Int J Legal Med (2004) 118 : 147–157
DOI 10.1007/s00414-004-0428-5

ORIGINAL ARTICLE

Peter M. Vallone · Rebecca S. Just · Michael D. Coble
John M. Butler · Thomas J. Parsons

A multiplex allele-specific primer extension assay for forensically informative SNPs distributed throughout the mitochondrial genome

Received: 25 August 2003 / Accepted: 6 January 2004 / Published online: 4 February 2004

Abstract The typing of single nucleotide polymorphisms (SNPs) located throughout the mitochondrial genome (mtGenome) can help resolve individuals with an identical HV1/HV2 mitotype. A set of 11 SNPs selected for distinguishing individuals of the most common Caucasian HV1/HV2 mitotype were incorporated in an allele specific primer extension assay. The assay was optimized for multiplex detection of SNPs at positions 3010, 4793, 10211, 5004, 7028, 7202, 16519, 12858, 4580, 477 and 14470 in the mtGenome. Primers were designed to allow for simultaneous PCR amplification of 11 unique regions in the mtGenome and subsequent primer extension. By enzymatically incorporating fluorescently labeled dideoxynucleotides (ddNTPs) onto the 3' end of the extension primer, detection can be accomplished with a capillary-based electrophoresis (CE) platform common in most forensic laboratories. The electrophoretic mobility for the extension primers was compared in denaturing POP4 and POP6 CE running buffers. Empirical adjustment of extension primer concentrations resulted in even signal intensity for the 11 loci probed. We demonstrate that the assay performs well for heteroplasmy and mixture detection, and for typical mtDNA casework samples with highly degraded DNA.

Keywords Mitochondrial DNA · Capillary electrophoresis · Allele-specific primer extension · Single nucleotide polymorphism · Multiplex PCR

Introduction

The increasing use of mitochondrial DNA (mtDNA) in forensic science is based on several well recognized advantages in comparison to nuclear DNA testing in cases of extreme sample degradation, shed hairs, or where comparisons between maternal relatives are necessary (a general review of mtDNA forensic testing is given by Holland and Parsons [1]). The human mitochondrial genome (mtGenome) contains approximately 16,569 base pairs (bps) of circular DNA, roughly 100,000 times less than is contained in nuclear DNA. This, plus the fact that mtDNA must be treated as a single locus (due to a lack of recombination), causes mtDNA testing to have much less power of discrimination than nuclear DNA. However, forensic mtDNA testing targets only a small fraction of the variation present in the mtGenome, and is usually restricted to ~610 base pairs encompassing hypervariable regions I and II (HV1/HV2) of the non-coding control region (CR).

Coble et al. have addressed the limitations of mtDNA's power of discrimination by identifying single nucleotide polymorphisms (SNPs) from the entire mtGenome that provide maximal discrimination among individuals who would otherwise match in HV1/HV2 [2]. These SNPs were identified by sequencing the entire mtGenome of 241 individuals matching the most common HV1/HV2 types in the population derived from West European Cau-

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Sources of support. The National Institute of Justice (NIJ) funded the work described here through an interagency agreement with the NIST Office of Law Enforcement Standards. This work was also supported by the National Institute of Justice grant 2000-1J-CX-K010 to T.J.P.

P. M. Vallone (✉) · J. M. Butler
Biotechnology Division,
National Institute of Standards and Technology,
100 Bureau Drive, Mail Stop 8311,
Gaithersburg, MD20899, USA
Tel.: +1-301-9754872, Fax: +1-301-9758505,
e-mail: peter.vallone@nist.gov

R. S. Just · M. D. Coble · T. J. Parsons
Armed Forces DNA Identification Laboratory,
Rockville, MD, USA

casians. From the large amount of variation thus detected, panels of selected SNP sites were proposed for application to specific common HV1/HV2 sequences to improve forensic discrimination. The targeted common HV1/HV2 types comprise ~21% of the West European Caucasian population, and the most common HV1/HV2 type alone accounts for ~7% of the population [2]. Therefore, development of efficient SNP assay protocols for these panels of discriminatory sites would find substantial practical application in improving the discrimination of mtDNA testing.

Due to the nature of mtDNA variation, existing primarily as point mutational polymorphisms, the mtDNA system is highly amenable to SNP assays [3, 4]. This is particularly true in the coding region of mitochondrial DNA, where polymorphisms are widely spaced. Such dispersal makes sequencing less practical (especially for degraded samples), and sequencing within the coding region would have the undesirable potential to reveal medically significant mutations. Currently, a variety of assays and technologies exist for the detection of SNPs. Specific to mtDNA, a linear hybridization array is currently available for a series of informative SNPs within HV1/HV2 [5]. Among the many other potential options available are: flow cytometry with bead-based hybridization arrays (Luminex), 5' nuclease (Taq Man), primer extension with MS detection (Mass Array Sequenom), and short fragment sequencing with pyrophosphate monitoring (Pyrosequencing) [6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16].

In choosing among various SNP assays, it is critical to keep in mind the practical necessities in forensic typing of mtDNA. Foremost, mtDNA testing is typically applied to highly degraded or trace samples, so an applicable assay must be able to deal with very limited extract quantities. This calls for extremely high sensitivity, short amplicon length, and the ability to multiplex. The latter reduces the number of separate amplifications that must be performed, and therefore the volume of DNA extract required. Secondly, the assay must be reliable for indicating the presence of mixture due to contamination or heteroplasmy. Lastly, the mtDNA genome is densely populated with genes critical to cellular energy production, and mutations in the coding region that alter amino acid composition of proteins or secondary structure of transfer and ribosomal RNAs have the potential for direct deleterious effect. For this reason, forensically relevant SNPs in the coding region should be chosen to involve neutral variants. We feel that SNP assays like pyrosequencing that reveal a substantial amount of surrounding sequence variation, or the simple sequencing of coding region genes [17], will not prove wise practical choices for reasons of medical genetic privacy and ethics.

We have selected a primer extension assay utilizing fluorescently labeled dideoxynucleotides (ddNTPs) to test its performance in a multiplex format as a practical forensic mtDNA typing system. In this assay, fluorescently labeled extension primers are separated and detected on a capillary electrophoresis (CE) instrument. In forensic laboratories electrophoresis is commonly employed for analyzing fluorescently labeled PCR products containing short tandem repeats (STRs) for human identification [18]. Having a detection instrument in common with STRs is an added advantage of this primer extension approach. We used the commercial reagent kit SNaPshot that is available for multiplex detection of SNPs. The SNaPshot kit contains the reagents required for fluorescent labeling of SNP primers, fluorescently labeled ddNTPs, buffer, and polymerase. The researcher is responsible for the design of PCR primers (i.e. for the generation of PCR amplicons) and the primer extension or "SNP primers" that interrogate the SNP sites. The PCR amplicons required by the primer extension assay can be pooled from singleplex PCR or generated using carefully designed primers in multiplex PCR. The identity of the base sequence at the SNP site directs the incorporation of the complementary ddNTP as in ordinary sequencing. However, the output of the SNaPshot typing bears greater visual and interpretational similarity to STR typing; a series of discretely spaced, colored peaks that indicate the polymorphic variant present at a series of sites. This assay is similar to a "minisequencing" assay developed previously for the mtDNA control region [19].

We report here development of a multiplex SNaPshot assay that targets the most useful of the discriminatory SNP panels identified by Coble et al. [2]. This panel ("A") targets 11 sites, 10 from the mtDNA coding region and 1 from the control region outside of HV1/HV2, that greatly increase the ability to distinguish among individuals matching the most common Caucasian HV1/HV2 type.

Materials and Methods

Mitochondrial SNP sites

The location and sequence variation for each SNP in panel A is listed in Table 1. The numbering of site position throughout this paper follows the revised Cambridge Reference Sequence (rCRS) [20].

PCR primer design

The 11 PCR primer pairs were selected using the publicly available web-based primer selection software Primer3 [21]. A template sequence (a subset of the rCRS) consisting of 150 base pairs

Table 1 The 11 mtSNP sites for separating the most common Caucasian HV1/HV2 type

Position	Sequence variation
477	T/C
3010	G/A
4580	G/A
4793	A/G
5004	T/C
7028	C/T
7202	A/G
10211	C/T
12858	C/T
14470	T/A
16519	T/C

The primary sequence variants are listed, with the rCRS variant listed first.
Site 14470 has been observed to also have a C variant.

upstream and downstream from a SNP site was input into the Primer3 program. The regions 30 bases upstream and downstream from the SNP site were excluded from being selected as PCR primer binding sites. The size of each amplicon was kept under 200 base pairs to increase success when typing degraded samples. Primer selections parameters (t_m, length, %GC, etc) were left on the Primer3 default settings. Each primer pair was selected independently (i.e. singleplex primer design). The final set of 22 PCR primers were screened for potential secondary structures such as primer-dimer and hairpin interactions. The screening was performed using previously described algorithms [22, 23].

Extension primer design

The 11 extension primers were designed using an in-house algorithm implemented in Visual Basic 6.0. The user input consists of template sequences containing the SNP site(s). Design parameter variables consist of the desired length and predicted t_m of an extension primer. Unless stated otherwise all t_m and thermodynamic (ΔG) values were calculated using nearest-neighbor thermodynamic parameters [24, 25]. Primer sequences upstream and downstream adjacent to the SNP site were selected that have the appropriate length and t_m characteristics. Extension primers were selected that had a predicted t_m of approximately 60°C. Extension primers were screened for hairpin and primer-dimer interactions as described for the multiplex PCR primers. Extension primers were screened versus the non-template PCR amplicons to avoid possible non-specific hybridization.

Oligonucleotide synthesis and purity

Oligonucleotides were purchased from Qiagen Operon (Alameda, CA). Oligonucleotides were delivered lyophilized and desalted. Stock solutions of 100 µM were prepared by adding the appropriate volumes of deionized water. The integrity of each oligonucleotide was confirmed by mass spectrometry [26].

Quantitation of oligonucleotides

The final concentration of each extension primer was determined using UV absorption measurements performed at 260 nm and extinction coefficients derived from nearest-neighbor values [27]. When comparing the measured concentration values to the vendor estimates, the average deviation was approximately 20% and in one case as high as 40%. The discrepancy in concentrations values suggests the need to experimentally confirm concentration readings especially when reporting concentrations for "balanced" multiplex assays PCR, extension or otherwise.

DNA samples and quantitation

Pristine DNA samples were extracted as in Coble et al. [2]. DNA sample quantity in the multiplex reactions ranged from 0.2 pg to ~1 ng total human genomic DNA. Sensitivity experiments were performed on serial dilutions of DNA quantified by spectrophometry at 260 nm. For mixture experiments, genomic DNA concentrations were verified by real-time PCR on an Applied Biosystems 7700 instrument (Foster City, CA) using the TaqMan assay with amplification primers and a probe specific to human Alu sequences (A. McClure, T.J. Parsons, unpublished results). Eight non-probative mtDNA casework extracts were obtained from the Armed Forces DNA Identification Laboratory (AFDIL) mtDNA section. These extracts were from degraded skeletal remains with a minimum postmortem interval of 25 years; the skeletal remains were extracted as in Gabriel et al. [28].

Multiplex PCR conditions

For testing with pristine genomic DNA, multiplex amplification of the 11 unique amplicons was carried out in a total volume of 15 µl. The reaction volume was increased to 25 µl for some trials with degraded casework samples.

Standard final PCR reagent concentrations were: 1 U of AmpliTaqGold DNA polymerase (Applied Biosystems, Foster City, CA), 1× Taq Gold PCR buffer, 250 µM dNTPs (Promega, Madison, WI), 5 mM $MgCl_2$, 0.16 mg/ml bovine serum albumin (BSA) fraction V (Sigma, St. Louis, MO), 0.5 µM of each amplification primer pair (22 total primers). The amount of AmpliTaqGold DNA polymerase was varied in trials with degraded casework samples. Thermal cycling for PCR and SNaPshot assays was carried out using the GeneAmp 9700 (Applied Biosystems) running in 9600-emulation mode (i.e. ramp speeds of 1°C/s).

Multiplex PCR thermal cycling conditions

The conditions for multiplex PCR were as follows: 95°C for 10 min, 3 cycles of 95°C for 30 s, 50°C for 55 s, 72°C for 30 s then 19 cycles of 95°C for 30 s, 50°C for 55 s and +0.2°C per cycle, 72°C for 30 s, 11 cycles of 95°C for 30 s, 55°C for 55 s, 72°C for 30 s, 72°C for 7 min and storage at 4°C.

PCR reaction clean-up

Following PCR thermal cycling, unincorporated primers and dNTPs were removed by adding 6 µl of ExoSAP-IT (USB, Cleveland, OH) to each 15 µl PCR reaction. Reactions were mixed briefly and incubated at 37°C for 90 min then 80°C for 20 min. An extended incubation at 37°C was required to ensure digestion of all unincorporated PCR primers.

Primer extension reaction conditions

Multiplex primer extension reactions were carried out in a total volume of 10 µl. Reaction components were: 2.5 µl of ABI Prism SNaPshot multiplex kit mix (Applied Biosystems), 0.5 µl of 10× AmpliTaqGold PCR buffer, 3 µl of multiplex PCR products, 3 µl of deionized water, and 1 µl of a stock solution of extension primers (an unbalanced stock solution contains 10 µM of each extension primer). Thermal cycling conditions for extension reactions were carried out as described in the SNaPshot multiplex kit manual: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s.

Unincorporated ddNTP removal

Excess fluorescently labeled ddNTPs were inactivated by addition of 1 unit of shrimp alkaline phosphatase (SAP). Reactions were mixed briefly and incubated at 37°C for 30 min then 80°C for 20 min.

Analysis on ABI 3100 (16 capillary) Genetic Analyzer

The ABI PRISM 3100Genetic Analyzer was used with filter set E5 in order to process the data from the 5 dyes dR110, dR6G, dTAMRA, dROX, and LIZ after an appropriate spectral matrix had been created using materials from the matrix standard set DS-02 (Applied Biosystems). Fluorescently labeled extension reactions were prepared for CE analysis by mixing 14 µl of Hi-Di formamide (Applied Biosystems), 0.4 µl of the LIZ-120 internal sizing standard (Applied Biosystems), and 0.9 µl of SAP-treated extension reaction. A 36 cm capillary array filled with either denaturing POP6 or POP4 performance optimized polymer (Applied Biosystems) was utilized for DNA fragment separation. A.C.E. (Amresco, Solon, OH) capillary electrophoresis running buffer was used in 1× concentration. Typical run module parameters were:

run temperature 60°C, capillary fill volume 184 steps, pre-run voltage 15 kV, pre-run time 60 s, injection voltage 1 kV, injection time 13 s, run voltage 15 kV, data delay 200 s, and run time 1200 s. Data analysis was performed using GeneScan 3.7 and Genotyper 3.7 software (Applied Biosystems). A macro based on fragment size and dye color was implemented using the Genotyper 3.7 software for automated allele calls.

Separation and sizing of PCR amplicons

PCR amplicons were detected and sized on a Bioanalyzer 2100 instrument (Agilent Technologies, Palo Alto, CA). The Bioanalyzer utilizes micro-fabricated chips for rapid DNA fragment sizing. The sample preparation and LabChip procedures included in the DNA 1000 LabChip kit (Agilent Technologies) were followed for sizing and quantifying singleplex PCR amplicons.

Results and discussion

SNP sites

The assay was designed to detect 11 SNP sites located in the mitochondrial genome. The 11 SNPs are mostly bialleic sequence variations, although 3 variants have been observed for site 14470, where an additional C allele occurs in some African mtDNAs (unpublished data). The location and sequence variation of the 11 mtSNP sites are listed in Table 1. Consistent with the known bias toward transitions over transversions in mtDNA polymorphisms, 10 of these SNPs represent transition variants with a single transversion pair at mtSNP 14470 T/A. Of the 11 SNP sites, 9 have variants that represent silent mutations in protein coding genes, with no potential for phenotypic effect on the protein gene products. Another SNP site is position 16519 in the control region; while residing in a region of relatively low variation in the CR, this particular site has the highest evolutionary substitution rate in the entire mtDNA genome (unpublished phylogenetic analysis, M.D. Coble and T.J. Parsons), and therefore has high utility for additional discrimination for multiple common HV1/HV2 types. The final site in the multiplex SNP panel A is 3010, that resides in the 16S rRNA gene. Variants within the 16S gene have potential to alter rRNA structure and function, and therefore are not categorically neutral; however, we have retained this SNP in our assay because it has long been identified as a widely spread, benign polymorphism [2, 29, 30].

PCR primer pairs

The 11 primer pairs were selected as described in the materials and methods section. To increase the chance for success with degraded samples, PCR primers were selected to produce amplicons that were less than 200 base pairs in length (Table 2). Of the 11 amplicons, 9 were kept under 150 bp in length. The average predicted melting temperature of the 22 primers was 59.8 ± 0.9°C [21]. PCR primers were screened for potential primer-dimer cross reactivity. The complete set of 22 primers was inter-compared resulting in 253 possible primer-primer comparison combinations. The top six interactions are listed in Fig. 1. The degree of potential primer-dimer interaction is shown along with a "score" value. The score value is determined by the number of Watson Crick base pairs (+1) combined with number of mismatches (–1). Note that gaps and loops were not included in the scoring due to the short length of

Table 2 PCR primer sequences for multiplex amplification of 11 mtSNP sites

Locus	PCR primer sequence	Length	t_m (°C)	Amplicon size (bp)
477-F	CTTTTGGCGGTATGCACTTT	20	60.1	122
477-R	GGTGTGTGTGTGCTGGGTA	19	58.9	
3010-F	GCGCAATCCTATTCTAGAGTCC	22	59.4	124
3010-R	TCACGTAGGACTTTAATCGTTGA	23	58.8	
4580-F	TCTTTGCAGGCACACTCATC	20	60.0	130
4580-R	GCAGCTTCTGTGGAACGAG	19	59.7	
4793-F	CAACCGCATCCATAATCCTT	20	59.8	186
4793-R	ATGTCAGAGGGGTGCCTTG	19	61.1	
5004-F	TCCATCATAGCAGGCAGTTG	20	59.8	124
5004-R	TGGTTATGTTAGGGTTGTACGG	22	58.8	
7028-F	GGCCTGACTGGCATTGTATT	20	60.0	125
7028-R	AAGCCTCCTATGATGGCAAA	20	59.7	
7202-F	ACGCCAAAATCCATTTCACT	20	59.4	126
7202-R	TTCATGTGGTGTATGCATCG	20	58.9	
10211-F	ACCACAACTCAACGGCTACA	20	59.2	143
10211-R	GGAGGGCAATTTCTAGATCAAA	22	59.6	
12858-F	ATGATACGCCCGAGCAGA	18	60.3	126
12858-R	TGTGGGTCTCATGAGTTGGA	20	60.1	
14470-F	CAAGACCTCAACCCCTGACC	20	61.9	129
14470-R	GGGGGAGGTTATATGGGTTT	20	58.9	
16519-F	ACCACCATCCTCCGTGAAAT	20	61.6	183
16519-R	AGACCTGTGATCCATCGTGA	20	59.1	

All primers are present at a final concentration of 0.5 μM for multiplex PCR.

Fig. 1 Primer-dimer screening results for 11 PCR primer pairs. Primer-dimer screening results for 22 PCR primers are illustrated. The score value was determined as described. A single interaction score of 9 with 5 additional interactions with a score of 6 were detected. After a prediction of thermodynamic stability, the potential interactions were determined to be insignificant at the temperatures used during thermal cycling

the primers (~20 bases). One potentially significant predicted primer-dimer interaction is between 7202 (forward) and 16519 (forward). This potential interaction may be considered especially significant due to the binding of 3' ends of the PCR primers to each other. Successful binding of the 3' end of the primer to the template is essential for primer extension and PCR amplification [31]. However, when predicting the stability of the duplex fragment 5' GTGAAAT 3', the t_m was calculated to be less than 10°C and therefore insignificant under PCR thermal cycling conditions. Additional screening of the 22 primers for intramolecular hairpins did not result in any significant interactions.

PCR thermal cycling conditions

The thermal cycling conditions represent a "reverse touchdown" approach. Cycling conditions are a slight modification of a protocol previously described for 12-plex amplification reactions [32]. The initial cycling is performed with an annealing temperature of 50°C, enabling PCR primer hybridization of the primary binding sites (and possibly others). In the next stage the annealing temperature increases by 0.2°C per cycle, this will gradually favor the production of amplicons with higher primer annealing temperatures. The amplification of extraneous PCR products can be tolerated to a degree with the caveat that they are not potential binding sites for the extension primers. However, the additional PCR products decrease the overall efficiency of the multiplex PCR by utilizing enzyme and dNTPs. The use of the described thermal cycling conditions allowed for the PCR primer pair concentrations to all be maintained at equimolar ratios at 0.5 µM. A comparison was made to a thermal cycling protocol with a constant annealing temperature (Ta=55°C), but which involved balancing of the PCR primer concentrations (data not shown) in order to obtain the same extension reaction results. Thus the necessity for adjusting PCR primer concentrations was avoided by employing the cycling conditions described.

Confirmation of singleplex PCR products

As a first step toward a multiplex assay, each of the 11 loci was amplified under singleplex PCR conditions. Singleplex reactions were run to confirm that the proper size PCR amplicons were being amplified and to detect any spurious PCR products. PCR conditions were identical to the multiplex conditions with the exception that only one primer pair was present. A PCR product of the correct size was confirmed for each singleplex PCR using the Agilent Bioanalyzer 2100 Lab on a Chip system.

Extension primers

The 11 extension primers listed in Table 3 were selected as described in the materials and methods section. The orientation of each extension primer is given with respect to the SNP site (forward or reverse). Primer-dimer and intramolecular hairpin screening did not indicate any potential non-desired interactions. Poly-thymidine (poly-T) "tails" were included on the 5' end of 10 of the extension primers as suggested in the SNaPshot multiplex kit manual. The purpose of the tails is to enable electrophoretic resolution of the extension primers. A spacing of four Ts between each of the extension primers was first attempted. However, initial experiments (data not shown) indicated overlap with the three shortest extension primers for loci 3010, 4793, and 10211 (originally 22, 26, and 30 nt, respectively). This resolution issue was remedied by altering the lengths of the first 2 extension primers to 19 and

Table 3 Extension primers for the multiplex detection of 11 mtSNPs

Locus	Primer sequence	Length	w/T-tail	t_m[a]	[µM]
3010-F	TGTTGGATCAGGACATCCC	19	19	59.3	0.4
4793-R	$(T)_4$–TCAGAAGTGAAAGGGGGC	18	22	59.7	11.5
10211-R	$(T)_{10}$–ACTAAGAAGAATTTTATGGA	20	30	49.2	15.5
5004-F	$(T)_{14}$–AGACCCAGCTACGCAAAATC[b]	20	34	60.8	12.4
7028-F	$(T)_{18}$–GACACGTACTACGTTGTAGC	20	38	58.5	5.8
7202-F	$(T)_{22}$–CCACAACACTTTCTCGGCCT	20	42	62.8	1.0
16519-R	$(T)_{24}$–TGTGGGCTATTTAGGCTTTATG	22	46	58.2	5.4
12858-F	$(T)_{27}$–GCAGCCATTCAAGCAATCCTATA	23	50	60.8	5.2
4580-R	$(T)_{29}$–TGGTTAGAACTGGAATAAAAGCTAG	25	54	58.2	6.0
477-F	$(T)_{38}$–CCCTCCCACTCCCATACTAC	20	58	60.9	5.6
14470-R	$(T)_{41}$–GGGAATGATGGTTGTCTTTGG	21	62	59.4	10.0

[a] t_m Prediction parameters: Na^+ 0.085 mM at 1.0 µM total strand concentration.
[b] Underlined base in 5004 is actually an "A" in the revised Cambridge reference mitochondrial sequence and did not effect primer extension.

22 bases, respectively. For shorter oligomers, properties of the fluorescent dye may have a greater effect on the electrophoretic migration in comparison to longer tailed oligomers. Table 3 lists the length of each extension primer and the length of the template-binding region. The predicted t_m for the binding region of each extension primer (not including the T-tail) is listed with an average of 58.9± 3.5°C.

Empirical optimization of the mtSNP 11-plex extension reaction

To optimize the multiplex assay, it was necessary to evaluate the results of the entire assay, run all the way through the primer extension, electrophoresis, and fluorescent typing steps. This was done, rather than simply balancing the amplification yield of the different amplicons, because of difficulty in resolving similarly sized products for 8 of the amplicons. These 8 amplicons fell within a 120–130 bp range. In addition, the quantity of PCR product generated may not necessarily correlate (linearly) with the efficiency or signal intensity observed in the extension reaction. Figure 2 shows the results for 2 of the 11-plex extension reactions using different extension primer concentrations. The extension reaction data from both panels were generated using aliquots of the same multiplex amplified PCR products. The upper panel of Fig. 2 shows the results of an equimolar mix of the extension primers at 1.0 µM. The signals for each of the 11 loci are well resolved from each another. Although all 11 of the mtSNPs were detected, it is readily apparent that the signal from 3010 dominates, followed by that of 7202. The signal for the loci 14470 is the weakest of the 11 loci.

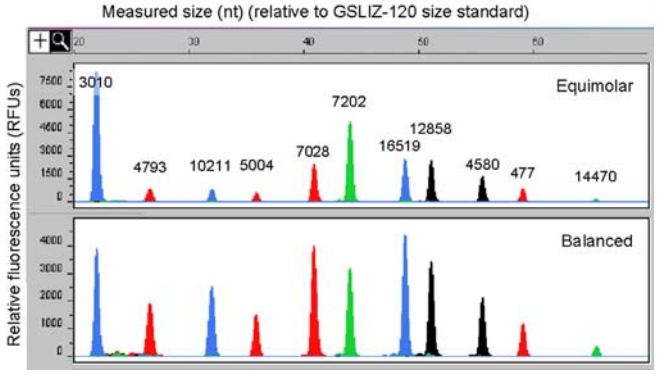

Fig. 2 Primer extension reactions representing eqimolar and balanced extension primer concentrations. Plots of size in nucleotides (nt) versus relative fluorescent units (RFUs). The *upper panel* represents the typical signal when using an unbalanced primer extension mix (all primers at 1.0 µM). The signal strength for locus 3010 dominates followed by 7202. The *lower panel* is a typical result after empirical optimization of the primer extension concentrations (concentrations listed in Table 3). The data for both plots were collected in POP6 running buffer under the described conditions. The orientation (forward/reverse) of the extension primers is listed in Table 3

Subsequent optimization of signal balance was an iterative process. Extension primer concentrations for loci exhibiting strong signal intensity were reduced and increased for the loci with the poorest signal. This process was repeated until further balancing did not significantly improve the signal balance. It should be noted that although only one comparison of an unbalanced and balanced experiment is shown in Fig. 2, the rationale for varying individual extension primer concentrations was evaluated after running 16 samples that exhibited various allele combinations (or haplotypes). This is important because the signal intensity was observed to vary based on the fluorescent dye labeled ddNTP being added, a point of particular relevance to heteroplasmy detection. For the 11 loci probed, only 3 different (out of a possible 6, assuming biallelic loci) fluorescent dye combinations were observed due to alleles present at these 11 SNP loci. The three dye combinations were: 6×C-T (black-red), 4×G-A (blue-green), and 1×T-A (red-green). The signal resulting from ddG incorporation was generally more intense than ddA whereas for ddC/ddT nucleotide incorporation the signal was more evenly balanced with a slight favoring of the ddT intensity. For the only T-A SNP, the signal was roughly equal. The lower panel of Fig. 2 represents an empirically balanced 11-plex mtSNP assay. The extension primer concentrations for the balanced 11-plex are given in Table 3. The loci are well represented in terms of signal intensity although the 14470 locus remains significantly lower than the others.

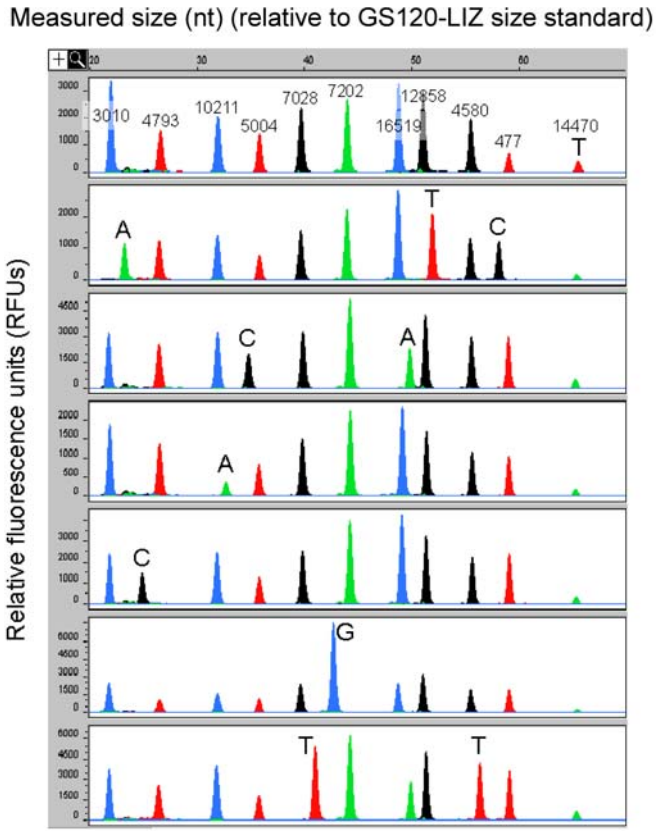

Fig. 3 Primer extension assays for 7 samples that exhibit variation at all loci. Plots of size (nt) versus RFUs for 7 unique samples exhibiting all possible alleles at the 11 mtSNPs examined. The typing of the 7 samples provided sizing information for each of the possible alleles. The "minor variants" are labeled in each plot. Assay data for all 7 plots shown were collected in POP6 running buffer under the described conditions

Following the first round of optimization, PCR primers for amplifying 14470 were redesigned to produce a different amplicon, but the signal for the 14470 locus remained the weakest. The forward orientation extension primer for 14470 was also investigated, but the 14470 locus still exhibited a relatively low intensity signal compared to the other 10 loci. The extension primer concentration for 14470 was raised as high as 20 µM, but a corresponding response in signal intensity was not observed. On average, the signal for 14470 was approximately 3 times lower than that for the next lowest locus 4793. However, in experiments with degraded casework extracts, higher amounts of Taq polymerase were used, significantly increasing the relative signal strength of 14470.

Figure 3 shows the electropherograms of a collection of 7 unique samples that, when combined, exhibit allele variation for each locus typed in the 11-plex assay. These "reference" samples were run to confirm accurate detection of the expected alleles. The data shown in Fig. 3 was collected in the POP6 polymer system. After smoothing and sizing using Genescan 3.7 software, the data were used to design a macro in Genotyper software for automated allele calling. The information in Table 4 provides

Table 4 Sizing of primer extension products in POP4 and POP6 separation media

Locus	Expected size (nt)	POP6		POP4	
		$\Delta nt_{allele1}$	$\Delta nt_{allele2}$	$\Delta nt_{allele1}$	$\Delta nt_{allele2}$
3010-F	20	1.8	3.2	4.1	5.8
4793-R	23	1.9	3.5	4.5	6.7
10211-R	31	0.9	1.7	2.8	3.7
5004-F	35	–0.2	0.8	1.2	2.5
7028-F	39	0.9	2.1	2.2	3.7
7202-F	43	0.0	1.3	0.9	2.3
16519-R	47	2.1	3.0	3.4	4.2
12858-F	51	0.4	1.3	1.5	2.6
4580-R	55	0.7	1.4	1.8	2.8
477-F	59	–0.7	0.1	0.4	1.5
14470-R	63	2.4	2.6	4.0	4.5

Δnt (estimated size–expected size) Sizing precision from one set of runs is less than 0.1 bp.

Table 5 Spacing of primer extension products in POP4 and POP6 separation media

Locus	Allele spacing[a]		Loci spacing[b]	
	POP6	POP4	POP6	POP4
3010-F	1.4 G/A	1.7	–	–
4793-R	1.6 C/T	2.2	1.7	1.7
10211-R	0.8 G/A	0.9	5.4	4.1
5004-F	1.0 C/T	1.3	2.1	1.5
7028-F	1.2 C/T	1.5	4.1	3.7
7202-F	1.3 G/A	1.4	1.9	1.2
16519-R	0.9 G/A	0.8	4.8	5.1
12858-F	0.9 C/T	1.1	1.4	1.3
4580-R	0.7 C/T	1.0	3.4	3.2
477-F	0.8 C/T	1.1	1.9	1.6
14470-R	0.2 T/A	0.5	6.3	6.5

[a]Allele spacing is defined as the absolute size difference between the extension products for a given locus.
[b]Loci spacing is defined as the absolute size difference between 2 adjacent loci=larger allele of locus N subtracted from the smallest allele of locus N–1.

the estimated extension primer size for the two alleles of each locus run in POP6 and POP4 polymer systems. Extension products separated in POP6 were sized more accurately when compared to POP4. On average, the difference between the estimated and expected extension primer length was 1.4 nucleotides (nt) versus 3.0 nt for fragments separated in POP6 and POP4, respectively. The peak separation for the two expected alleles for each locus is given in Table 5. In this case POP4 allows a slightly greater separation than POP6 (1.1 versus 1.3 nt, respectively) between the two alleles for a given locus. The separation between each adjacent locus was evaluated by calculating the distance between allele 2 and allele 1 of the following adjacent locus. Results indicate that POP6 has a slight advantage over POP4 in terms of spatial resolution. Sizing variation for extension primers was dependent on sequence length, sequence content, and attached dye.

Generally as the length of the primer increased so did the accuracy of the sizing as defined by the observed size matching the actual size of the extension product. A comprehensive set of extension primers that vary in length, sequence content/context, and incorporated dye would have to be examined in order to provide rigid rules for improved assay design in term of uniform spacing between extension products.

Detection of mixtures/heteroplasmy

To test assay performance with mixed samples (and therefore simulating heteroplasmy as well), a set of five different mixture ratios were amplified and extended. The two samples involved in the mixture series varied from each other at four positions: 4793, 7028, 16519, and 4580. Samples were adjusted for equivalent genomic DNA concentrations, then mixed in various ratios ranging from 90:10 to 10:90 mixtures. Figure 4 shows representative results from the mixture study. Each allele for each position was clearly detected over the ratio range that was examined. This level of sensitivity for detecting a minor component compares favorably to fluorescent dye-terminator sequencing, where ~20% minority component is generally regarded as the threshold for unambiguous detection [33].

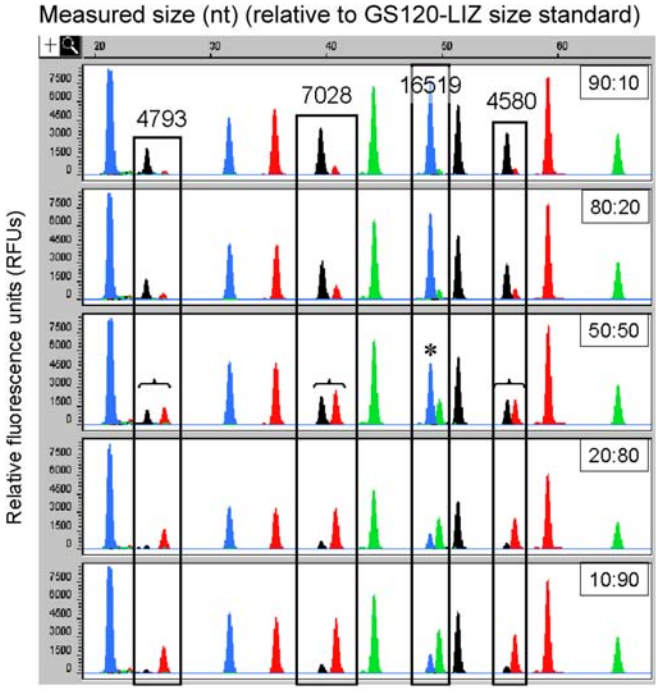

Fig. 4 Primer extension assay performance with mixed templates. Plots of size (nt) versus RFUs for two different samples mixed in five ratios (as indicated). The samples were co-amplified by multiplex PCR and typed in multiplex using the primer extension assay. The 4 variant loci 4793, 7028, 16519, and 4580 are boxed. Data indicates the potential for the detection of simple mixtures by using a multiplex primer extension assay. Samples were run in POP6 separation media

A primary issue in detecting heteroplasmy or low level mixture in mtDNA sequencing is the difficulty in distinguishing between spectral background signal and signal that originates from a low level alternative base. Moreover, with dye terminator sequencing the fluorescent signal from different base variants can change in intensity from site to site [34], further complicating the determination of heteroplasmy or mixture [35]. The SNaPshot assay has an advantage over sequencing in that the two allele variants are slightly displaced relative to one another, due to minor differences in mobility depending on the particular fluorescent dideoxy terminator that has been incorporated. Therefore, issues of spectral resolution do not affect the determination of heteroplasmy/mixture as they do with sequencing. Overall, the relative signal intensity of the two alleles is a reasonable, if rough, indication of the relative proportion of the two molecular species. The largest exception to this occurred at position 16519 (Fig. 4) where the C allele typed with an intensity roughly twice that of the T allele. An intriguing observation is that this same signal bias for 16519 is evident using a completely different SNP assay, a real time TaqMan assay that uses fluorogenic hybridization probes specific for each variant (data not shown). It may be that the secondary structure of the single stranded PCR product differs significantly between the two variants, causing differential probe binding efficiency in this case. Precise determination of heteroplasmy/mixture ratios is notably problematic with most assays, and in the case of SNaPshot would require locus-specific signal imbalance to be quantified through standard curve experiments, to determine correction factors. However, it is not generally a forensic requirement that heteroplasmic ratios be exactly quantified. Our results indicate that the SNaPShot assay represents an improvement over sequencing in terms of heteroplasmy/mixture detection, and performs at least as well as sequencing in estimating relative proportions (for discussion of issues of heteroplasmy quantitation by sequencing, see Tully et al. [35]).

Sensitivity of the 11-plex assay

Serial dilutions of quantitated DNA samples were used to indicate the sensitivity and dynamic range of the assay. The dilution series concentrations ranged from 2000 to 0.2 pg of total genomic DNA isolated from whole blood (mtDNA concentrations were not determined), and were subjected to the identical PCR, extension reaction, and electrophoresis conditions. Excellent results were obtained through this range down to a 1–2 pg cut-off for robust reproducibility. Sub-picogram amounts of template DNA most often gave identifiable peaks, but objective interpretation was compromised by low peak intensity, and the need to distinguish between true peaks and low level artifact peaks that sporadically occur just above baseline. This study was performed on multiple samples (n=5) of varying haplotypes to ensure reproducibility (data not shown). The dynamic range of the assay proved to be extremely high, spanning template amounts of less than a

single cell's worth of DNA through at least 3 orders of magnitude higher concentration.

Typing of degraded casework samples

To assess the performance of the 11-plex mtSNP assay on actual mtDNA casework samples, a series of 8 non-probative extracts from degraded skeletal remains were typed. The extracts were typical of mtDNA cases handled at the Armed Forces DNA Identification Laboratory (AFDIL), for which standard nuclear STR typing typically gives no results. Initial experiments showed the standard PCR and primer extension conditions using 15 µl volumes and 1 unit of Taq polymerase (0.07 units/µl final concentration) were rather susceptible to inhibition with casework samples (Fig. 5a), so comparisons were performed using either 15 µl or 25 µl reactions with 0.07 units/µl or 0.27 units/µl of Taq, on a range of extract volumes from 2 µl to the equivalent of 0.05 µl. Figure 5a–d shows typical results using 0.5 µl of extract and indicates that increasing the concentration of DNA polymerase in the reaction dramatically improved the success of the assay. The profiles shown in Fig. 5a–d were from a comparatively "easy" mtDNA case in terms of the amount of template DNA and number of amplifications required for standard mtDNA sequencing, but was particularly inhibitory. For the 11-plex mtSNP assay, only the lowest dilution of this extract gave complete typing for all 11 sites, while complete typing was obtained over a range of extract volumes (0.5 µl is shown in Fig. 5) when increased levels of Taq polymerase were used. The 25 µl reaction gave complete typing for all volumes of extract examined, and the larger volume seems overall slightly more robust in experiments with other extracts (data not shown), although this trend was not definitive. The increased levels of Taq polymerase also significantly improved the peak heights of position 14470, reducing the signal imbalance relative to the other SNP sites (see Fig. 5d). This was noted for both pristine positive controls and degraded samples. Overall we examined eight non-probative mtDNA casework extracts, and obtained complete typing from all, but only when increased levels of polymerase were used. The casework extracts were selected to represent a range of difficulty in terms of the number of amplifications and amount of extract needed for successful mtDNA amplification and sequencing in the original AFDIL casework processing.

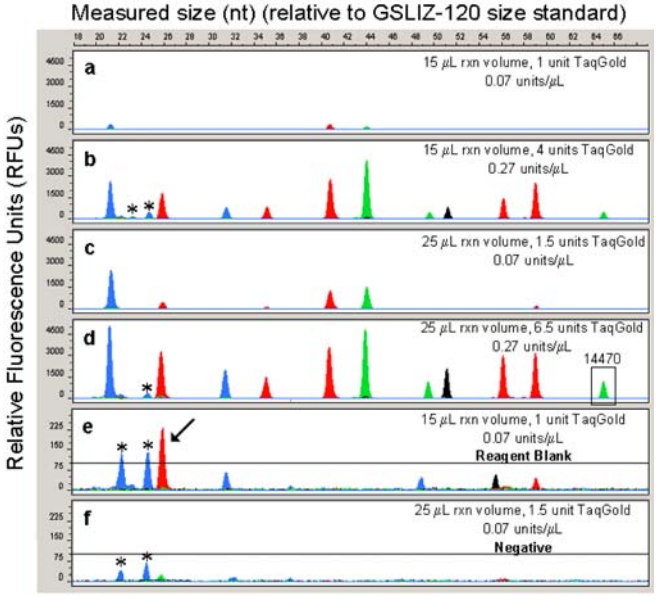

Fig. 5a–f Multiplex assay performance with degraded casework samples. Plots of size (nt) versus RFUs for a single mtDNA casework sample of 0.5 µL extract (**a–d**), 0.5 µL of reagent blank (**e**), and an amplification negative control with water only (**f**). Results from differing reaction volumes and final concentration of Taq polymerase are shown, as indicated on the panels. Asterisks (*) indicate artifact peaks that do not correspond to a target SNP site, and the arrow in panel (**e**) indicates a specific SNP peak that exceed the indicated 100 RFU cut-off. Note that the RFU scale of panel (**e**) is much smaller than panels (**a–d**). Assay data for all five plots shown were collected in POP6 running buffer under the described conditions

As is required in mtDNA typing (Holland and Parsons), we also tested "reagent blank" extraction controls that were associated with the casework extracts we investigated [1]. Reagent blanks are simply mock extractions run with the same reagents as the sample extractions, but with no sample added. Laboratories with practical experience in mtDNA typing all experience contamination in some percentage of their reagent blanks, and must have reporting criteria that properly take this into consideration. For example, obtaining the same typing results from a reagent blank as from a sample is an obvious criterion for dismissing the sample results for that amplification. Such considerations necessarily apply to SNP typing as well. So far in our trials with the 11-plex assay we have run 59 separate reactions on various amounts of 7 different reagent blanks associated with the casework samples. None of these reagent blank reactions exhibited results that resembled those similar to full typing. Occasionally small peaks were present that correspond in color and position to one of the target SNP sites (Fig. 5e). Applying a relative fluorescent unit (RFU) cut-off of 100, the maximum number of such peaks observed in reagent blanks was 2, while 57% of the reactions showed no peaks. In addition to these faint, sporadic primer extension peaks, the reagent blank results are typified by larger than normal background or artifact peaks that do not correspond to target SNPs.

As in automated fluorescent STR typing [18], it is necessary with this assay to establish criteria for distinguishing peaks that result from actual mtDNA typing from background or other artifact peaks. Figure 5e shows several prominent artifact peaks (indicated by *) that were regularly quite pronounced in the absence of sample or template DNA, and often present at greatly reduced intensity in reactions containing sample or control DNA as well. These particular artifacts peaks appeared to be specific to a particular lot of extension primers used for the casework typing, and their cause is unknown. So far in our experience, a cut off of 100 RFUs has been a useful threshold for interpretation. Degraded casework samples

giving full or strong partial results had peaks well above 100 RFUs while background peaks of reagent blanks and negative controls rarely approached this value.

As noted above, isolated SNP-specific peaks in reagent blanks occasionally exceeded 100 RFUs. This presents the question of how to treat a situation where a single small peak from a reagent blank matches a common SNP variant that is also present in the corresponding sample, but the sample itself gives a strong, full multiplex profile. The situation is directly analogous in standard mtDNA sequence testing to obtaining one or a few very low quality sequence electropherograms from a faint reagent blank amplicon. Even if the low clarity and high background of the sequence cause it to be rejected as uninterpretable and unreliable, it would nevertheless often be possible to align short stretches of sequence with corresponding portions of human mtDNA. These short fragments would most likely match the sequence from any associated sample. However, to our knowledge most laboratories, including AFDIL, have reporting criteria that allow robust, reproducible sample data to be reported under these circumstances based on the fact that, overall, a reliably interpretable type could not be recovered from the reagent blank. In any case, a cornerstone criterion of authenticity in difficult cases will always be the reproducibility of results from independent extractions. In cases of a partial match between a sample and reagent blank, the most prudent course would be to replicate the sample results, comparing to a new reagent blank as well. Validation of the SNP multiplexes will require each laboratory to develop similar operational guidelines to be established regarding comparison of negative controls to sample profiles, as well as the determination of RFU thresholds for peak calling, as in nuclear STR typing.

Conclusion

We have developed a multiplex assay capable of typing 11 SNPs spanning the mtGenome. The assay amplifies 11 unique regions of the mtGenome in single tube PCR. The extension primers were designed to work in multiplex and varied in length for electrophoretic separation. In our hands the assay has shown to be robust and indicates potential for mixtures and low copy number and degraded samples. The information gained during the development of this 11-plex assay will be applied to the design of additional multiplex mtSNP assays for separating other common HV1/HV2 mitotypes (see for example, Coble et al. [2].

Acknowledgements We thank Suzanne M. Barritt of AFDIL for discussion and comments on the manuscript, as well as for providing non-probative casework samples. Jill Appleby, Tracey Johnson, Danielle Goldstein, and Jennifer Kappeller of AFDIL also assisted in providing casework samples. Jennifer O'Callaghan, Jessica Saunier, Allison McClure, and Devon Pierce, AFDIL, provided technical laboratory or data analysis assistance. We thank Brion C. Smith, James C. Canik, and Kevin S. Carrol, AFDIL, for administrative, logistical, and moral support, and James P. Ross for computer support.

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