTAGGTATTTA	200
		Rev: CTATGGTTTCATAAAGGGAAGG	200		ACTTGCTCTCATTTACTACGGGAGTAGGAAG	400
RAB31 (chr 18)	rs9951171 (A/G)	For: ACGGTTCTGTCCTGTAGG	40	184	GCTTTATGG**A**TTGCCCTGCC	200
		Rev: AAAGAAAGAATGAATCAAAGGG	200		AGTGAACAGGTCCCAGCATGAAAGC	400
LY9 (chr 1)	rs560681 (C/T)	For: CACTCTAAAGGGCTCTCACC	40	192	GTGACCTGAGTAAAC**A**GAGATGGAGAGAAA	200
		Rev: CAACCTCACATGCACAGC	200		AACAGATGTTCTCAGAAAGAAACTGGTGGG	400
ZFXY (X and Y)	NA (G/C and T/C)	For: TGTTCTGGCATAGACATTGAGG	25	245	ACTGTGCA**G**TGTGGT**T**AAGAAACTTCT	200
		Rev: CATAACTTTGTTCCTATGACC	400		AGTTACATCTGAGTCCAGCACTTG	400

For, forward; Rev, reverse. Nucleotides in boldface refers to SNP.

performed and analyzed using the Corbett Robotics Rotorgene software (currently the RG6000 Series software, Version 1.7, Build 61). Experiments performed in the MX3005P were run using the MxPro software Version 3.00, Build 311. Results were also analyzed using the Plexor Desktop Analysis software v1.1.4 from Promega as the MxPro software does not have the capability of analyzing quenching curves. The Plexor technology is also a quenching system and in order for this technology to be used on all real-time instruments, Promega has developed the Plexor software to import data from many instruments including the MX3005P.

Polymerase chain reaction conditions for the RG3000/RG6000 were: 95°C 15 min then 45 cycles of 94°C 15 sec, 56°C 30 sec, and 72°C 30 sec (fluorescence was read during the 56°C step) then one cycle of 94°C 15 sec, 40°C 60 sec, and 50°C for 60 sec. The melting phase was carried out from 50°C to 80°C rising by 1°C/step, waiting for 5 sec for each step. These conditions were obtained from the Corbett Research application note "Mutation detection using FRET analysis," version 2.8 (T. Deppe, personal communication). PCR conditions for the MX3005P were 95°C 15 min then 45 cycles of 94°C 30 sec, 56°C 30 sec, and 72°C 15 sec (fluorescence was read during the 56°C step) then one cycle of 94°C 30 sec, 40°C 60 sec, and 50°C for 60 sec. The only change from the Corbett settings was an increase of 15 sec for the denaturation step. The melt was carried out over a default ramp from 50°C to 80°C.

For the RG6000, FAM was read in the Green channel (Gain 5.67), CAL Fluor Orange 560 was read in the Yellow channel (Gain 8.0), CAL Fluor Red 610 was read in the Orange channel (Gain 8.67), Quasar 670 was read in the Red channel (Gain 8.0), Quasar 705 was read in the Crimson channel (Gain 5.33), and Biosearch Blue was read in the Blue channel (Gain 1.0). The dyes, channels, and gains were the same for the RG3000, except that it does not have the Crimson and Blue channels. For the MX3005P, FAM was read in the FAM channel (Gain x2), CAL Fluor Orange 560 was read in the Cy3 channel (Gain x2) (it can also be read in the HEX channel [Gain x2] but gives higher relative fluorescence units (RFUs) in the Cy3 channel), CAL Fluor Red 610 was read in the ROX channel (Gain x1), and Quasar 607 was read in the Cy5 channel (Gain x1). It is very important to set the gain correctly because if the gain is too high, the RFUs will be over 100% (at saturation) and the drop in fluorescence will be undetectable while if it is set too low, it will be difficult to see the data above background.

Once the assays were developed, they were tested with several parameters including with and without the addition of bovine serum albumin (BSA) to a final BSA concentration of 160 μg/mL and in 10 μL versus 20 μL volumes on the RG6000 to determine instrument sensitivity. The 10 μL reaction was exactly half the 20 μL reaction (5 μL of 2x master mix, half the primers of the 20 μL reaction [still the same final concentrations], 1 μL of input DNA and H_2O to 10 μL). The 20 μL reaction was used for all other experiments in the RG6000. All assays in the MX3005P in the 96-well plates were performed in 20 μL volume as signal intensity was not sufficient with 10 μL samples. In addition, selected samples or the standard DNAs were serially diluted to test both dropout of alleles at low concentration (to determine a minimal input DNA) and the potential of the assay for human DNA quantitation.

Real-Time Quantitation Assay

For comparative purposes, a subset of the samples analyzed with the multiplex SNP assay were also quantitated using the TaqMan™ duplex (*Alu* plus gender) assay as described in Nicklas and Buel (35) with the exception that it was run in the RG6000 in a 10 μL volume with only the *Alu* primers and probe.

Sequencing

The SNP genotypes of the 10 test individuals were confirmed by sequencing of the PCR products. PCR products were treated with ExoSAP-IT (USB Scientific, Cleveland, OH) according to the manufacturer's directions. Sequencing was performed by the Vermont Cancer Center DNA Analysis Facility on an Applied Biosystems 3130XL. Electropherograms were viewed and printed using Chromas Lite freeware (Technelysium Pvt. Ltd., Tewantin, Qld, Australia, http://www.technelysium.com.au/chromas_lite.html).

Results

In initial testing, some SNPs did not work well as there was little discrimination in melting temperature between the alleles. Occasionally this could be remedied by making new probes to the other allele. These switched probes often had very different melting characteristics than the original set. Probes with G:T mismatches were always avoided. In total, the 73 low Fst SNPs in ALFRED were reviewed to select 27 $p = q = 0.5$ SNPs. Beacon Designer found probe/primer sets for ~16 of these. Probes and primers were actually made for 12 of these to select the 7 SNPs in Table 1. For the gender SD, two different positions in the ZFXY genes were selected with one additional swap of strand to obtain the chosen probe/primer set. Primer and probe concentrations were optimized for each assay (see Table 1) based on findings made by Szilvási et al. (24) which indicated that the melting SNP assay is very sensitive to the ratio of forward to reverse primer and that optimal concentrations need to be in the range of 20–60 and 200–400 nM, respectively. Table 2 gives the genotype results for all 10 individuals for the seven SNPs and one SD developed in this study. In

TABLE 2—*Genotype results for the 10 test individuals.*

Sample	ZFXY	THSD2*	FLJ43720*	RAB31*	LY9*	ATP13A4*	PALLD*	A2BP1*
Individual #1	Female	CC	CA	AA	TT	CC	AC	GC
Individual #2	Female	AA	AA	GG	CT	CC	AA	CC
Individual #3	Male	CA	CA	GG	TT	TT	AA	CC
Individual #4	Male	CA	CA	GG	CT	CC	AC	GG
Individual #5	Male	AA	CC	GA	TT	CC	AC	GC
Individual #6 (9947)	Female	CC	CC	AA	CT	CC	AA	CC
Individual #7	Female	AA	AA	AA	TT	CT	CC	GC
Individual #8	Male	CA	AA	AA	CC	CC	AA	CC
Individual #9	Male	AA	CA	GG	CT	TT	AA	GC
Individual #10	Male	CC	CA	AA	CT	CC	AA	CC

*Genotype results from SNP assay and sequencing data.

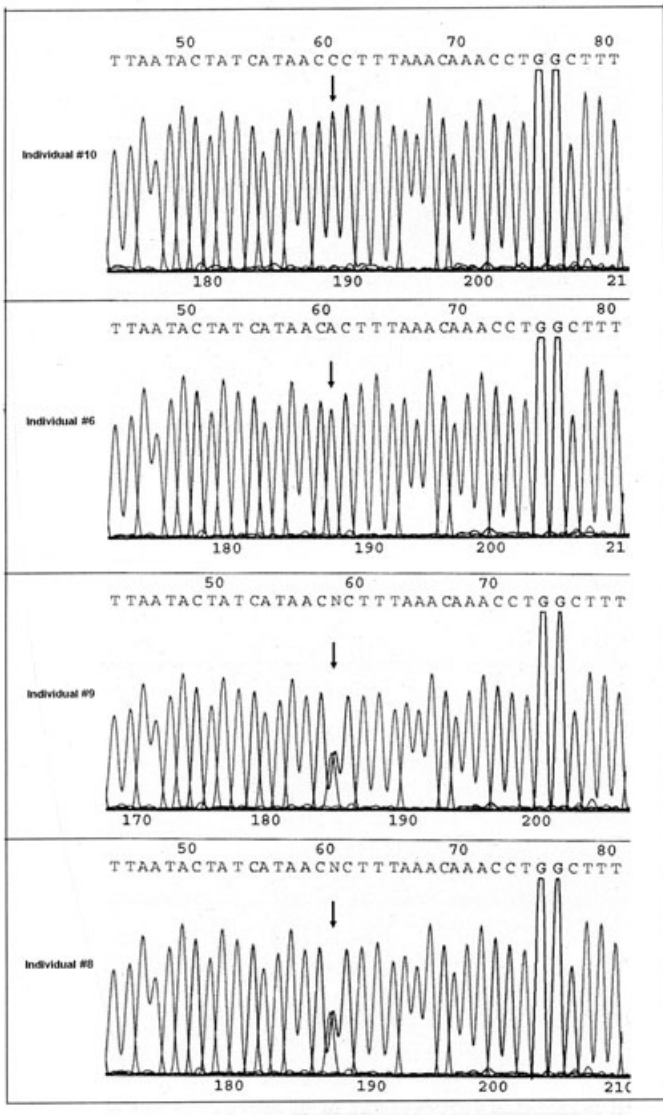

FIG. 2—*Sequence analysis of the PCR product for the PALLD SNP for four individuals (one CC homozygote, one AA homozygote, and two heterozygotes). The arrows highlight the SNP position.*

order to confirm the genotypes observed in the SNP assays, DNA sequencing was performed for all 10 individuals at the seven SNP loci. Figure 2 shows an example of sequencing results for the PALLD SNP for four individuals. There was complete concordance in genotyping results with the two methods. The results for the ZFXY locus confirmed the identified gender for the 10 individuals.

Once the SNPs were tested individually, the primer ratios optimized, and the assay appeared robust, several multiplex assays were developed (Table 3). These were: two 6-plexes for the RG6000, one including five SNPs and the SD gender marker (6-plex A) and the other with six SNPs, i.e., an autosomal SNP replacing the SD (6-plex B) and a two well (4-plex #1 and #2) seven total SNP plus gender assay for four/five color real-time instruments.

Figure 3 shows melting curves of five individuals (picked from the 10 test individuals to give an example of both homozygotes and a heterozygote at each SNP and include both male and female) with 6-plex A on the RG6000. Data was interpreted using the Corbett software (plot graphs the change in fluorescence vs. temperature [d*F*/d*T*]). The Corbett software automatically calls the genotypes once the bins are assigned and the genotypes are defined based on the bins. Both types of homozygotes and the heterozygotes are clearly differentiated. Results using the 4-plex #1 on the Stratagene MX3005P for three of the four individuals represented in Fig. 3 are shown in Fig. 4. Results are interpreted manually on the Stratagene MxPro software. Data for the other assays are not shown.

The 6-plex A results with and without the addition of BSA (160 μg/mL) were essentially identical except that the background for the FAM/Green channel was higher with BSA (data not shown). Thus, BSA can be added to the assay if needed to reduce or counteract the adverse effect of potential PCR inhibitors. To evaluate instrument performance, the 6-plex A assay was tested on eight samples at both 20 and 10 μL volumes in the RG6000 in the 0.1 mL tubes; both results were generally comparable although some heterozygotes were a bit more difficult to detect at 10 μL (data not shown). Obviously at lower concentrations, the 20 μL reaction will fare better as it has twice as much input DNA (2 μL of DNA vs. the 1 μL input of the same DNA in the 10 μL reaction).

Additional experiments were performed to determine the sensitivity of the 6-plex A assay. Four single source samples (individuals #3, #5, #6, and #7) were diluted to ~72, 18, and 4.5 pg total input DNA in a 20 μL reaction (based on quantitation performed using a TaqMan™ duplex [*Alu* plus gender] assay [35]) and eight replicates of the 6-plex A assay performed. Four samples were required to guarantee at least one heterozygote at each locus (Table 4). The homozygote assay started to fail at 18 pg (10% failed) and failed 46% of the time at 4.5 pg. Both heterozygote alleles were observed ~67% of the time at 18 pg while at 4.5 pg both alleles were observed only 9.5% of the time.

Of interest, the assays also appear able to quantitate the amount of DNA present when run with a standard curve. While the melting curve determines genotype, the real-time amplification can be used to determine the amount of DNA. Although for this technique, the curves are inverted (because fluorescence decreases during

TABLE 3—*Description of loci and dyes used in each multiplex assay.*

SNP/SD	6-plex A	6-plex B	4-plex #1	4-plex #2
A2BP1				Quasar 670
ATP13A4	CAL Fluor Red 610	CAL Fluor Red 610	CAL Fluor Red 610	
FLJ43720	CAL Fluor Orange 560	CAL Fluor Orange 560	CAL Fluor Orange 560	
LY9	Biosearch Blue	Biosearch Blue		CAL Fluor Orange 560
PALLD		FAM		FAM
RAB31	Quasar 705	Quasar 705		CAL Fluor Red 610
THSD2	Quasar 670	Quasar 670	Quasar 670	
ZFXY	FAM		FAM	

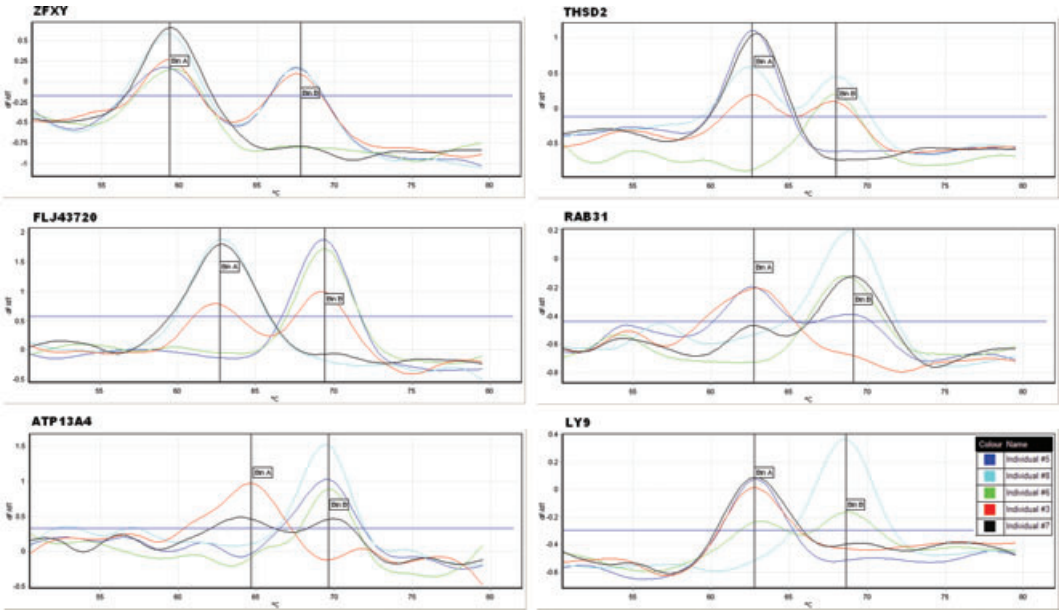

FIG. 3—*Analyzed melting results (plot of the change in fluorescence versus the change in temperature [dF/dT] for the 6-plex A assay for five individuals (#3 [red], #5 [blue], #6 [green], #7 [black], and #8 [turquoise]) generated on the Corbett RG6000 (at least one of each homozygote and a heterozygote are represented).*

amplification), the Cts are still proportional to the amount of DNA present. Figure 5 shows the analyzed data (inverted curves shown) for a set of standard curve samples (concentration of the 2 μL of input DNA of 64–0.0039 ng/μL) of the RAB31 locus of a 6-plex A assay performed on the RG6000 analyzed by the Corbett software. Table 5 lists the R^2 values, reaction efficiencies, and the calculated concentration for a known sample for each locus of the 6-plex A assay. The R^2 values are all close to 1.0 and the reaction efficiencies are all very close to the expected 100%. For comparison, the last column of the table gives the concentration of each

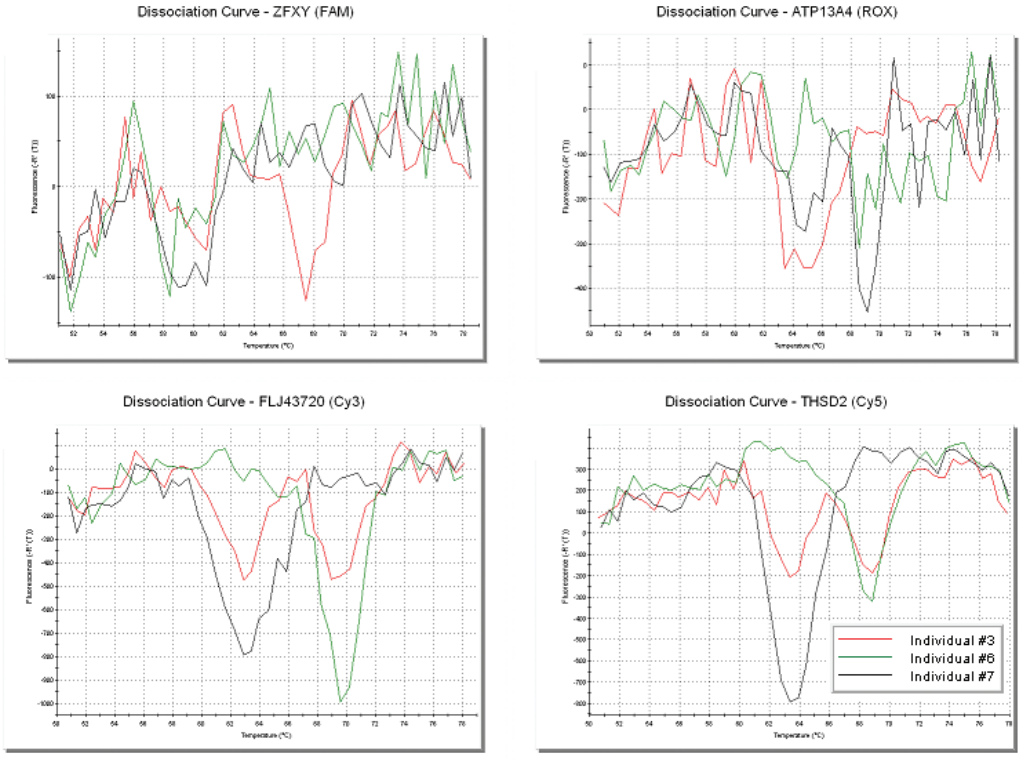

FIG. 4—*Analyzed melting results for the 4-plex assay #1 with gender for three individuals (#3 [red], #6 [green], and #7 [black]) on the Stratagene MX3005P (one of each homozygote and a heterozygote are represented). The results are not as smooth as with the Rotorgene RG6000 but, with practice, genotype calls can be made manually. Using slower melting conditions can reduce some of the noise.*

TABLE 4—*6-plex A assay results using samples diluted to low concentrations*.*

	# Observed	% Observed (%)
Homozygote: 72 pg		
One allele present	102/102	100
No alleles present	0/102	0
Homozygote: 18 pg		
One allele present	92/102	90
No alleles present	10/102	10
Homozygote: 4.5 pg		
One allele present	55/102	54
No alleles present	47/102	46
Heterozygote: 72 pg		
Both alleles present	37/42	88.1
One allele present	5/42	11.9
No alleles present	0/42	0.0
Heterozygote: 18 pg		
Both alleles present	28/42	66.7
One allele present	12/42	28.6
No alleles present	2/42	4.8
Heterozygote: 4.5 pg		
Both alleles present	4/42	9.5
One allele present	20/42	47.6
No alleles present	18/42	42.9

*This data combines the results for all four individuals at all six loci using six replicates ($4 \times 6 \times 8 = 144$ total tests). Based on the genotypes of the tested individuals, 102 of these tests should give a determination of homozygote and 42 should give a determination of heterozygote. For a homozygote, only two possible assay results are possible; either one allele or no alleles (failure) are observed while for a heterozygote, three assay results could be obtained: both alleles, one allele (dropout), or no alleles (failure). Column 2 gives the observed frequencies of the different occurrences for homozygote or heterozygote at three different DNA inputs.

sample as determined by a TaqMan™ *Alu* quantitation method published previously (35). While the SNP assay is not as sensitive as *Alu*-based quantitation methods (single copy detection vs. multiple-copy marker detection), it still provides a good indication of the DNA concentration of a sample. Values measured by the two assays did vary by up to 2.5 fold (sample #1); however, most values were similar and sufficiently close to allow a dilution which would generate an acceptable DNA concentration within the input range for STR typings (note: for studies performed in the MX3005P, quantitation of DNA concentration requires download of the results into the Plexor [Promega] software which can handle the inverse curves).

Discussion

These multiplex FRET assays using quenching probes can determine the genotype of an individual for five to seven SNPs plus gender determination in a fast (2 h), accurate manner using a real-time PCR instrument. The 6-plex A assay was sensitive, giving correct typings at comparable template amounts to that required for STR analysis using standard typing kits. The assay did fail to give the correct genotype for heterozygotes at low template amounts, detecting only one or no alleles. One allele still may be helpful as a screening procedure where the data is meant only to sort between a limited number of individuals. Using a single tube or well test (or two wells for the double 4-plex), the assays are effective down to ~50 pg of DNA.

Theoretically, using five SNPs (with $p = q = 0.5$) plus gender, two samples coming from two different random individuals will only be identical 0.37% of the time while using seven SNPs plus gender this will only occur 0.05% of the time (1 in 1,900). The percent identical matches for this assay will, of course, vary depending on racial group and this is only an approximation of what might be expected. These percentages are sufficient for an initial forensic screening technique and certainly compare well with not so ancient techniques. This assay could also be used in a medical setting where questions occasionally arise as to the possibility of a tissue swap or contamination.

There could be concerns that the assays may have limitations when relatives are involved. If the relatives are of different genders, then this will be immediately detected by the gender SD so the concern would be same-sex pairs. If one considers two same-sex siblings for a single SNP with $p = q = 0.5$, the probability of being identical at one locus is 19/32 and being non-identical is 13/32 so for a 4-plex assay with three SNPs and the gender assay, the probability of being identical is $(19/32)^3$ or 21%, high but still not identical >75% of the time (Appendix B). For the seven SNP plus gender with seven SNPs, this falls to only 2.6% or over 97% non-identical. In the case of a same gender, parent–child pair, the chance of being identical for a single SNP is 1/2, for three SNPs this falls to 1/8 (12.5%), and for seven SNPs to 1/128 or 0.78% (Appendix C). While obviously not as robust as for unrelated individuals, this assay still will exclude relatives at a high rate.

This assay is not really designed for use with mixtures as with only two alleles at every locus, mixtures would be very hard to detect especially if the inputs were unequal. A mixture could give a different profile than either sample alone assuming near equal input from the two input DNAs, except in the case where both individuals typed identically (same probability as above). If the question was whether two samples were the same (not to be STR tested) or different (need testing), the result would be to test the sample and the mixture would become evident on STR analysis.

This SNP assay is the first report using six SNPs in one multiplex assay in a real-time instrument. Other real-time methodologies such as TaqMan™ can perform at most three assays in a multiplex

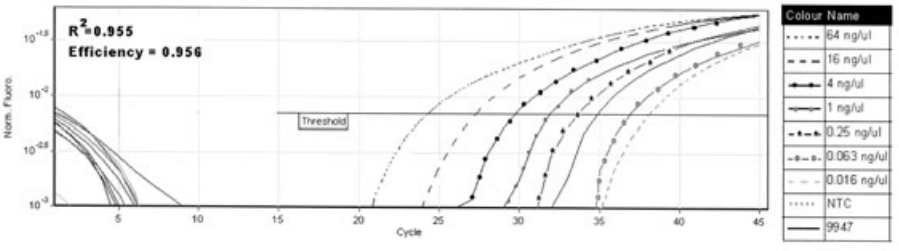

FIG. 5—*Real-time amplification (quantitation) results using a standard curve and GM9947A on the RG6000. Results for RAB31 are shown. The expected and observed concentrations for GM9947A are 0.1 and 0.088 ng/μL, respectively.*

TABLE 5—*Quantitation results for 6-plex A assay in RG6000.*

Sample	ZFXY	ATP13A4	THSD2	FLJ43720	Ly9	RAB31	Mean
R^2	0.986	0.977	0.998	0.989	0.974	0.976	0.983
Efficiency (%)	97.7	109.2	98.6	104.0	95.2	107.2	102.0

Individual	ZFXY	ATP13A4	THSD2	FLJ43720	Ly9	RAB31	Mean ± SD	TaqMan™ *Alu* assay
#2	0.0014	1.16	1.06	0.98	1.71	0.64	0.93 ± 0.52	2.48
#3	0.68	0.88	0.99	0.85	0.79	1.07	0.88 ± 0.13	2.08
#4	0.12	0.12	0.19	0.18	0.12	0.13	0.14 ± 0.03	0.34
#5	5.37	5.33	3.95	5.63	20.22	7.12	7.94 ± 5.57	5.89
#6	–	0.05	0.06	0.05	0.13	0.11	0.08 ± 0.03	0.11
#7	0.01	1.79	1.33	1.60	1.62	2.92	1.55 ± 0.85	1.67
#8	33.60	45.21	88.11	89.22	76.96	144.45	79.6 ± 35.8	94.8
#9	32.07	42.55	31.09	47.70	65.83	26.47	40.9 ± 13.2	38.2

because of the limit of using two colors (dye channels) per SNP-one color for each allele. The melting method allows use of only one dye (channel) per SNP. While a FRET assay using excitation of one dye and emission of the second (as opposed to the assay used here which uses the excitation of one dye and quenching by the other), could theoretically be multiplexed, it was thought that cross-talk of multiple dyes might become a problem and that the quenching technique should be cleaner. Although assays that generate fluorescence are typically considered more sensitive than those that monitor its decrease, the work reported here shows that the assay works at template concentrations similar to other forensic DNA assays and the quenching approach apparently does not limit the usefulness of the assay.

In addition to sample identification, the assay could be used simultaneously as a DNA quantitation measure if a standard curve is processed at the same time. A good estimate of sample concentration could be made by taking a mean of the results for the six loci. Running a standard curve using a mixed sample also gives melt curves with both alleles at various concentrations, making interpretation of sample results easier.

Additional SNPs could be added to the assays by duplexing two SNPs in one color (15,18,28,29). This requires picking SNPs where the melting peaks of the two perfect matches and two mismatches are all distinct. Alternatively, two tightly linked SNPs under a single probe (preferably not in significant linkage disequilibrium) can give four different melting peaks for the four haplotypes (14). Pont-Kingdon and Lyon (28,29) have also described spanning probes which span two somewhat distant SNPs by looping out the DNA in between.

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Additional information and reprint requests:
Eric Buel, Ph.D.
Vermont Forensic Laboratory
103 S. Main St
Waterbury, VT 05671
E-mail: ebuel@dps.state.vt.us

Appendix

A SNP Identity Percentage Calculation

Assume $p = q = 0.5$ for alleles A and B comprising a SNP.
Genotypes are: AA (freq 0.25), AB (freq 0.50), and BB (freq 0.25) by Hardy–Weinberg.
Determine frequency of two people being identical: AA + AA (freq = $0.25 \times 0.25 = 0.0625$) plus AB + AB (freq = $0.5 \times 0.5 = 0.25$) plus BB + BB (freq = $0.25 \times 0.25 = 0.0625$). Total = 0.375.
For n independent SNPs = $(0.375)^n$; in particular for four SNPs = $(0.375)^4 = 0.01978$ or $\sim$2% or for 8 SNPs = $(0.375)^8 = 0.00039$ or 0.04% or $\sim$1 in 2500.

B Sharing of SNP Genotypes by Same Gender Siblings

One has to consider all the possible parental pairs and the children they produce. Consider a SNP with two alleles a and b, each with frequency 0.5.

Parents	Freq of Mating	Child Genotypes	Freq of Two Identical Siblings
AA × AA	0.25 × 0.25	AA	1
BB × BB	0.25 × 0.25	BB	1
AB × AA	0.5 × 0.25	AB, AA	0.5
AA × AB	0.25 × 0.5	AA, AB	0.5
AB × BB	0.5 × 0.25	AB, BB	0.5
BB × AB	0.25 × 0.5	BB, AB	0.5
AA × BB	0.25 × 0.25	AB	1
BB × AA	0.25 × 0.25	AB	1
AB × AB	0.5 × 0.5	AA, 2AB, BB	0.375

Sum of identical sibs = Σ(freq mating) × (freq of ID sibs) = 19/32

C Sharing of SNP Genotypes by Same Gender Parent and Child

Again one has to consider all the possible parental pairs and the children they produce. Consider a SNP with two alleles a and b, each with frequency 0.5.

Parents	Freq of Mating	Child Genotypes	Freq of Child and First Parent Identical
AA × AA	0.25 × 0.25	AA	1
BB × BB	0.25 × 0.25	BB	1
AB × AA	0.5 × 0.25	AB, AA	0.5
AA × AB	0.25 × 0.5	AA, AB	0.5
AB × BB	0.5 × 0.25	AB, BB	0.5
BB × AB	0.25 × 0.5	BB, AB	0.5
AA × BB	0.25 × 0.25	AB	0
BB × AA	0.25 × 0.25	AB	0
AB × AB	0.5 × 0.5	AA, 2AB, BB	0.5

Sum of identical parent child combos = Σ(freq mating) × (freq of ID parent + child) = 0.5