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Development of a Multiplex PCR and Linear Array Probe Assay Targeting Informative Polymorphisms within the Entire Mitochondrial Genome

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Abstract:

The hypervariable regions I/II (HVI/II) of the mitochondrial genome are routinely targeted by forensic DNA laboratories for the analysis of limited and/or degraded samples. However, there are limitations to targeting only the HVI/II regions independent of the method of analysis. The power of discrimination is limited for all population groups as a result of a few common HVI/II sequences. Most notably, 7% of Caucasians share the same common HVI/II sequence. To increase the informativeness of mtDNA analysis, additional sequence polymorphisms outside the HVI/II regions need to be targeted.

To address this issue, we have developed a highly sensitive, easy to use 5-plex and 10-plex PCR and linear array assay for simultaneous analysis of polymorphic regions in the non-coding and coding regions. This assay targets 61 polymorphic sites distributed throughout the mitochondrial genome using 15 primer pairs and a total of 105 sequence-specific oligonucleotide probes immobilized in lines on a nylon membrane. The 5-plex PCR is used to amplify five regions ranging in size from 314-444 bp; the 10-plex PCR is used to amplify 10 regions ranging in size from 103- 183 bp. The 5-plex probe panel consists of 59 probes and targets variation at 14 HVI sites, 11 HVII sites, eight coding region (CR) sites, and two variable region I (VRI) sites. The 10-plex probe panel consists of 46 probes and targets 17 CR sites, four VRI sites and five VRII sites.

A population study was conducted in order to determine the power of discrimination for the new expanded HV+ array. A total of 674 samples from four population groups (US Caucasian (197), US Hispanic (197), African American (193), and Japanese (86)) were amplified with the 5-plex and 10-plex primers and typed with the corresponding probe panels. The discrimination power was greatly increased for all populations and a significant increase in the power of discrimination was observed for both the US Caucasian (0.9946 from 0.9768) and the US Hispanic (0.9893 from 0.9449). Also, preliminary data show that the new expanded array is more informative than sequencing the HVI/II regions for individuals in the US Caucasian population.

In order to determine the assay limitations, a developmental validation study was conducted. Studies to address the DAB and TWGDAM guidelines were conducted at in our laboratory at Children's Hospital Oakland Research Institute (CHORI) and at the National Center for Forensic Science (NCFS). A sensitivity study was conducted and it was shown that both the 5-plex and 10-plex assays are highly sensitive assays, with the 10-plex being more sensitive. Typeable results were observed with ~5 pg of DNA input for the 5-plex at 34 cycles and ~1pg input for the 10-plex. At 38 cycles, ~0.5 pg of DNA input yielded typeable results for both the 5-plex and 10-plex assays. A mixture study was conducted and it was shown that a minor component present at ~10% is reliably detected with the 5-plex assay and ~5% with the 10-plex assay. Species specificity studies were conducted at the NCFS and results showed that the probes for both the 5-plex and 10-plex probe panels are primate specific. In addition, all primers were shown to be primate specific except the pair targeting the 16s rRNA region. Various case type samples (including low copy number samples) were also successfully amplified and typed by the NCFS. Additional developmental studies, including reproducibility, precision and accuracy, and environmental samples were also completed by the NCFS.

Lastly, a procedure was validated for automating the typing assay and scanning and interpretation software were modified for use with the 5-plex and 10-plex assays. Up

to 48 samples can be typed manually or automated in less than two hours and the data can now be quickly analyzed using the Strip Scan Mitotyper software.

Executive Summary:

Introduction

Mitochondrial DNA (mtDNA) is an informative and useful target for the forensic genetic analysis of limited and/or degraded samples. However, there are some inherent limitations to targeting only the hypervariable regions I/II independent of the method of analysis (sequencing or LINEAR ARRAY typing). The power of discrimination is limited for all population groups as a result of a few common HVI/II sequences. Therefore, additional sequence polymorphisms outside the HVI/II regions need to be targeted to increase the power of discrimination of mtDNA analysis in addition to the HV regions.

The HV regions of the human mitochondrial genome have a high sequence diversity and accordingly have been the primary area of study for identification purposes. Several systems targeting the hypervariable regions (HVI and HVII) of the mitochondrial genome are currently being used by forensic laboratories for the analysis of highly degraded or limited DNA, including the HVI/HVII linear array probe panel developed with previous NIJ grant support. It has been widely reported that some HVI/HVII types are common among all populations (Parsons & Coble 2001), even though the overall distribution of mtDNA HVI/II sequences is highly skewed towards rare types. Approximately 7% of Caucasians share the most common HVI/II sequence differing from the rCRS at 263G and 13 additional HV sequences are shared among >0.5% of the population (Parsons & Coble 2001). A greater percentage of individuals share the same mtDNA linear array types (mitotypes) because not all sites within the HVI/II regions are targeted with the HVI/HVII probe panel. Approximately 10% of Caucasians share the

most common HVI/HVII mitotype (1111111111). Sequencing the entire HVI/II region can discriminate somewhat between these mitotypes but the problem posed by relatively common sequences remains. Therefore, as Parsons and Coble (2001) have noted, the greatest limitation for current mtDNA testing, based on HVI/II, lies within the small number of common mtDNA types which cannot be further discriminated.

To increase the discrimination power of mtDNA testing and to further distinguish between individuals who share common HVI/II sequences, additional sequence variation in the coding and variable regions needs to be identified and assays targeting this variation need to be developed. The primary goal of this research was to develop a sensitive, robust multiplex PCR and Linear Array typing system that would target a set of highly informative polymorphic sites distributed throughout the mtDNA genome. To achieve this goal, we identified a set of highly informative polymorphic sites from published and unpublished mitochondrial genome sequence data as well as data collected by our laboratory. These sites are targeted and simultaneously amplified with a multiplex PCR and typed with a panel of probes immobilized on a nylon membrane. To increase the discrimination power of the panel of 31 HVI/HVII probes, primers and probes targeting polymorphic sites that subdivide common HV types as well as rapidly evolving polymorphic sites that subdivide multiple haplogroups (homplasic sites) were added to the existing system. Additionally, probes that target sequences within HVI/HVII regions to reduce the number of '0' and weak signal types were added to improve the robustness of the assay. This assay allows for simultaneous amplification and genotyping of polymorphic sites distributed throughout the mtDNA genome and greatly enhances the informativeness of current mtDNA typing systems.

Research Goals and Objectives

The primary goal of this research was to develop a rapid, sensitive, and robust multiplex PCR and immobilized probe panel with increased discrimination power compared to HVI/HVII mtDNA analysis. The following objectives were met: 1) select a panel of informative polymorphisms, 2) design a multiplex PCR amplification system, 3) design immobilized oligonucleotide probes targeting informative sites, 4) generate a population database to determine the informativeness of the selected sites and complete developmental validation studies and 5) validate a high-throughput method for typing and develop a scanning and interpretation software for data analysis.

Indentifying a Panel of Informative Polymorphic Sites

To improve the discrimination power, we first identified sites to subdivide the most common HVI/HVII types by targeting variation outside the HV regions from published, unpublished and data collected internally (Just *et al.* 2004). Since a large number of individuals having a few common HV types primarily accounted for the lower discrimination power we primarily focused on improving the discrimination power for the US Caucasian and Hispanic populations. The complete mtDNA sequence for 32 US Caucasian samples with the two most common HVI/HVII types was determined using Sanger sequencing. The mitochondrial genome was amplified in 24 reactions resulting in amplicons ~800 bp in size (Ingman & Gyllensten 2001). Candidate polymorphic sites were identified by maximum parsimony analysis. The overall frequency of the variable sites in the mtDB sequence database (Ingman & Gyllensten 2006) was also considered

when selecting candidate sites. In addition, the complete mtDNA sequence of ~65 US Hispanic samples with the two most common HVI/HVII types was determined using the Affymetrix mtDNA resequencing Chip (data reported elsewhere). Candidate polymorphic sites were indentified by phylogenetic analysis and the overall frequency of the candidate sites in the global population was also considered when selecting these sites.

The location of the position and base substitution's effect on amino acid were also considered when selecting the candidate polymorphic sites as well as the final panel of sites. Whenever possible, silent (synonymous) substitutions over replacement (non-synonymous) mutations were selected. Polymorphic sites with known disease associations were avoided. All polymorphic sites in the final panel are synonymous substitutions, do not code for amino acids, or otherwise not reportedly associated with any disease. Finally, polymorphic sites were chosen to minimize the number of targeted regions to reduce the number of required primer pairs in the multiplex.

Final panel of Sites: 5-plex and 10-plex assay (105 probes)

A total of 61 polymorphic sites are targeted with the final 5-plex and 10-plex assays. The final 5-plex probe panel consists of 59 probes and targets variation at 14 HVI sites, 11 HVII sites, eight coding region (CR) sites, and two variable region I (VRI) sites. The final 10-plex probe panel consists of 46 probes and targets 17 CR sites, four VRI sites and five VRII sites. The probe designations and the targeted sequence variation are listed in the Table1a (5-plex) and Table 1b (10-plex) below.

Table 1a.

5-plex	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
HVI	16093 1	A T T T C
	16093 2	• • C • •
	16111 1	A G C C A
	16111 2	• • T • •
	IA1	T G T A C G G T
	IA2	T • C • • • • •
	IA3	T • • • • A • •
	IA4	C • • • • • • •
	IC1	A G T A C A T A G T A C A T A A A G C C C A
	IC2	• • C • • • • • • • • • • • • • • • • • • •
	IC3	• • • • • • • • • C • • • • • • • • • • • •
	IC4	• • • • • • • G • • • • • • • • • • • • • •
	IC5	• • • • • • • • • • • • • • • • • • A • • •
	IC6	• • • • • • • • • • • • • • • • • • • T • •
	ID1	C G T C C
	ID2	• • C • •
	IE1	C T C A C C C A C T A G G A T A C C A
	IE2	• • • • • • • • • • • • • • • • • T • •
	IE3	• • • • • • • • T • • • • • • • • • • •
	IE4	• • T • • • • • • • • • • • • • • • • •
VRI	64 1	G T C T G
	64 2	• • T • •
	72	G G T A T
		• • C • •

5-plex	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
HVIII	IIA1	G T A T G (73: A)
	IIA2	• • G • •
	IIB1	C C T C A T C C T A T T (146: T; 150: C; 152: T; 153: A)
	IIB2	• • C • • • • • • • • •
	IIB3	• • • • • • • • C • • •
	IIB4	• • C • • • • • C • • •
	IIB5	• • • • • • T • • • • •
	IIB6	• • • • • • T • C • • •
	IIB7	• • C • • • T • C • • •
	IIB8	• • C • • • • • • G • •
	IIB9	• • C • • • • • C G • •
	IIC1	G A A C A T A C T T A C T A A A (189: A; 195: T; 198: C; 200: A)
	IIC2	• • • • • • • • C • • • • • •
	IIC4	• • • • • • • • C • • T • • •
	IIC5	• • G • • • • • • • • • G • •
	IID1	T T G A A T G (247: G; 249: A)
	IID2	• • A • • • •
	IID3	• • • • – • •
	189 1	G A A C A (189: A)
	189 2	• • G • •
CODING REGION	4769 1	A T A G C (4769: A)
	4769 2	• • G • •
	4793 1	A T A G C (4793: A)
	4793 2	• • G • •
	5004 1	T C T T A (5004: T)
	5004 2	• • C • •
	6776 1	C A T A T (6776: T)
	6776 2	• • C • •
	7028 1	G C C C A (7028: C)
	7028 2	• • T • •
	11719 1	G G G C T (11719: G)
	11719 2	• • A • •
	11794 1	T C T C A (11794: T)
	11794 2	• • C • •
	11914 1	A C G T T (11914: G)
	11914 2	• • A • •

Table 1B.

10-Plex	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
CODING REGION		709 710
	709 1	C C G T T C
	709 2	• • A • • •
	709 3	• • A C • •
		731
	731 1	T A A A T
	731 2	• • G • •
		750
	750 1	G G A A C
	750 2	• • G • •
		1719
	1719 1	T A G C C
	1719 2	• • A • •
		1811
	1811 1	T A A C C
	1811 2	• • G • •
		1888
	1888 1	G A G C C
	1888 2	• • A • •
		3010
	3010 1	C C G A T
	3010 2	• • A • •
		4580
	4580 1	A T G C T
	4580 2	• • A • •
	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
CODING REGION		4769
	4769 1	A T A G C
	4769 2	• • G • •
		4793
	4793 1	A T A G C
	4793 2	• • G • •
		5004
	5004 1	T C T T A
	5004 2	• • C • •
		6308
	6308 1	A A C T A
	6308 2	• • T • •
		6776
	6776 1	C A T A T
	6776 2	• • C • •
		7028
	7028 1	G C C C A
	7028 2	• • T • •
		10394 10397 10398 10400
	10394 1	G A C T G A A C C G A
	10394 2	• • T • • • • • • • •
	10394 3	• • • • • G G • T • •
	10394 4	• • • • • • G • • • •
	10394 5	• • • • • • G • T • •
		11719
	11719 1	G G G C T
	11719 2	• • A • •

10-Plex	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
CODING REGION		11794
	11794 1	T C T C A
	11794 2	? ? C ? ?
		11914
	11914 1	A C G T T
	11914 2	? ? A ? ?
		14766 14770
	14766 1	A A C T A A C C C
	14766 2	? ? T ? ? ? ? ? ?
	14766 3	? ? ? ? ? ? T ? ?
		14793 14798
	14793 1	C C A C T C A T T C
	14793 2	? ? G ? ? ? ? ? ? ?
	14793 3	? ? ? ? ? ? ? C ? ?
VRI		16519 16524 16526 16527
	16519 1	G G T C A T A A A G C C T
	16519 2	? ? C ? ? ? ? ? ? ? ? ? ?
	16519 3	? ? ? ? ? ? ? G ? ? ? ? ?
	16519 4	? ? C ? ? ? ? G ? ? ? ? ?
	16519 5	? ? ? ? ? ? ? ? ? A ? ? ?
	16519 6	? ? C ? ? ? ? ? ? A ? ? ?
	16519 7	? ? ? ? ? ? ? ? ? ? T ? ?
	16519 8	? ? C ? ? ? ? ? ? ? T ? ?
	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
VRI		477 482
	477 1	A C T A A T C T C A
	477 2	? ? C ? ? ? ? ? ? ?
	477 3	? ? ? ? ? ? ? C ? ?
		489 493
	489 1	A A T A C A A C C
	489 2	? ? C ? ? ? ? ? ?
	489 3	? ? C ? ? ? G ? ?
		523 524 524.1 524.2
	523,524 1	A C A C - - C G
	523,524 2	? ? ? ? A C ? ?
	523, 524 3	? ? - - - - ? ?

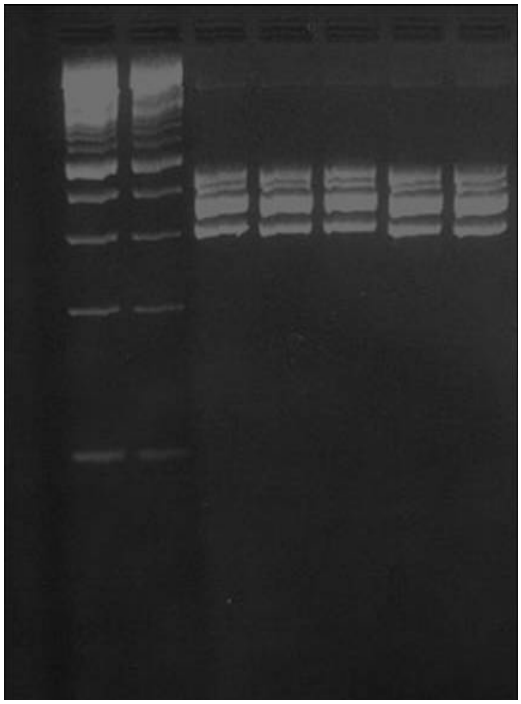
Design and Optimization of Final Multiplex PCR Systems

Multiplex PCR reaction conditions were optimized to amplify multiple targets simultaneously maximizing sensitivity through the minimization of primer dimer formation, unequal amplification, and non-specific products. Minimization of primer dimer formation and amplification of non-specific products is important because these by-products may interfere with amplification yield and/or typing. Modified bases and hot start polymerases to enhance specificity were used. To reduce non-specific product formation, repetitive regions were avoided and potential primers were searched against the human genome for sequence similarity.

The following eight published primer sequences were used in the final multiplex: 3010-F, 3010-R, 4580-F, 4580-R, 5004-R, 7028-R, 16519-F, and 16519-R (Vallone *et al.* 2004). All other primers were designed using Primer 3 software (Vieux *et al.* 2002). Primers were designed in conserved regions to minimize the chance of a polymorphism under the primer disrupting amplification and reducing efficiency and success. Primers were designed with a melting temperature (Tm) of ~65° C, ~5-6° C above the targeted annealing temperature (59° C). Primers were designed to work with the existing HVI/HVII primers and under the same PCR conditions. The distribution of informative polymorphic sites allowed for primer sets to be designed to target multiple polymorphic sites, decreasing the number of amplicons. Although 61 sites are targeted, only 15 primer pairs were required. However, because the VRI/II regions overlap with the HVI/HVII regions, it was necessary to design two multiplex PCRs. Three primer pairs designed to amplify larger targets ~314-375 were added to the duplex PCR, resulting in a 5-plex PCR. Ten additional primer pairs were needed to target the rest of the selected sites for

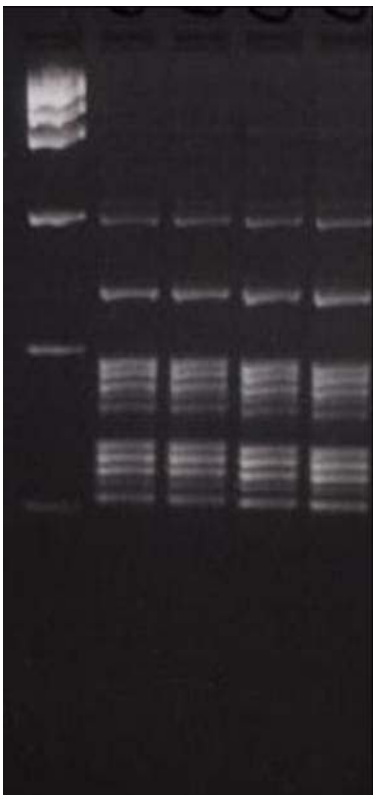
the final 10-plex assay. To increase sensitivity and amplification success of degraded samples, these ten primer sets were designed to minimize the size of the PCR product, aiming for amplicons between 100 and 200 bp. This range of amplicon sizes allows for 'laddering' of all products in the 5 and 10 plex to determine amplification success. Using a 3% Nusieve/ 1% seakem agarose gel system, fragments differing by 7-10 bp can be resolved. Amplicon sizes are noted for each target shown in the gel images below (Figures 1A-1B). We found that a 1X TAE solution resulted in better resolution for the 5-plex whereas a 1X TBE solution resulted in better resolution for the 10-plex, because of the amplicon size.

Figure 1A. Final 5-plex PCR



Region	Size (bp)
HVI	444
HVII	415
6776/7028	375
4769/4793/5004	362
11719/11794/11914	314

Figure 1B. Final 10-plex PCR



Region	Size (bp)
16519/16524/16526/16527	183
709/710/731/750	173
477/482/493/498	165
1811/1888	156
14766/14770/14793/14798	141
4580	130
3010	124
10394	117
1719	110
6308	103

During the design and testing stages, each primer set was tested individually for formation of primer dimer and non-specific amplification products prior to combining primer pairs into the multiplex. If primer dimer was observed in the single-plex, the primers were redesigned. Once each individual reaction was optimized, primer pairs were then combined and again checked for balance and non-specific amplification products.

Design and Optimization of probes

The method for mtDNA typing is based on the analysis of the co-amplified DNA using Sequence-Specific Oligonucleotide (SSO) probes immobilized in lines on a strip of nylon membrane (referred to as a linear array probe panel). A panel of probes was

designed to target informative sites chosen based on the added value to the HVI/HVII probe panel. SSO Probes are used to "capture" PCR products that contain sequence complementary to individual probes. Probes were designed to minimize cross-hybridization and obtain balanced signals while maintaining a high level of sensitivity. This specificity can be achieved by centering the target polymorphism within the designed probe and by choosing the complementary strand of the target sequence with the most destabilizing mismatch to the variant (e.g. C/A mismatch is less stable than a G/T mismatch). Specificity and signal intensity was also altered by increasing or decreasing the length of the probe (increasing or decreasing the melting temperature) or shifting the probe spanning region to achieve a different GC skew. In some cases, it was necessary to use modified bases to achieve optimal specificity and signal intensity. Also, probe signal intensity was decreased or increased by adjusting the concentration of the probe applied to the nylon membrane. Probes were designed to work under the same hybridization and wash conditions as the HVI/HVII linear array.

Over the course of this granting period, three probe panels were developed. A panel of 56 probes targeting 32 sites, 83 probes targeting 48 sites and two panels targeting 61 sites which utilize 105 probes. Representative results from the 56 probe panel, 83 probe panel, and final 5-plex and 10-plex probe panels are shown in the Figures 2A-2C below. Population studies were conducted using the 56 and 83 probe panels to determine the discrimination power of the added probes and polymorphic sites and these data were considered when selecting the final panel of polymorphic sites. A larger population study and developmental validation was conducted using the final HV+ assay and results are summarized below.

Figure 2A. 56 Probe Panel

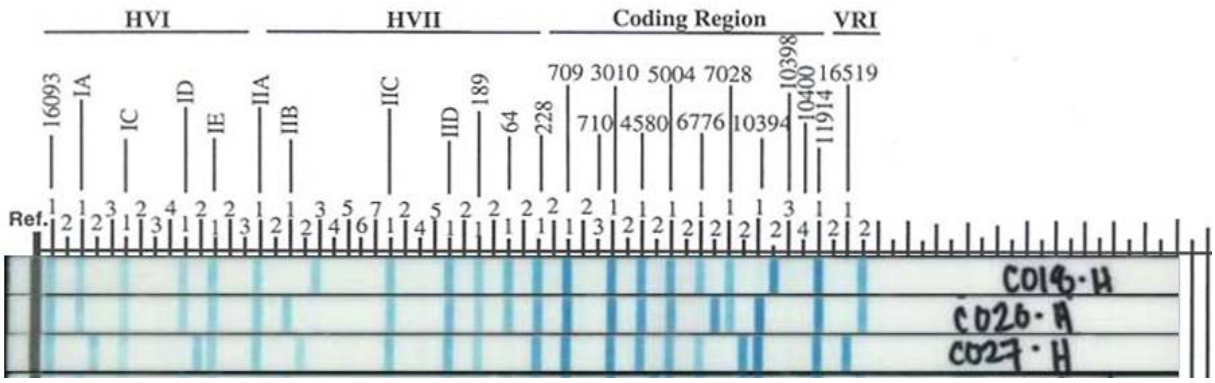


Figure 2B. 83 Probe Panel

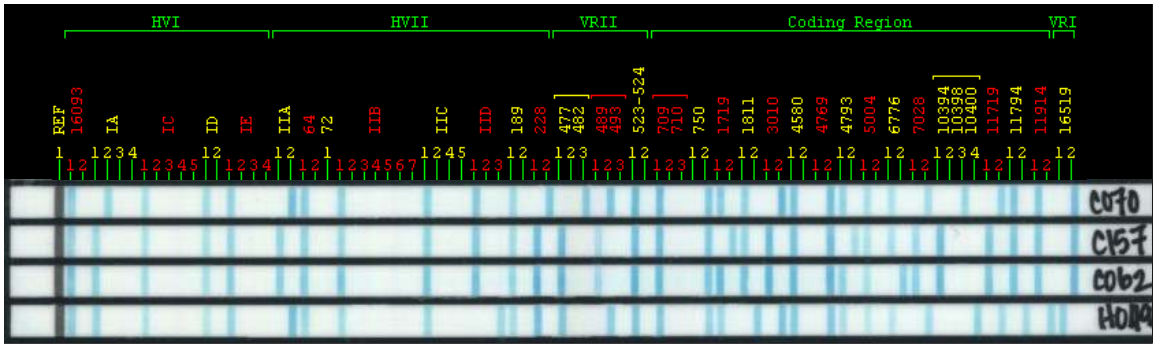
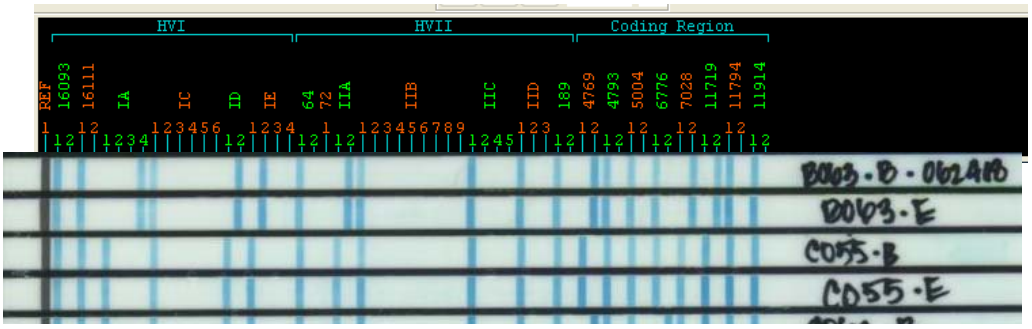
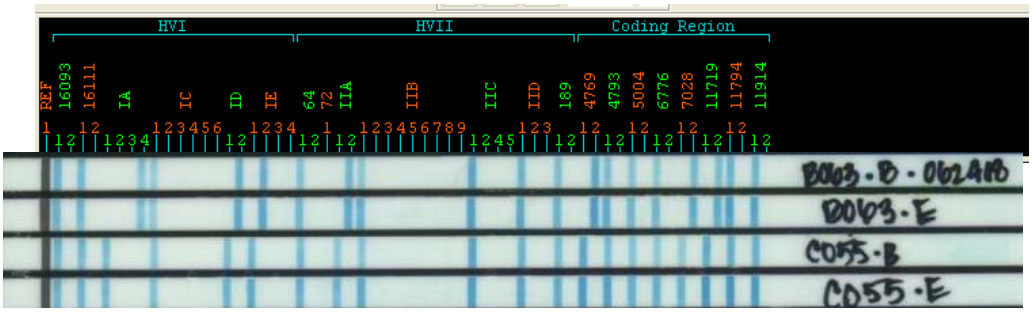


Figure 2C. Final Assay: 5-plex probe panel



Final Assay: 10-plex probe panel



Generation of a Population Database Using the Final 5-plex and 10-plex System

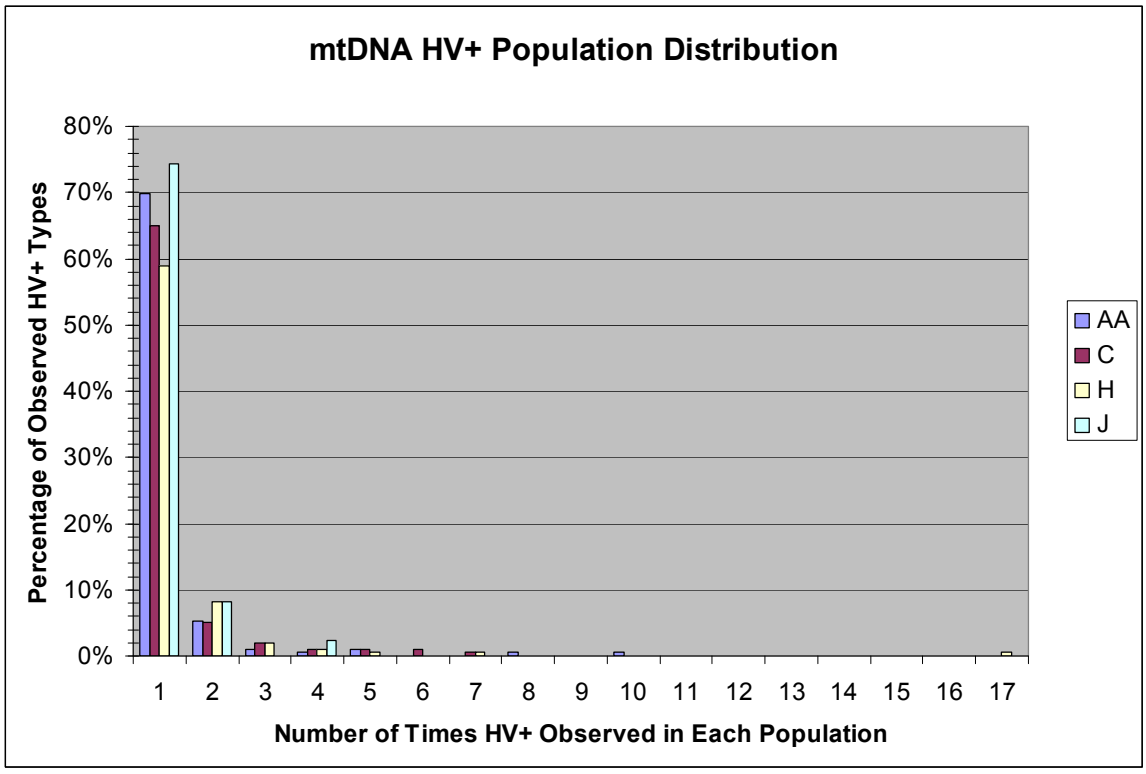
A population database was generated by amplifying and typing 674 samples from four population groups (194 African American, 197 US Caucasian, 197 US Hispanic and 86 Japanese) with the final 5-plex and 10-plex PCR and probe panels. The genetic diversity values ($h = (1-\sum freq^2) * (n/1-n)$) were calculated for each of the population groups and were compared to the values obtained with the HVI/HVII linear array assay. The h values are reported in Table 2 below for each population group for both the HVI/HVII assay and the new HV+ assay. The discrimination power was greatly improved for the Japanese, US Hispanic, and US Caucasian populations with a slight increase for the African American population for the new HV+ assay compared to the HVI/HVII assay. The discrimination power for the US Caucasian population was increased from 0.9768 with the HVI/HVII assay to 0.9946 with the HV+ assay. The discrimination power was increased from 0.9449 to 0.9893 for the US Hispanic population using the HV+ assay. The number of observed and unique mitotypes increased for all populations as well, with the greatest increases for the US Hispanic and US Caucasian populations; there was an ~35% increase in the number of observed types and an ~43% increase in the number of unique types for both population groups.

Table 2. Power of Discrimination and Number of Unique Types

Population Group (n)	# observed mitotypes			# unique mitotypes			h value	
	HVI/ HVII	HV+	% increase	HVI/ HVII	HV+	% increase	HVI/ HVII	HV+
African American (194)	137	**153**	10.5%	111	**136**	18.4%	0.993	**0.9938**
U.S. Caucasian (197)	99	**149**	33.6%	73	**128**	43.0%	0.9768	**0.9946**
U.S. Hispanic (197)	91	**141**	35.5%	67	**116**	42.2%	0.9449	**0.9893**
Japanese (86)	58	**73**	20.5%	48	**64**	25.0%	0.9806	**0.9948**

The distribution of HV+ mitotypes was also determined for each population group (see graph below). The percentage of observed HV+ types occurring one to 17 times is reported for each of the four population groups. Approximately 60-75% of the HV+ types were unique (occurring only 1 time in the specified population), ~5-8% of the HV+ types occurred twice and less than 2% of the HV+ types occurred three or more times.

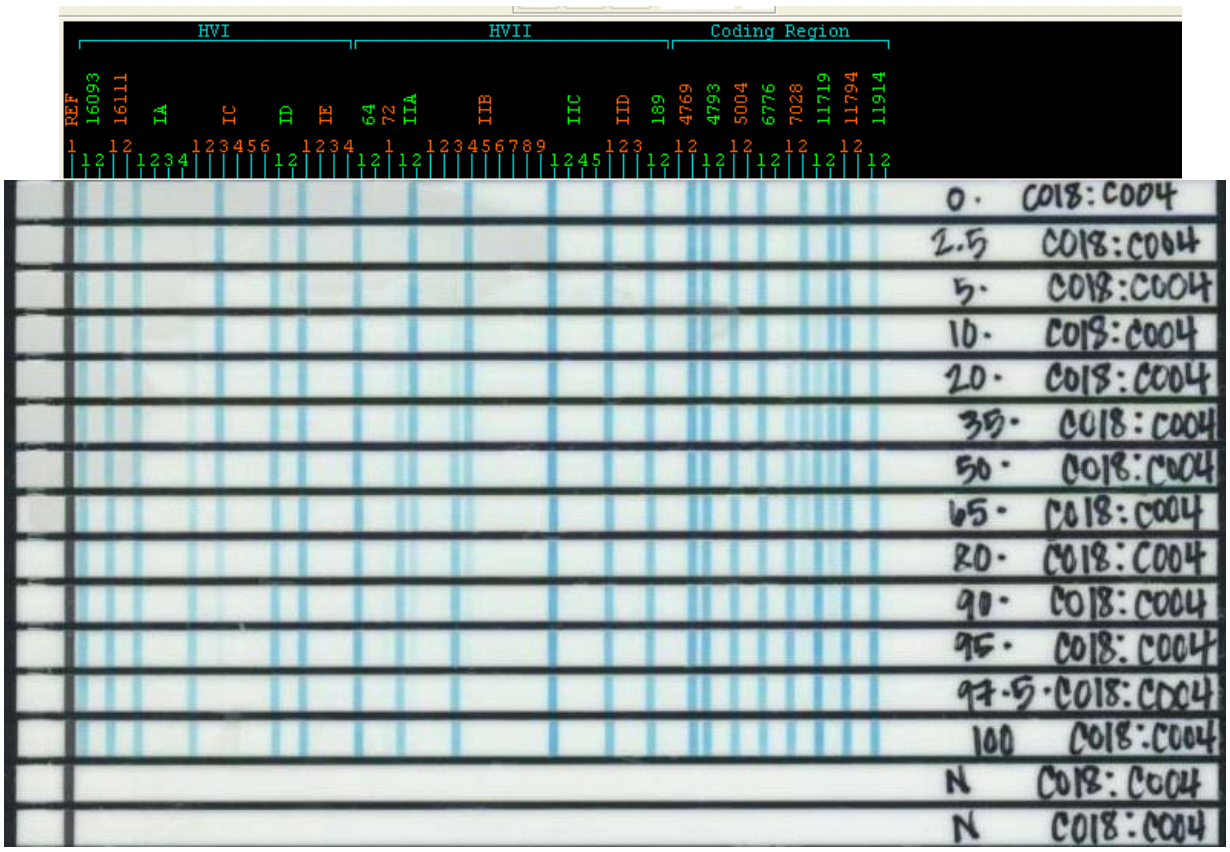
Distribution of HV+ Types in 674 Individuals from 4 Population Groups



Summary of Developmental Validation

A mixture study was conducted and it was shown that a minor component present at ~10% is reliably detected with the 5-plex assay and ~5% with the 10-plex assay. Minor components present at 5-10% were detectable with the 5-plex assay and 2.5-5% with the 10-plex, depending on the probe, with some probes being more sensitive than others (See Figures 3A-3B below).

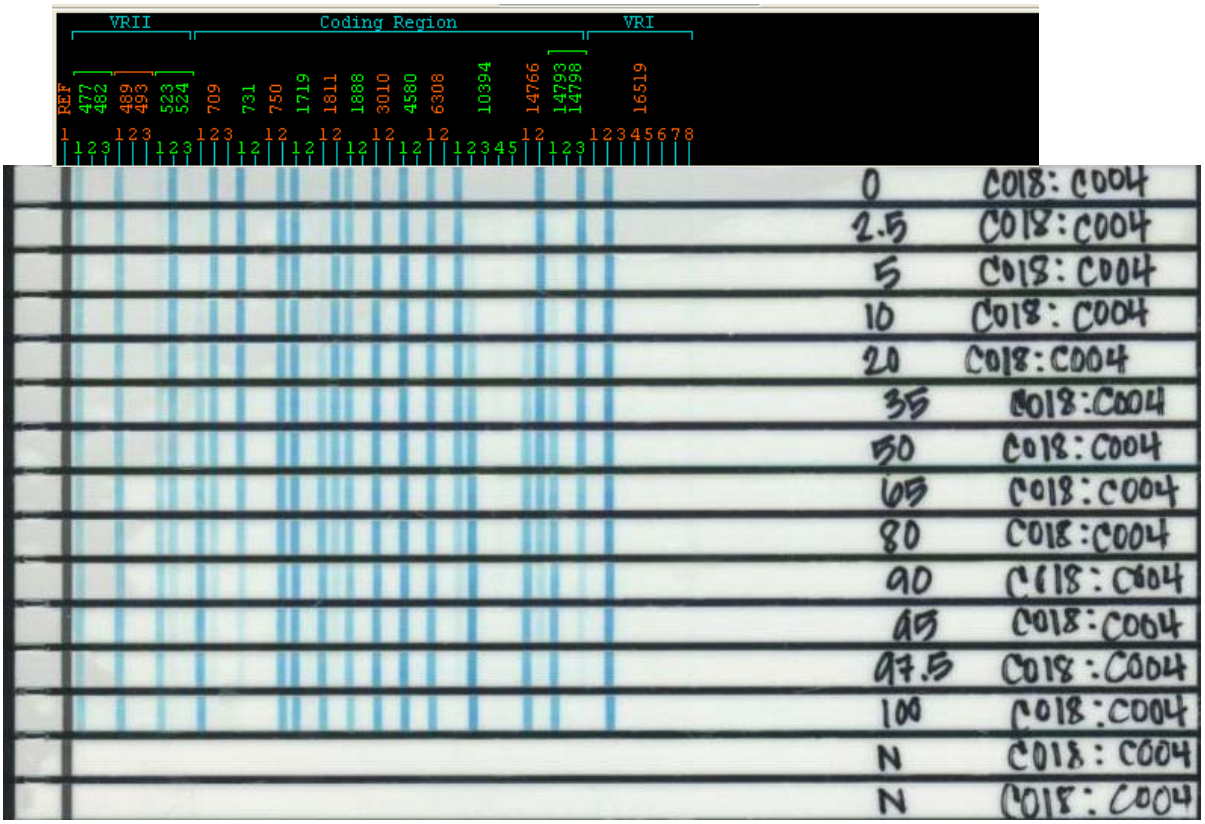
Figure 3A. 5-Plex Mixture Study Typing Results



5 Plex Mixture Study MitoReport

	HVI						HVII						Coding Region									
	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
0	1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1
2.5	1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1
5	1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1
10	1	1	1	1w,3	1	1	1	0	1w,2	3w,4	1	1	1	2	1	1	1	1w,2	1w,2	1	1
20	1	1	1	1w,3	1	1	1	0	1w,2	3w,4	1	1	1	2	1	1	1	1w,2	1w,2	1	1
35	1	1	1	1,3	1	1	1	0	1,2	3,4	1	1	1	2	1	1	1	1,2	1,2	1	1
50	1	1	1	1,3	1	1	1	0	1,2	3,4	1	1	1	2	1	1	1	1,2	1,2	1	1
65	1	1	1	1,3	1	1	1	0	1,2	3,4	1	1	1	2	1	1	1	1,2	1,2	1	1
80	1	1	1	1,3w	1	1	1	0	1,2w	3,4w	1	1	1	2	1	1	1	1,2w	1,2w	1	1
90	1	1	1	1,3w	1	1	1	0	1,2w	3,4w	1	1	1	2	1	1	1	1,2w	1,2w	1	1
95	1	1	1	1,3w	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1
97.5	1	1	1	1	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1
100	1	1	1	1	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 3B. 10 Plex Mixture Study Typing Results



10-Plex Mixture Study MitoReport

	VRII		Coding Region													VRI	
	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519	
0	1	1	1w,2	2	1	2	1	2	1	1	1	1	1	2	3	2	
2.5	1	1	2	2	1	2	1	2	1	1	1	1	1	2	3	2	
5	1	1	2	1w,2	1	2	1	2	1	1	1	1	1	2	3	2	
10	1	1	1w,2	1w,2	1	2	1	1w,2	1	1	1	1	1w,2	1w,2	1w,3	2	
20	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2	
35	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2	
50	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2	
65	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2	
80	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2	
90	1	1	1,2w	1,2w	1w	2	1	1,2w	1	1	1	1	1w,2	1,2w	1,3w	2	
95	1	1	1,2w	1,2w	1w	2	1	1, 2w	1	1	1	1	1w,2	1,2w	1,3w	2	
97.5	1	1	1	1	1w	2	1	1	1	1	1	1	2	1	1,3w	2	
100	1	1	1	1	0	2	1	1	1	1	1	1	2	1	1	2	
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

A sensitivity study was conducted and it was shown that both the 5-plex and 10-plex assays are highly sensitive assays, with the 10-plex being more sensitive. Typeable results were observed for 5 pg at 34 cycles (~730 mtDNA copies), 1 pg at 36 cycles (~150 mtDNA copies) and 0.5 pg at 38 cycles (~75 mtDNA copies) for the 5-plex assay. For the 10-plex assay, typeable results were observed for 1 pg at 34 cycles (~150 mtDNA copies), 0.5 pg at 36 cycles and 38 cycles (~75 mtDNA copies). mtDNA copy number was estimated using the quantitative PCR assay as described by (Hudlow *et al.* 2008). A subset of the typing results are shown in Figures 4A-4D below.

Figure 4A. 5-plex Sensitivity Study Results (34 cycles)

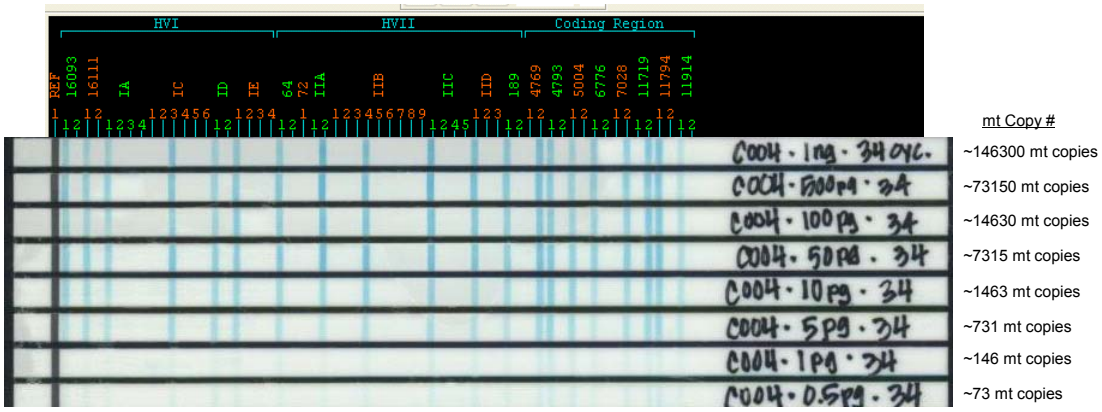


Figure 4B. 10-plex Sensitivity Study Results (34 cycles)

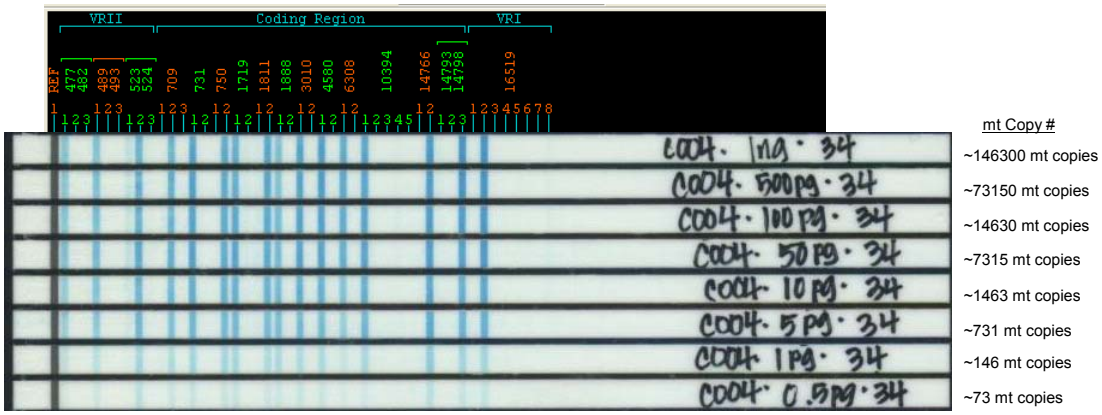


Figure 4C. 5-plex Sensitivity Study Results (38 cycles)

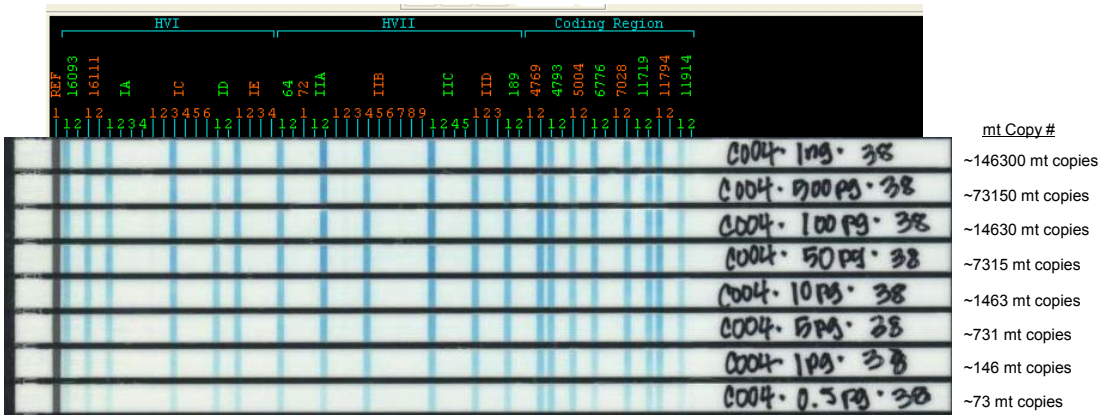
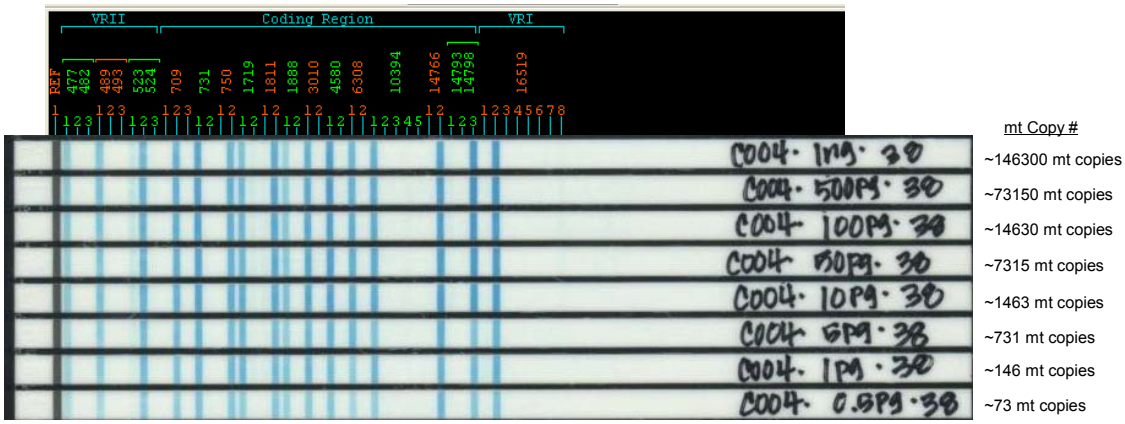


Figure 4D. 10-plex Sensitivity Study Results (38 cycles)



Summary of Developmental Validation Studies Conducted at NCFS

A subset of the studies required to satisfy the developmental validation requirement for a new PCR based assay as outlined in the SWGDAM guidelines were completed at the National Center for Forensic Science (NCFS). Validation studies completed by NCFS include species specificity, reproducibility, analysis of case type samples, precision and accuracy and environmental studies to assess stability of DNA.

Reproducibility study was conducted between the NCFS and CHORI lab. For each sample, similar amplification yields were observed between labs. The expected probe signals were observed for all regions for each of the samples typed at both labs. Additionally, similar probe signal intensities were observed for the PCR products generated at CHORI and at NCFS and typed at NCFS. Typing results were concordant between labs.

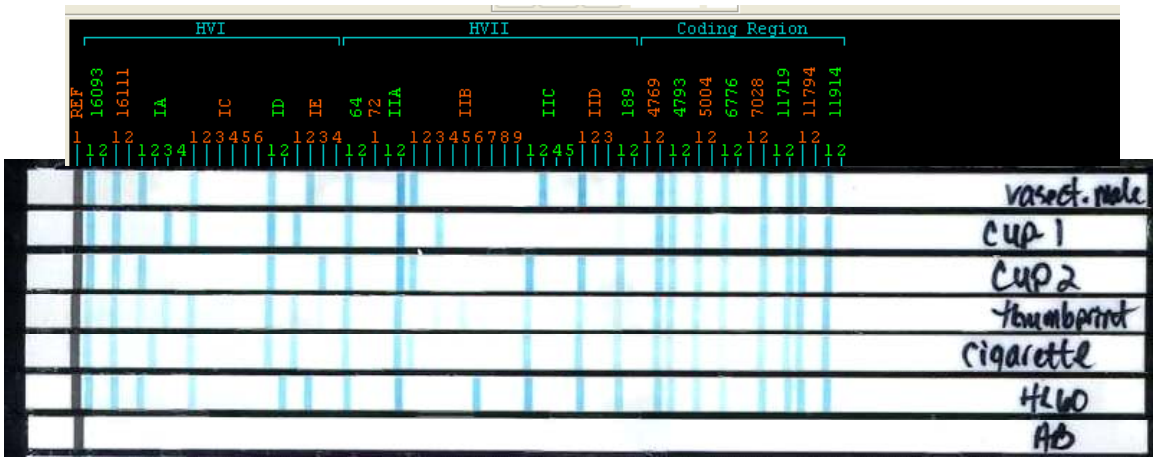
A precision and accuracy study was conducted at NCFS. All 15 targets amplified using the 5-plex and 10-plex PCR assays observed by gel electrophoresis for all five replicates from the five different individuals. Typeable results were obtained using the 5-plex and 10-plex assays for all samples. Typing results were consistent for each of the replicates for all five samples.

A species specificity study was conducted and blood derived DNA from pig, dog, rabbit, goat, mouse, cow, tortoise, ferret, and 19 primates were amplified and typed. Results for the animal and primate specificity studies showed that the probes for both the 5-plex and 10-plex probe panels are primate specific. Additionally, the primers for the 5-plex and 10-plex PCRs, with the exception of the pair targeting the 16s rRNA region (which includes site 3010) are also primate specific.

DNA extracts from samples collected from various sources including semen from a vasectomized male, menstrual blood, post coital swab and swabbed objects, skin, and fingerprint were amplified and typed using the 5-plex and 10-plex PCR and linear array probe panel. Amplification and typing was successful for the tested casework samples, including low copy number samples such as DNA swabbed from cups and thumbprints. As expected, mtDNA analysis was not useful for the analysis of sperm fractions from post-coital samples when collected using the differential extraction method as only the female type was observed. Typing results for a subset of these samples are shown in the Figures 5A-5B below.

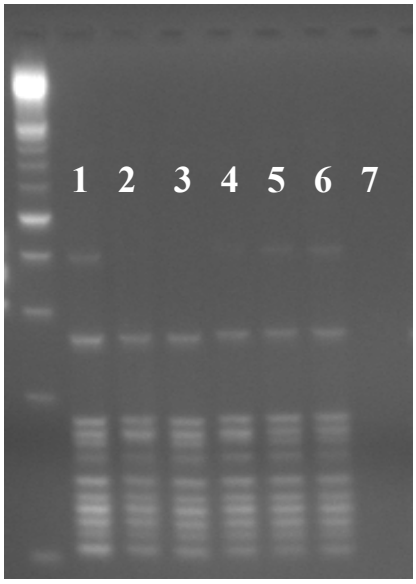
Figure 5A. Contact Samples, Vasectomized Male 5-plex Results

1. Semen, vasectomized male
2. Beverage container lid (1)
3. Beverage container lid (2)
4. Thumbprint
5. Cigarette butt
6. HL60
7. HL60

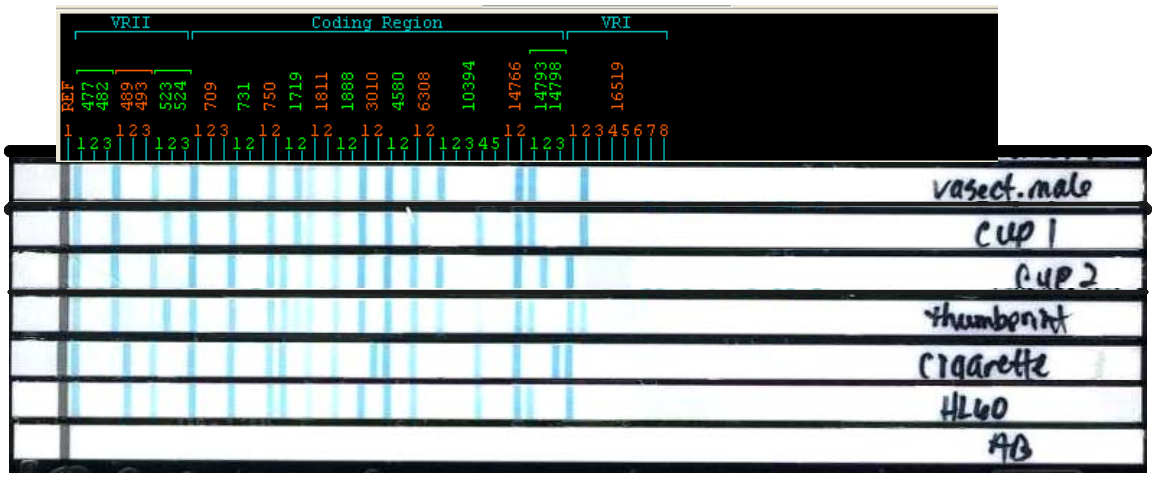


	HVI						VRI		HVII					Coding Region							
Vasect. Male, Contact Samples	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
Vasect. Male	1	1	1	1	1	2	1	0	2	1	2	1	1	2	1	1	1	2	2	1	1
Cup 1	1	1	3	1	1	1	1	0	2	3	0	0	1w	2	1	1	1	2	2	1	1
Cup 2	1	1	1	0	1	3	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
Thumbprint	1	1	1,3	1	1	1,3w	1	0	2	3w,5w	1	1	1	2	1	1	1	2	2	1	1
Cigarette	1	1	2	1	1	1	1	0	2	1	1	1	1	2	1	1	1w	2	2	1	1
HL60	1	1	1	1	2	2	1	0	2	6	1	1	1	2	1	1	1	2	2	1	1

Figure 5B. Contact Samples, Vasectomized Male 10-plex Results



1. Semen, vasectomized male
2. Beverage container lid (1)
3. Beverage container lid (2)
4. Thumbprint
5. Cigarette butt
6. HL60
7. Blank



	VRII			Coding Region												VRI
Vasect. Male, Contact Samples	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
Vasect. Male	1	1	1	1	1	2	2	1	1	1	1	1	1	2	1	2
Cup 1	1	1	2	1	1	2	2	1	1	1	1	1	4	2	1	2
Cup 2	1	1	1	1	1	2	1	1	1	1	1	1	1	2	2	1
Thumbprint	1	1,2w	1w,2	1	1	2	1,2	1	1	1	1	1	1,4w	2	1	1,2
Cigarette	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1
hl60	1	2	1	1	1	2	1	1	1	1	1	1	4	2	1	1

High-Throughput Alternatives: Automate Typing and modify Interpretation Software

Several options for automating the typing steps of the linear array procedure were explored and a protocol for use with the Tecan ProfiBlot T24 or T48 was optimized and validated procedure (Table 3).

Table 3. Typing Procedure for Use with Tecan T24 Profiblot

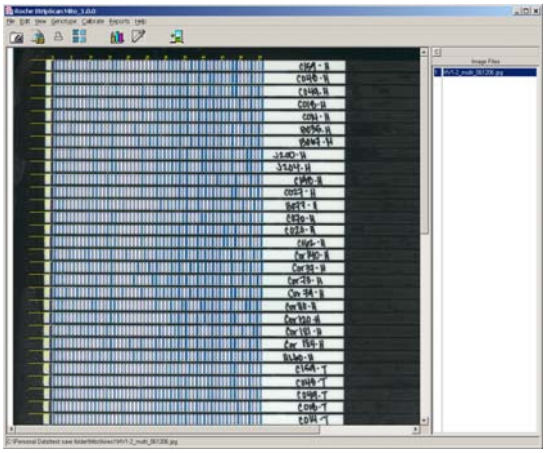
Step	File	Channel	Volume	Time	Temp
1	Temp				55
2	Disp	4	1000ul		
3	Disp	4	3000ul		
4	Pause				
5	Inc			15 min	
6	Asp				
7	Disp	2	1000ul		
8	Disp	2	3000ul		
9	Asp				
10	Disp	1	1000ul		
11	Disp	1	3000ul		
12	Inc			5 min	
13	Asp				
14	Disp	2	1000ul		
15	Disp	2	3000ul		
16	Asp				
17	Disp	2	1000ul		
18	Disp	2	3000ul		
19	Inc			12 min	
20	Asp				
21	Cool				
22	Disp	2	1000ul		
23	Disp	2	3000ul		
24	Asp				
25	Disp	5	1000ul		
26	Disp	5	3000ul		
27	Inc			5 min	
28	Asp				
29	Disp	6	1000ul		
30	Disp	6	3000ul		

Step	File	Channel	Volume	Time	Temp
31	Inc			15 min	
32	Asp				
33	Disp	3	3000ul		
34	Asp				
35	Disp	3	3000ul		
36	Asp				
37	Disp	3	3000ul		
38	END				

Channel 1 = conjugate solution
Channel 2 = wash buffer
Channel 3 = distillied water
Channel 4 = wash buffer
Channel 5 = Citrate buffer
Channel 6 = Color Development solution

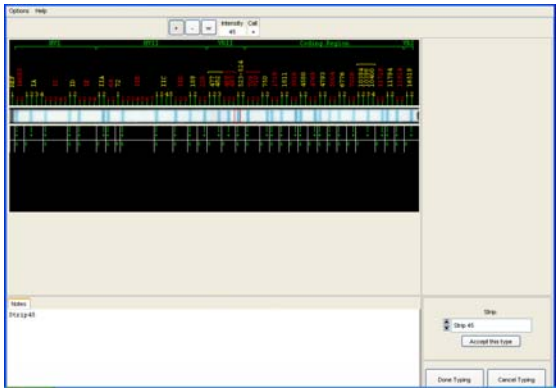
Existing StripScan software for scanning and interpreting the linear array typing results obtained from the 5-plex and 10-plex assays was also modified. An overview of the software is provided in the Figure 6 below. Several features were added to the software including a modification to allow the user to change the colors of the reference guide and backgrounds. Also, a feature to generate a mitoreport in excel was added.

Figure 6. StripScan MitoTyper 1.0 Software Overview



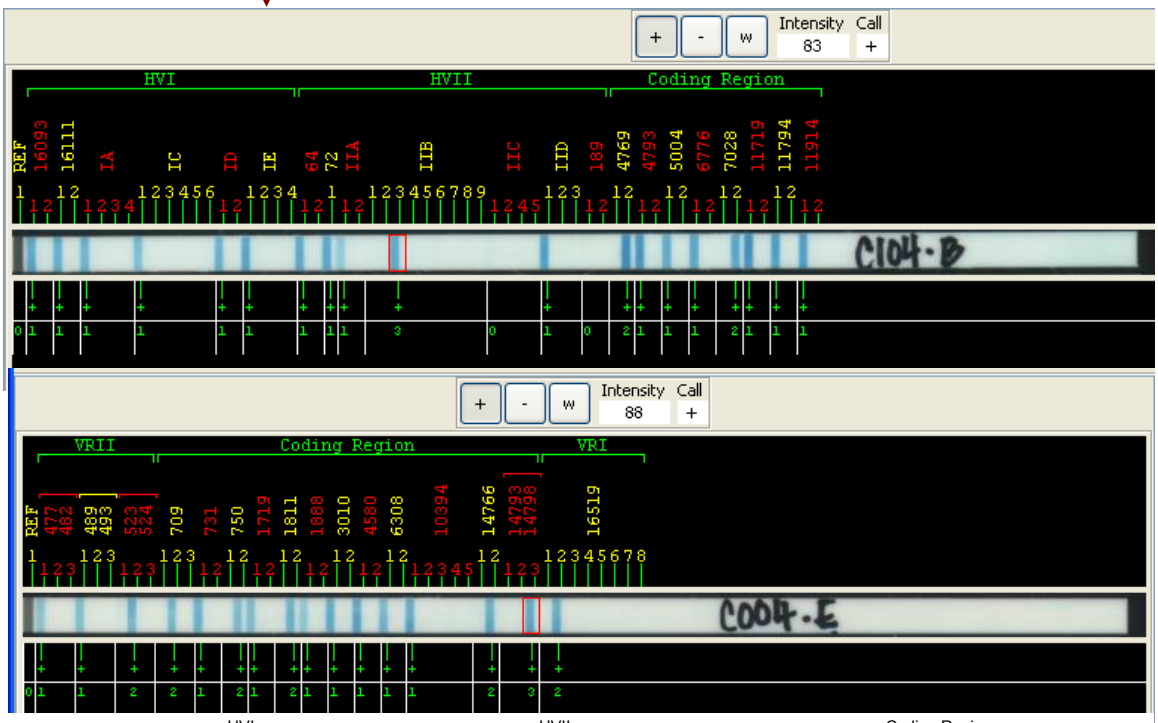
1. Batches of up to forty-eight strips are assembled on a custom holding plate, and digitally imaged using a common flatbed scanner

2. StripScan measures the signal intensity for each probe on the strip then interprets the probe signals using genotyping algorithms



3. The probe patterns are reviewed by lab personnel experienced in typing, and can be overidden as needed

4. After all strips in an image are reviewed, custom designed reports are generated indicating signal intensities and selected probe calls for each strip



	HVI								HVII					Coding Region							
	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
B063-B	1	1	4	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
B063-E	1	1	4	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
C055-B	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
C055-E	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
C062-B	1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	2	1	1	1	1
C062-E	1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	2	1	1	1	1
C070-B	1	1	2	1	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
C070-E	1	1	2	1	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1

Conclusion

We successfully identified a highly informative set of polymorphic sites and developed a highly sensitive, easy to use 5-plex and 10-plex PCR and linear array assay for simultaneous analysis of polymorphic regions in the non-coding and coding regions. A population study was conducted in order to determine the power of discrimination for the new expanded HV+ array and results show that the informativeness was improved for all population groups tested. Also, a significant increase in the power of discrimination was observed for both the US Caucasian (0.9946 from 0.9768) and the US Hispanic (0.9893 from 0.9449) and the discrimination power approached that of or exceeded HVI/HVII sequencing. A sensitivity study was conducted and it was shown that both the 5-plex and 10-plex assays are highly sensitive assays, with the 10-plex being more sensitive. Typeable results were observed with ~5 pg of DNA input for the 5-plex at 34 cycles and ~1pg input for the 10-plex. At 38 cycles, ~0.5 pg of DNA input yielded typeable results for both the 5-plex and 10-plex assays. Results from a mixture study show that a minor component present at ~10% is reliably detected with the 5-plex assay and ~5% with the 10-plex assay. The 5-plex and 10-plex probe panels were shown to be primate specific and all primers were shown to be primate specific except the pair targeting the 16s rRNA region. Various case type samples (including low copy number samples) were also successfully amplified and typed by the NCFS. Additional collaborative studies are ongoing and include analysis of bone and hair samples. A procedure was also validated for automating the typing assay and scanning and interpretation software were modified for use with the 5-plex and 10-plex assays. Up to

48 samples can be typed manually or automated in less than two hours and the data can now be quickly analyzed using the Strip Scan Mitotyper software.

1. Main Body

I. Introduction

A. Statement of the Problem:

PCR-based DNA typing tests for the detection of nuclear DNA (e.g. AmpFLSTR Identifiler, Profiler, and Cofiler and AmpliType® HLA DQA1 and PM) have been particularly useful in the analysis of limited and/or degraded samples. However, in some cases the nuclear DNA is too degraded or limited for even the most robust of nuclear gene amplification and detection systems, including miniSTRs (Holland *et al.* 1993). In these cases, mtDNA analysis can be a valuable tool. Both sequence based and probe based systems targeting the hypervariable regions (HVI and HVII) of the mitochondrial genome are currently being used by forensic laboratories (Gabriel *et al.* 2003; Divne *et al.* 2005; Melton *et al.* 2005).

While mtDNA is an informative and useful marker, there are some inherent limitations to targeting only the HVI and HVII regions independent of the method of analysis. To overcome the limited power of discrimination that primarily results from a few relatively common HVI/HVII sequences, additional sequence variation in the variable and coding regions needs to be identified and assays targeting this variation need to be developed. To address this need, we identified a set of highly informative polymorphic sites distributed throughout the mitochondrial genome and developed a sensitive, robust multiplex PCR and Linear Array typing system. This system allows for simultaneous analysis of hypervariable region *and* coding region sites in a single assay. Primers and probes targeting these sites were designed and optimized to work under the

existing amplification and typing conditions of the HVI/HVII linear array assay. The final version of the HV+ assay consists of 15 primer pairs and 105 immobilized probes targeting 61 polymorphic sites. Polymorphic sites to subdivide common HVI/HVII types as well as some rapidly evolving polymorphic sites were targeted to help improve the discrimination power. Also several HVI and HVII sites were added to improve the robustness of the HVI/II Linear Array Assay by reducing the number of '0' and 'weak' signal types caused by destabilizing probe mismatches.

B. Review of Relevant Literature

a. Mitochondrial DNA

Mitochondria are organelles, present in multiple copies in the cellular cytoplasm, which are responsible for cellular respiration. Multiple copies of the mtDNA genome are contained within each organelle (mitchondrion). Alleles of the nuclear genes typed by the existing PCR-based tests are present in only one (spermatozoa and eggs) or two copies per cell, whereas mtDNA sequences can be present 100-1000 copies per cell (Robin & Wong 1988). Since PCR amplification generally requires that the region of DNA containing the sequence of interest be intact (i.e. not degraded), the chances of obtaining a result from a severely degraded sample are significantly increased when mtDNA is typed, simply because of its abundance relative to single copy nuclear DNA markers. The high copy number of the mtDNA genome also frequently allows a result to be obtained from samples that have too little DNA to yield a result for a nuclear gene marker (e.g. telogen hairs). On occasion in some cells, more than a single unique mtDNA sequence can be observed within a cell or group of cells of a single individual. This

phenomenon is known as heteroplasmy. The level and extent of heteroplasmy has been extensively characterized in normal individuals (Bendall *et al.* 1997; Calloway *et al.* 2000; Tully *et al.* 2000; Stewart *et al.* 2001; D'Eustachio 2002) and these studies have provided valuable information in overcoming interpretation challenges for forensics (Butler & Levin 1998).

Another unique feature of the mtDNA genome that makes it particularly suitable for the analysis of biological remains, hairs, bone, teeth, and extremely limited or degraded DNA samples is that it is maternally inherited (Giles *et al.* 1980). This mode of inheritance makes it a valuable genetic marker for the investigation of missing person cases because the subject's mother and siblings, as well as the mother's siblings (uncles and aunts), will all carry the same mtDNA sequence as that of the subject. Consequently, samples from maternally related individuals can be used as reference samples for the missing person.

The mitochondrial genome is a small, circular molecule of about 16,569 bp. Unlike the human nuclear genome, the mtDNA genome is predominately coding sequence, approximately 15400 bp (greater than 90%). Additionally, the sequence shows extreme economy in that the genes have none or only a few noncoding bases between them, and in many cases the termination codons are not coded in the DNA but are created post- transcriptionally by polyadenylation of the mRNAs. Genes for 2 rRNAs (12S and 16S), 22 tRNAs, and 13 proteins (including cytochrome c oxidase subunits I, II and III, ATPase subunit 6, cytochrome b) make up the entire mitochondrial coding region. The remainder of the genome, the control region (or D-loop region), is an ~1120 bp region of noncoding DNA which contains one origin of replication and both origins of

transcription, as well as additional transcription and replication control elements (Anderson et al. 1981).

The sequence variation in the mtDNA genome has been well characterized. Since the first sequence was determined in 1981 (Anderson et al. 1981), thousands of HVI/II sequences (Budowle *et al.* 1999; Imaizumi *et al.* 2002) and more recently thousands of full mitochondrial genome sequences have been collected (Herrnstadt *et al.* 2002; Ingman & Gyllensten 2006). The mtDNA nucleotide sequence evolves six to 17 times faster than comparable nuclear DNA gene sequences, reflecting a replication apparatus of lower fidelity (Howell *et al.* 1996). Consequently, the entire mtDNA genome is highly polymorphic. However, some parts of the genome are more mutable and evolve faster than others, leading to an unequal distribution of polymorphisms. The non-coding control region has the highest rate of evolution and evolves approximately 10 times the rate of the gene-coding region (Parsons & Coble 2001). For this reason, the greatest concentration of sequence polymorphism lies within the control region, specifically within two hypervariable regions, HVI and HVII (Aquadro & Greenberg 1983; Greenberg *et al.* 1983). The HVI and HVII regions are typically targeted for forensics identification purposes because of the high density of sequence variation.

b. Approaches for Mitochondrial DNA Analysis

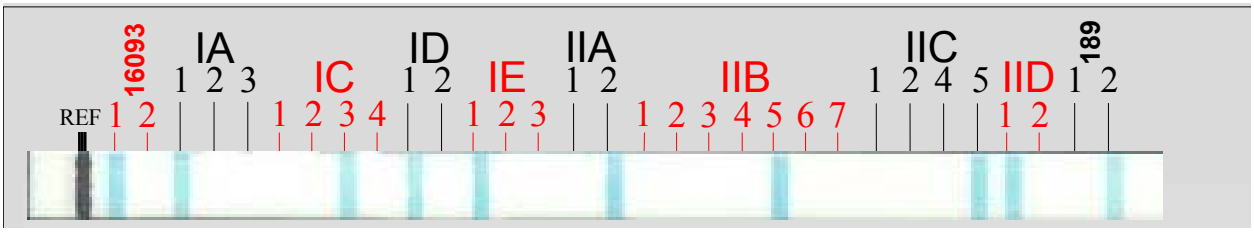
Several approaches have been used to analyze the HVI/II regions of mtDNA for forensics and include sequence based methods: Sanger sequencing and pyrosequencing (Wrischnik *et al.* 1987; Sullivan *et al.* 1991; Ginther *et al.* 1992; Sullivan *et al.* 1992; Holland *et al.* 1993; Budowle *et al.* 1999) and probe based methods: conventional dot blot, Luminex and LINEAR ARRAY (Allen *et al.* 1998; Reynolds *et al.* 2000; Gabriel *et*

al. 2001a). Sanger sequencing is currently the most widely used method for mtDNA analysis in Forensic cases. This procedure requires amplification of both HVI and HVII regions and is currently performed separately in two to four amplification reactions depending on the quality of DNA. If the DNA is extremely degraded, some laboratories take an approach to amplification and sequencing that has been used for studies of ancient DNA, which uses a series of primers that target very small overlapping segments of the HVI and HVII regions (Gabriel *et al.* 2001b). This strategy may involve as many as eight or more reactions and consumes an even greater amount of the often limited sample material.

c. LINEAR ARRAY mtDNA HVI/HVII Region-Sequence Typing Kit

The LINEAR ARRAY mtDNA HVI/II Region-Sequence Typing Kit (Roche Applied Science, Indianapolis, IN) is an alternative approach to mtDNA analysis which uses multiplex PCR and the immobilized probe technology. The HVI/HVII linear array assay consists of two primer pairs for co-amplification of HVI and HVII PCR products and 33 probes immobilized in 31 lines for detection of sequence variation at 18 positions spanning both hypervariable regions. The mtDNA linear array typing results for one individual (with mitotype 1131125512) are shown below in Figure 1.

Figure 1. HVI/HVII Linear Array Typing Results



Unlike standard amplification procedures for mtDNA sequencing analysis, the HVI/II regions are co-amplified, thereby reducing the amount of DNA material consumed during amplification by 50 to 75% as well as PCR set-up time. In addition, less than 50% (~20 of the 50 μL) of the PCR product generated with the assay is consumed during the quantification and hybridization steps, leaving the remainder of the PCR product for subsequent sequence analysis if needed (enough for forward and reverse sequencing of both HVI and HVII regions). High quality DNA sequence has been consistently obtained using PCR product generated from the duplex reaction (Date Chong *et al.* 2005). This PCR system has been optimized to ensure both products amplify with approximately equal efficiency, primer dimer formation is minimized, and non-specific products are rarely generated. A higher amount of DNA polymerase is used in the amplification to increase sensitivity and reduce the effect of potential PCR inhibitors. Probes were optimized to minimize cross-hybridization, increase sensitivity and give approximately equal signal intensity within a hypervariable region.

One advantage of using the linear array technology is that it requires only a thermal cycler and a rotating water bath and can be implemented by any laboratory since it is simple to adopt and provides a low-budget solution to mtDNA typing compared to DNA sequencing. Significant advances in automation of typing as well as 'reading' of the linear arrays have been made. For high-throughput laboratories, automated instrumentation can be used to improve throughput. To improve throughput of data analysis, software and hardware for scanning the developed "strips" and inferring a genotype are also available. Thus, screening samples with the linear array probe panel manually or using automated instrumentation could provide useful information within a

day and decrease the number of specimens that need to be sequenced (Kline *et al.*; Gabriel *et al.* 2003).

The mtDNA HVI/II linear array can be used as a screening tool to eliminate suspects quickly and to identify samples requiring further genetic analysis (i.e. sequencing). For example, suspects and specimens (e.g. multiple hairs) associated with a specific case can be rapidly screened with immobilized sequence-specific oligonucleotide (SSO) probes. Samples from suspects who cannot be excluded and specimens that match the reference sample by the immobilized probe linear array could then be sequenced, if desired. This mtDNA HVI/II amplification and linear array system and strategy has been used successfully in Sweden for casework samples for several years (Allen *et al.* 1998). This assay has proven to be an effective tool for analyzing the origin of hairs shed at a crime scene and reducing the number of samples necessary for sequencing. The system also has been used successfully to identify remains from a mass grave site in Croatia (Gabriel *et al.* 2001b). While this HVI/HVII typing system has proven to be useful for forensic applications, further improvements can be made by adding primers and probes to increase the informativeness of the assay.

C. Rationale for Research

While targeting the HVI and HVII regions of the mtDNA genome is highly informative, there are some inherent limitations regardless of the method of analysis. Although the overall distribution of mtDNA HVI/II sequences is highly skewed towards rare types, there are some common types among all populations (Parsons & Coble 2001). Approximately 7% of Caucasians share the same common HVI/II sequence (differing from the Anderson reference sequence at 263G) and 13 additional sequences are shared

among >0.5% of the population (Parsons & Coble 2001). A greater percentage of individuals share the same mtDNA linear array types (mitotypes) because not all sites within the HVI/II regions are targeted with the linear array kit. Approximately 10% of Caucasians share the most common mitotype (1111111111). Sequencing the entire HVI/II region can discriminate somewhat between these mitotypes but the problem posed by relatively common sequences remains. Therefore, as Parsons and Coble (2001) have noted, the greatest limitation for current mtDNA testing, based on HVI/II, lies within the small number of common mtDNA types for which the power of discrimination is low. To increase the discrimination power of mtDNA testing and to further distinguish individuals who share a common HVI/II sequence, we and others have proposed to target regions outside the hypervariable D-loop regions.

Although the greatest concentration of polymorphisms lies within HVI/II regions, the remainder of the genome is much larger, making up greater than 90% of the genome and therefore contains a large portion of the total mtDNA variation. The remainder of the non-coding control region, referred to as the variable regions VRI and VRII, contains additional polymorphic stretches. This region has been targeted by sequence analysis by laboratories when the common HVI/II sequence has been observed. Again, additional sequence reactions require additional sample material, which may be limited in availability. Sequence analysis has revealed that several sites within the VR regions (specifically 16519 and 72) are highly informative and can be targeted by probes utilized in a linear array assay (Gabriel *et al.* 2001a). While sequencing the VRI/II regions has proven to increase discrimination and aid in distinguishing individuals with the common

haplotype, a large number of individuals share the entire non-coding region (which includes both HV and VR regions) (Gabriel *et al.* 2001a; Parsons & Coble 2001).

To complement HVI/HVII sequence analysis, coding region single nucleotide polymorphism (SNP) assays have been developed using the SNaPshot technology (Vallone *et al.* 2004; Brandstatter *et al.* 2006; Kohnemann *et al.* 2008; Parson *et al.* 2008). This primer extension technology can be used for the simultaneous analysis of ~10-20 SNPs (Brandstatter & Parson 2003). However, the technology is primarily limited to targeting SNPs or sequence regions with a low density of polymorphism and therefore, SNaPshot assays targeting sites within the hypervariable regions would be challenging to develop. For this reason, an initial sequence analysis of the HVI/II regions is required prior to analysis with one or more coding region SNaPshot assays. Also, because of the technology limitation, multiple SNaPshot assays would be required to target the same number of SNPs as the Linear Array.

II. Research Design and Methods

A. Research Goal

The primary goal of this research was to develop a highly sensitive multiplex PCR amplification system and linear array probe panel to target the most polymorphic regions of the mitochondrial genome. This assay would allow simultaneous amplification and genotyping of polymorphisms within multiple coding and non-coding regions of the mtDNA genome enhancing the power of discrimination of current mtDNA systems. The specific aims of this project were to 1) finalize a panel of informative polymorphic sites, 2) design and optimize multiplex PCR amplification system, 3) design and optimize linear array probe panel, 4) generate a population database and conduct

validation studies, and 5) optimize protocols for automated typing instruments and modify existing scanning and interpretation software for use with this assay.

III. Summary of Results

A. Identifying a Panel of Informative Polymorphic Sites

Our first objective was to identify polymorphic sites outside of the commonly targeted hypervariable regions of the mtDNA genome that would increase the discrimination power of the HVI/II probe panel for all major US population groups. The power of discrimination (h value) for the mtDNA HVI/II probe panel was relatively high for the African American and Japanese populations. The lowest discrimination power was observed for the US Hispanic and Caucasians groups. For this reason, we primarily focused on identifying sites to improve the discrimination power for these two population groups.

A large number of individuals having a few common HV types primarily accounted for the lower discrimination power in these two populations. Therefore, we first focused our efforts on identifying sites to help subdivide the most common HVI/HVII types by targeting variation outside the HV regions. To determine sites to improve the discrimination power for the US Caucasian population, the complete mtDNA sequence for 32 samples with the two most common HVI/HVII types was determined using Sanger sequencing. The amplification and sequencing strategy taken by Ingman et al. (2001) was used to generate full mitochondrial genome sequence data. The mitochondrial genome was amplified in 24 reactions resulting in amplicons ~800 bp in size. Candidate polymorphic sites were identified by maximum parsimony analysis. The

overall frequency of the variable sites in the mtDB sequence database (Ingman and Gyllensten 2006) was also considered when selecting candidate sites. In addition, the complete mtDNA sequence of ~65 US Hispanic samples with the two most common HVI/HVII types was determined using the Affymetrix mtDNA resequencing Chip (data reported elsewhere). Candidate polymorphic sites were indentified by phylogenetic analysis and the overall frequency of the candidate sites in the global population was also considered when selecting these sites.

Additional candidate polymorphic sites were identified from the published and unpublished full mtDNA sequence data provided by Armed Forces DNA Identification Laboratory (AFDIL) with the goal of maximizing the power of discrimination for the most common HV types as well as for a global population (Just *et al.* 2004). Rapidly evolving sites were also selected (homoplasic sites) in order to further subdivide multiple haplogroups, resulting in an increased discrimination power for the global population. Additional sites targeting sequence variation in the hypervariable regions were also targeted in order to decrease the number of 'blanks' or weak signal types found in the HVI/HVII region and to improve the robustness of the assay.

B. Other Considerations for Final Polymorphism Selection

The location of the position and base substitution's effect on amino acid were considered when selecting the candidate polymorphic sites as well as the final panel of sites. Whenever possible, silent (synonymous) substitutions over replacement (non-synonymous) mutations were selected. Polymorphic sites with known disease associations were avoided. All polymorphic sites in the final panel are synonymous

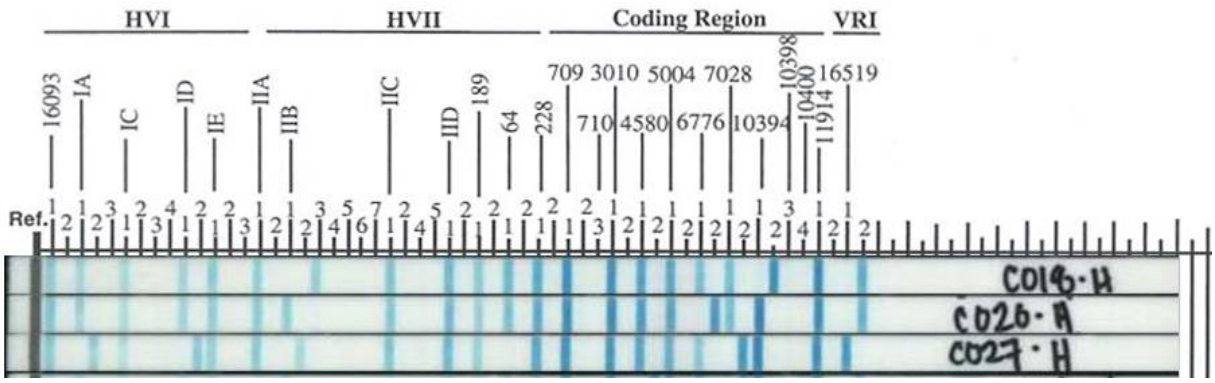
substitutions, do not code for amino acids, or otherwise not reportedly associated with any disease.

Finally, polymorphic sites were chosen to minimize the number of targeted regions to reduce the number of required primer pairs in the multiplex. For example, if two SNPs were equally informative (linked), but one could be co-amplified with a SNP already chosen for the final panel while the other would require an additional primer pair in the multiplex PCR, the SNP close in proximity to another selected SNP was chosen.

C. Sites Targeted by the 56 Probe Panel

Candidate polymorphic sites were identified from the analysis of complete mtDNA genome sequence data collected in our lab, by AFDIL and published data. Additional SNPs sites were identified from the analysis of our HVI/HVII sequence database. From these candidate sites, 14 polymorphic sites were selected for this first version of the assay for their potential to increase the discrimination power of the HVI/HVII linear array assay. A majority of these 14 polymorphic sites were selected for their ability to further distinguish samples with common Caucasian HVI/II types. A total of 25 probes are required to capture the variation for these 14 sites or nine regions (in addition to the HVI/HVII regions). Of the 14 sites, two are located in the HVII region (64, 228), one is located in the VRI region (16519) and 11 are located in the Coding Region (CR) (709, 710, 3010, 4580, 5004, 6776, 7028, 10394, 10398, 10400, and 11914). To capture the sequence variation at sites 709 and 710 requires three probes and for 10394, 10398, and 10400 four probes are required, all other sites require only two probes. Representative typing results for the 56 probe panel are shown in Figure 2 below.

Figure 2. 56 Probe Panel Typing Results



a. Power of Discrimination of the 56 Probe Panel

A total of 120 samples from both the US Caucasian and US Hispanic populations were typed using the 56 probe panel. Genetic diversity values as well as the number of different and unique types for each population group are reported in Table 1 below.

Table 1. Genetic Diversity Values for US Caucasian and US Hispanic Populations

U.S. Caucasian (n=120)	# different mitotypes	# unique mitotypes	h value
HVI/HVII mitotype	67	46	0.9775
56 probe panel (HVI/HVII mitotype + coding region)	88	69	0.9934
HVI/HVII sequence	94	76	0.9947
56 probe panel + HVI/HVII sequence	100	85	0.9965

U.S. Hispanic (n=120)	# different mitotypes	# unique mitotypes	h value
HVI/HVII mitotype	52	35	0.929
56 probe panel (HVI/HVII mitotype + coding region)	72	51	0.983

Results from this population study show that the overall genetic diversity (h value) can be increased following the addition of the 25 new probes. When typed with only the HVI/HVII mtDNA linear array an h value of 0.978 is obtained for this US Caucasian population. Using the 56 probe panel assay, the h value is increased to 0.993, which is nearly as informative as the h value obtained from HVI/HVII sequencing (0.995) for US Caucasians. Results from this study also show that with the 56 probe panel, the two most common Hispanic HVI/HVII types can be further distinguished and genetic diversity (h value) can be increased. When the 56 probe panel assay is used, the most common Hispanic HVI/HVII type can be further subdivided into ten groups. Similarly, the second common Hispanic HVI/HVII type can be further subdivided into five smaller groups. Results also indicate that when these 120 US Hispanic samples are typed with only the LINEAR ARRAY HVI/HVII assay (31 probes) an h value of 0.929 is obtained. However, with the 56 probe panel assay, the h value is increased to 0.983. While the discrimination power was greatly improved by the addition of the 25 probes for the US Caucasian population, additional sites were required to further improve the discrimination power for the global population (specifically for the US Hispanic population).

D. Sites Targeted with the 83 Probe Panel

A total of 83 probes and one reference line can be deposited on the nylon membrane with the current striping technology. Therefore, 27 probes could be added to the 56 probe panel described above. Additional polymorphic sites were identified from the analysis of HVI/HVII and complete mtDNA genome sequence data collected in our

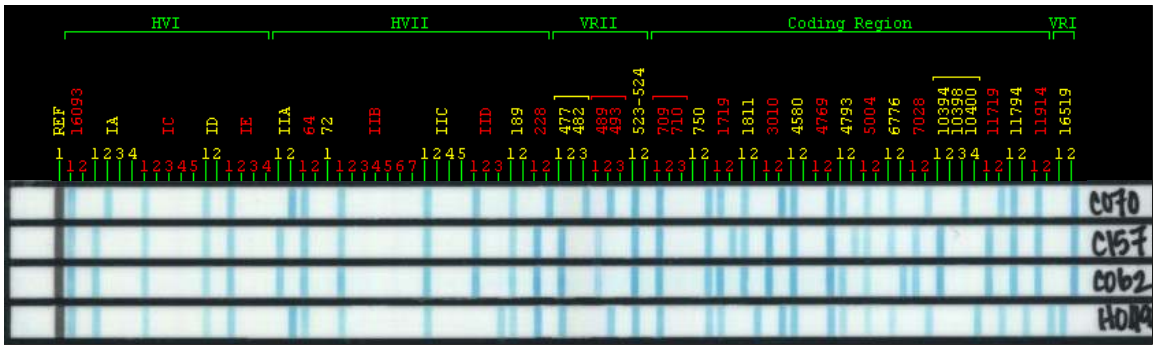
lab, by AFDIL and published data. Twelve additional sites to further subdivide common HV types observed in the Caucasian or Hispanic population (common haplogroups) were identified and added to the probe panel as well as several homoplasic sites (fast sites which have mutated multiple times and are variant in more than one haplogroup). Of these 12 sites, five are located in the VRII region (477, 482, 489, 493, 523-524) and seven are located in the coding region (750, 1719, 1811, 4769, 4793, 11719, 11794). A total of 22 probes were required to capture the variation at these 12 sites. To capture the sequence variation at sites 477 and 482 as well as sites 489 and 493 requires three probes which span the two sites; all other sites require two probes. All probes target a point mutation with the exception of the probes targeting the CA repeat region (523-524). With this version of the assay a CA deletion results in a positive 2 probe, a CA insertion results in a weak 1 probe and no change results in a positive probe 1. Probe sequence modifications as well as the addition of a CA insertion probe were made to the probes in this region for the final assay to increase specificity.

An additional five probes targeting four new sites were added to the existing HVI/II probe panel in order to decrease the frequency of weak and no signal (types 0's) which can be observed when a destabilizing mismatch is present within the probe binding region. These five probes were designed to target sequence variants commonly observed in the IA, IC, IE, IIA, or IID region which produce a weak or no signal. The IA4 probe targets a new variant site 16126 (previously IA 0) and the IC5 probe targets a new variant site 16319 (previously IC 0). The IE4 probe targets a sequence variant having both the 16270 and 16278 mutations (previously IE 0). The IID3 probe targets a

deletion at position 249 (previously IID 0). A probe to distinguish a 72C sequence variant was also added (previously IIA w1).

This 83 probe panel targets a total of 48 sites: 40 probes targeting 24 sites in HVI/HVII (HVI: 16093, 16124, 16126, 16129, 16270, 16278, 16304, 16309, 16311, 16319, 16362; HVII: 64, 72, 73, 146, 150, 152, 189, 195, 198, 200, 228, 247, 249), eight probes targeting five sites in VRII (477, 482, 489, 493, 523-524), 33 probes targeting 18 sites in the coding region (709, 710, 740, 1719, 1811, 3010, 4580, 4769, 4793, 5004, 6776, 7028, 10394, 10398, 10400, 11719, 11794, 11914), and two probes targeting one site (16519) in the VRI region. Representative typing results are shown in Figure 3 below.

Figure 3. 83 Probe Panel Typing Results



a. Power of Discrimination of the 83 Probe Panel

To evaluate the 83 probe typing system and to determine the overall discrimination power of the assay, 120 US Caucasian and 120 US Hispanic samples were typed. Results show that the power of discrimination of mtDNA linear array typing was significantly improved for both populations with the additional probes and primers (see

Table 2 below). For the US Hispanic population, the h value was significantly increased from 0.936 with the HVI/HVII linear array to 0.986 with the 83 probe panel and for US Caucasians, the h value was increased from 0.979 to 0.996 with the 83 probe panel. We also found that the 83 probe panel was more informative than HVI/HVII sequencing for this Caucasian population based on the higher h value observed with the 83 probe panel (h= 0.996 compared to h=0.995). Overall, this 83 probe assay was proven to be much more informative than current mtDNA HVI/HVII assays.

Table 2. Genetic Diversity Values for US Caucasian and US Hispanic Populations

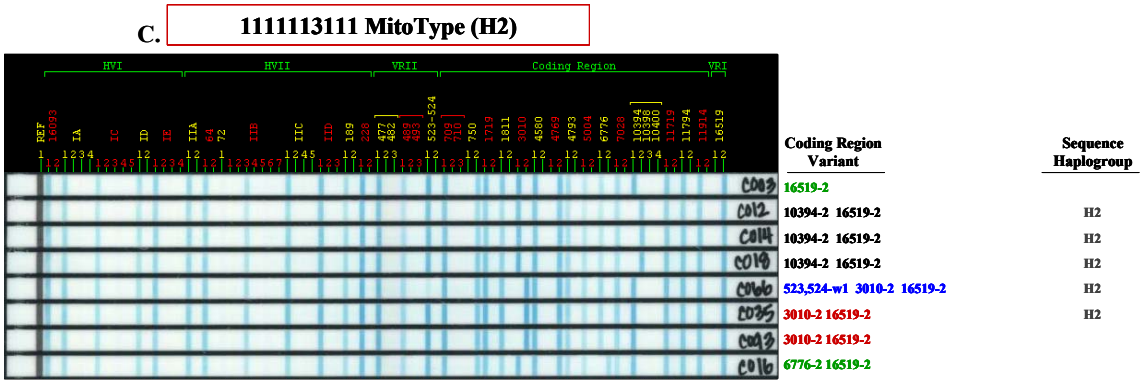
U.S. Causcasian	# different mitotypes	# unique mitotypes	h value
HVI/HVII mitotype	67	46	0.9793
83 probe panel	100	86	0.9964
HVI/HVII sequence	94	76	0.9947
83 probe panel + HVI/HVII sequence	106	94	0.9978

U.S. Hispanic	# different mitotypes	# unique mitotypes	h value
HVI/HVII mitotype	56	40	0.9361
83 probe panel	82	65	0.9864
HVI/HVII sequence	97	85	0.9941
83 probe panel + HVI/HVII sequence	103	93	0.9945

The three figures below show that HVI/HVII common mitotypes can be subdivided with the 83 probe panel. Panel (A) shows the common Caucasian HVI/II mitotype (1111111111), shared by 11% of Caucasians. When additional probes targeting informative polymorphic sites outside the HVI/II region are added, this type can be further subdivided into eight types. Panel (B) shows that the common 112111111 mitotype can be subdivided into four unique types and that sequence haplogroup H5 can be divided into three unique coding region variant types. Also, Panel (C), the second most common mitotype in Caucasians, can be further subdivided into five separate coding region variant types and we can further subdivide sequence haplogroup H2 into three separate coding region variant types. Sequence haplogroups refer to those identified by Coble et al. (2004).

Figures 3A-C. Subdivision of Common Mitotypes Using the 83 Probe Panel

Figures 3A-C. Subdivision of Common Mitotypes Using the 83 Probe Panel (cont.)



Panel (D) shows that 1111111111 HVI/HVII mitotypes can be further subdivided into five unique coding region variant types. Also, the H1 haplogroup can be further subdivided into two unique coding region variant types (Figure 3D). Panel (E) shows that common Hispanic HVI/HVII mitotype (1111121131) shared by 15% of Hispanics, can be further subdivided into seven coding region variant types. Also, the C1-2 haplogroup, as determined by HVI/HVII sequencing, can be further subdivided into three unique coding region variant types (Figure 3E). Sequence haplogroups refer to those identified by Coble et al. (2004).

Figure 3D. Subdivision of H1 Sequence Haplogroup Using the 83 Probe Panel

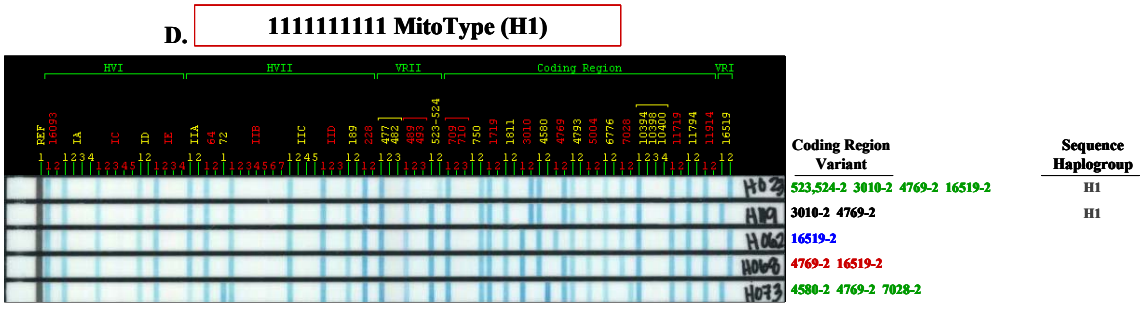
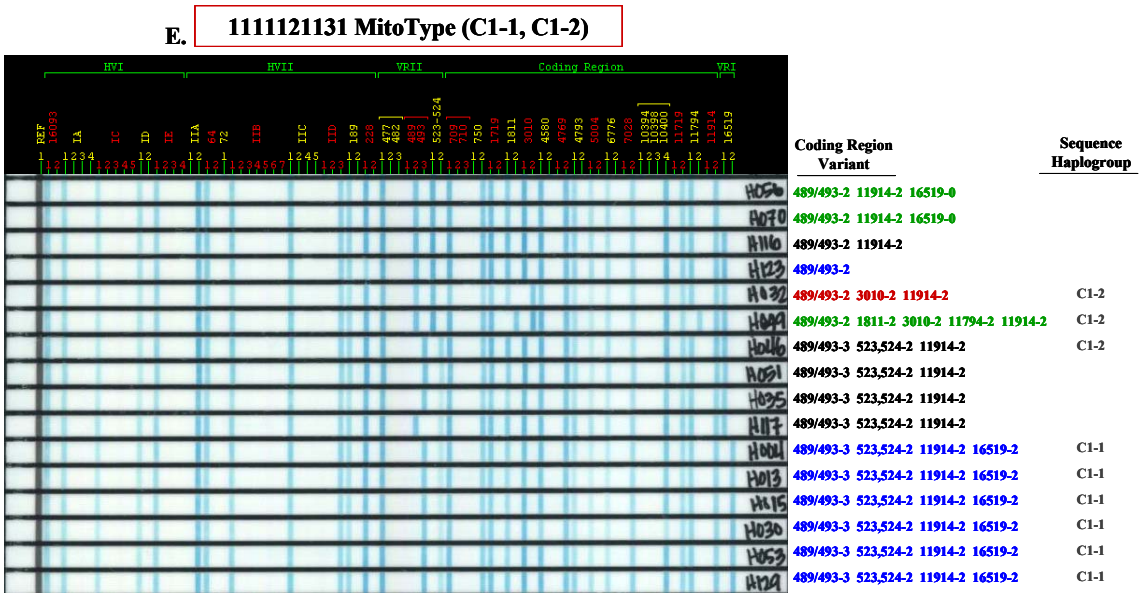


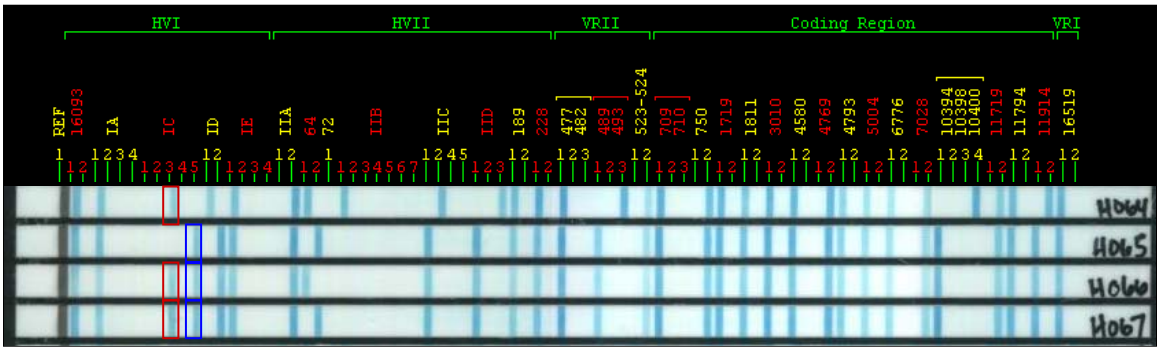
Figure 3E. Subdivision of C1-2 Sequence Haplogroup Using the 83 Probe Panel



Also, some HVI/HVII sites were added to improve the robustness of the assay by reducing the number of '0' and weak signal types caused by destabilizing probe mismatches. An additional five probes targeting four new sites were added to the existing HVI/II probe panel in order to decrease the frequency of weak and no signal (types 0's) which can be observed when a destabilizing mismatch is present within the probe binding region. These five probes were designed to target sequence variants commonly observed in the IA, IC, IE, IIA, or IID region which produce a weak or no signal. The IA4 probe targets a new variant site 16126 (previously IA 0). The IE4 probe targets a sequence variant having both the 16270 and 16278 mutations (previously IE 0). The IID3 probe targets a deletion at position 249 (previously IID 0). A probe to distinguish a 72C sequence variant was also added (previously IIA w1). The IC5 probe targets a new variant site 16319 (previously IC 0). Examples of the IC5 probe results are shown in Figure 4 (strips 1-4). Strip 1 shows the IC3 (16311C) probe

signal type, Strip 2 shows the IC5 (16319A) probe signal type and Strips 3 and 4 show IC3/5 (16311C, 16319A) probe signal types.

Figure 4. IC5 Probe Targets New Variant Site 16319



E. Final Panel of Informative Polymorphic Sites: 5-plex and 10-plex Assay (105 probes)

A total of 61 polymorphic sites are targeted with the 5-plex and 10-plex assay. Additional sites were selected based on sequence data as well as published data for their potential to increase the power of discrimination. These additional polymorphic sites were identified through analysis of the literature as well as the analysis of partial and whole mitochondrial genome data. Several sites to further decrease the number of observed weak and '0' signal types were identified by sequence analysis and added to the final version of the assay, including 16524, 16526, 16527, 143, 153, 10397, and 16320. Also, in an effort to further improve the discrimination power for the US Hispanic population, sites 6308, 1888 and 16111 were added. Polymorphic site 731 was added to help further subdivide a common type observed in African American population. Probes and primers targeting the polymorphic region spanning 14700 (14766, 14793 and 14798) was added to provide additional haplotyping information as well as to generally improve the discrimination power. The polymorphic sites in this region (14700) were identified

by the analysis of full mitochondrial genome data collected using an Affymetrix mtDNA resequencing chip in collaboration with the University of California, Davis, California Department of Justice, and Affymetrix. We selected and provided DNA samples from 68 Hispanic individuals representing the two most common HVI/HVII mitotypes and 72 Caucasian individuals representing a random population for the collaborative sequencing project. Full mitochondrial genome sequence data was provided and used for selecting the final panel of polymorphic sites.

The final 5-plex probe panel consists of 59 probes and targets variation at 14 HVI sites, 11 HVII sites, eight coding region (CR) sites, and two variable region I (VRI) sites. The final 10-plex probe panel consists of 46 probes and targets 17 CR sites, four VRI sites and five VRII sites. The probe designations and the targeted sequence variation are listed in the Table3a (5-plex) and Table 3b (10-plex) below.

Table 3a. Probe Designations and Targeted Sequence Variation for the 5-plex Probe Panel

5-plex	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
HVI	16093 1	16093: A T T T C
	16093 2	• • C • •
	16111 1	16111: A G C C A
	16111 2	• • T • •
	IA1	16124: T G 16126: T A C 16129: G G T
	IA2	T • C • • • • •
	IA3	T • • • • A • •
	IA4	C • • • • • • •
	IC1	16304: A G T A C A T 16309: A G 16311: T A C A T A A A 16319: G 16320: C C A
	IC2	• • C •
	IC3	• • • • • • • • • C • • • • • • • • • • • • •
	IC4	• • • • • • • G • • • • • • • • • • • • • • •
	IC5	• • • • • • • • • • • • • • • • • • • A • • •
	IC6	• T • •
	ID1	16362: C G T C C
	ID2	• • C • •
	IE1	16264: C T C A C C C A 16270: C T A G G A T A 16278: C C A
	IE2	• • • • • • • • • • • • • • • • • • T • •
	IE3	• • • • • • • • T • • • • • • • • • • •
	IE4	• • T • • • • • • • • • • • • • • • • •
VRI	64 1	64: G T C T G
	64 2	• • T • •
	72	72: G G T A T
		• • C • •

Table 3a (cont).

5-plex	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
HVII	IIA1	G T **73** A T G
	IIA2	. . G . .
	IIB1	C C **146** T C A T **150** C C **152** T **153** A T T
	IIB2	. . C . . . .
	IIB3	. . . . C . . .
	IIB4	. . C . . . . . C . . .
	IIB5	. . . . . . T . . . . .
	IIB6	. . . . . . T . C . . .
	IIB7	. . C . . . T . C . . .
	IIB8	. . C . . . . . . G . .
	IIB9	. . C . . . . . C G . .
	IIC1	G A **189** A C A T A C **195** T T A **198** C T **200** A A A
	IIC2	. . . . C . . . .
	IIC4	. . . . C . . T . . . .
	IIC5	. . G . . . . G . .
	IID1	T T **247** G A **249** A T G
	IID2	. . A . . . . . .
	IID3	. . . . – . .
	189 1	G A **189** A C A
	189 2	. . G . .
CODING REGION	4769 1	A T **4769** A G C
	4769 2	. . G . .
	4793 1	A T **4793** A G C
	4793 2	. . G . .
	5004 1	T C **5004** T T A
	5004 2	. . C . .
	6776 1	C A **6776** T A T
	6776 2	. . C . .
	7028 1	G C **7028** C C A
	7028 2	. . T . .
	11719 1	G G **11719** G C T
	11719 2	. . A . .
	11794 1	T C **11794** T C A
	11794 2	. . C . .
	11914 1	A C **11914** G T T
	11914 2	. . A . .

10-Plex	PROBE DESIGNATION	SEQUENCE VARIATION DETECTED
CODING REGION	709 1	C C G T T C
	709 2	. . A . . .
	709 3	. . A C . .
	731 1	T A A A T
	731 2	. . G . .
	750 1	G G A A C
	750 2	. . G . .
	1719 1	T A G C C
	1719 2	. . A . .
	1811 1	T A A C C
	1811 2	. . G . .
	1888 1	G A G C C
	1888 2	. . A . .
	3010 1	C C G A T
	3010 2	. . A . .
	4580 1	A T G C T
	4580 2	. . A . .
	6308 1	A A C T A
	6308 2	. . T . .
	10394 1	G A C T G A A C C G A
	10394 2	. . T . . . .
	10394 3	. . . . . G G . T . .
	10394 4	. . . . . . G . . .
	10394 5	. . . . . G . T . .
	14766 1	A A C T A
	14766 2	. . T . .
	14793 1	C C A C T C A T T C
	14793 2	. . G . . . .
	14793 3	. . . . C . .
VRI	16519 1	G G T C A T A A A G C C T
	16519 2	. . C . . . .
	16519 3	. . . . G . . . . .
	16519 4	. . C . . . . G . . . . .
	16519 5	. . . . A . . .
	16519 6	. . C . . . . . . A . . .
	16519 7	. . . . T . .
	16519 8	. . C . . . . T . .
VRII	477 1	A C T A A T C T C A
	477 2	. . C . . . .
	477 3	. . . . C . .
	489 1	A A T A C A A C C
	489 2	. . C . . . . . .
	489 3	. . C . . . G . .
	523,524 1	A C A C – – C G
	523,524 2	. . . . A C . .
	523, 524 3	. . – – – – . .

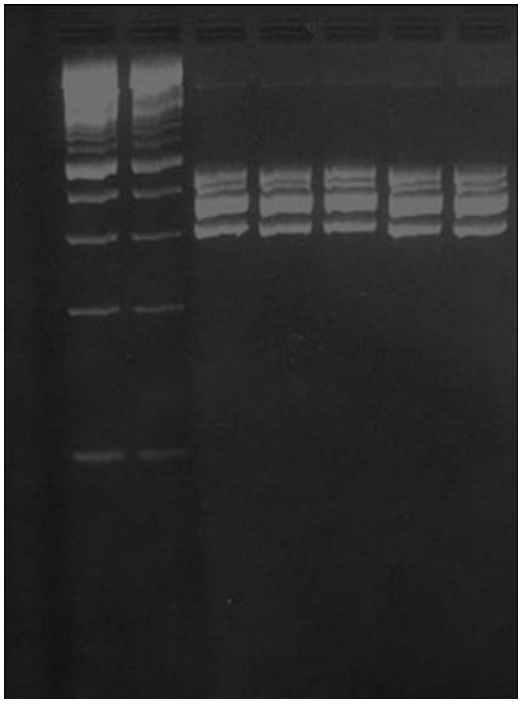
a. Design and Optimization of Final Multiplex PCR Systems

Multiplex PCR reaction conditions were optimized to amplify multiple targets simultaneously maximizing sensitivity through the minimization of primer dimer formation, unequal amplification, and non-specific products. Minimization of primer dimer formation and amplification of non-specific products is important because these amplification products may interfere with amplification yield and/or typing. Modified bases and hot start polymerases to enhance specificity were used. To reduce non-specific product formation, repetitive regions were avoided and potential primers were searched against the human genome for sequence similarity.

The following eight published primer sequences were used in the final multiplex: 3010-F, 3010-R, 4580-F, 4580-R, 5004-R, 7028-R, 16519-F, and 16519-R (Vallone *et al.* 2004). All other primers were designed using Primer 3 software (Vieux *et al.* 2002). Primers were designed in conserved regions to minimize the chance of a polymorphism under the primer disrupting amplification and reducing efficiency and success. Primers were designed with a melting temperature (Tm) of ~65° C, ~5-6° C above the targeted annealing temperature (59° C). Primers were designed to work with the existing HVI/HVII primers and under the same PCR conditions. The distribution of informative polymorphic sites allowed for primer sets to be designed to target multiple polymorphic sites, decreasing the number of amplicons. Although 61 sites are targeted, only 15 primer pairs were required. However, because the VRI/II regions overlap with the HVI/HVII regions, it was necessary to design two multiplex PCRs. Three primer pairs designed to amplify larger targets ~317-374 were added to the duplex PCR, resulting in a 5-plex PCR. Ten additional primer pairs were needed to target the rest of the selected sites for

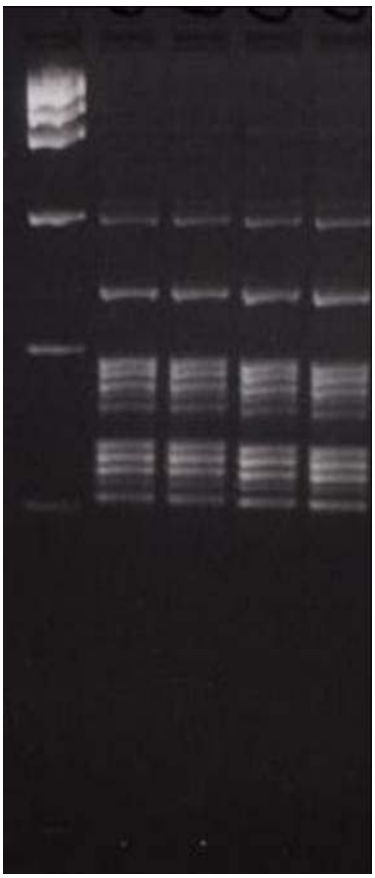
the final 10-plex assay. To increase sensitivity and amplification success of degraded samples, these ten primer sets were designed to minimize the size of the PCR product, aiming for amplicons between 100 and 200 bp. This range of amplicon sizes allows for 'laddering' of all products in the 5 and 10 plex to determine amplification success. Using a 3% Nusieve/ 1% seakem agarose gel system, fragments differing by 7-10 bp can be resolved. Amplicon sizes are noted for each target shown in the gel images below (Figures 5a and 5b). We found that a 1X TAE solution resulted in better resolution for the 5-plex whereas a 1X TBE solution resulted in better resolution for the 10-plex because of the amplicons sizes.

Figure 5a. Final 5-plex PCR on Agarose Gel



Region	Size (bp)
HVI	**444**
HVII	**415**
6776/7028	**374**
4769/4793/5004	**362**
11719/11794/11914	**314**

Figure 5b. Final 10-plex PCR on Agarose Gel



10 Plex

Target	Size
477F+709R *	395 bp
1719F+1811/1888R *	266 bp
16519	183 bp
709	173 bp
477	163 bp
1811/1888	156 bp
14700	141 bp
4580	130 bp
3010	124 bp
10394	117 bp
1719	110 bp
6308	102 bp

* Products formed by F an R primer of targets in close proximity

During the design and testing stages, each primer set was tested individually for formation of primer dimer and non-specific amplification products, prior to combining primer pairs into the multiplex. If primer dimer was observed in the single-plex, the primers were redesigned. Once each individual reaction was optimized, primer pairs were then combined and again checked for balance and non-specific amplification products.

b. Design and Optimization of Probes for Final 5-plex and 10-plex Probe Panels

The method for mtDNA typing is based on the analysis of the co-amplified DNA using Sequence-Specific Oligonucleotide (SSO) probes immobilized in lines on a strip of nylon membrane (referred to as a probe linear array). A panel of probes were designed to target informative sites chosen based on the added value to the HVI/HVII probe panel. SSO Probes are used to "capture" PCR products that contain sequence complementary to individual probes. Probes were designed to minimize cross-hybridization and obtain balanced signals while maintaining a high level of sensitivity. This specificity can be achieved by centering the target polymorphism within the designed probe and by choosing the complementary strand of the target sequence with the most destabilizing mismatch to the variant (e.g. C/A mismatch is less stable than a G/T mismatch). Specificity and signal intensity was also be altered by increasing or decreasing the length of the probe or shifting the probe spanning region to achieve a different GC skew to increase/decrease the melting temperature. In some cases, modified bases were used to achieve optimal specificity or signal intensity. Probe signal intensity also was decreased or increased by adjusting concentration of the probe when applied to the nylon membrane. This approach to probe design was taken for all sites, and representative results from probes designed to target site 6776 are shown in Figure 6. By adjusting length and probe concentration (shortening the length and decreasing the concentration), the cross-hybridization observed for probes shown in Figure 6a and 6b was minimized (Figure 6c).

Figure 6a.

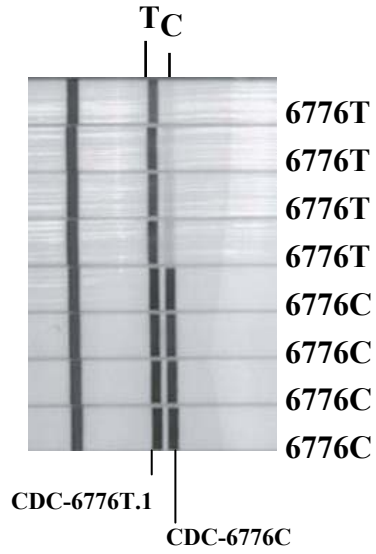


Figure 6c.

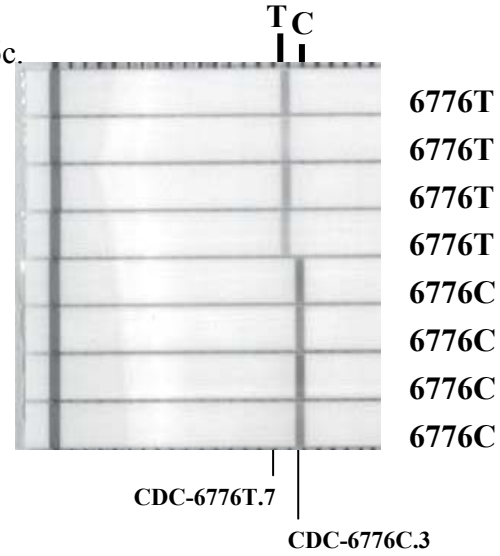
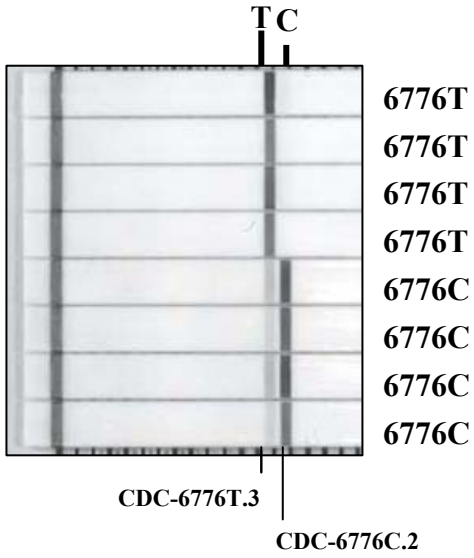


Figure 6b.



Probes were designed to work under the same hybridization and wash conditions as the HVI/HVII linear array. Representative typing results from the final 5-plex and 10-plex probe panels are shown in the Figures 7a and 7b below.

Figure 7a. Final 5-plex Probe Panel Typing Results

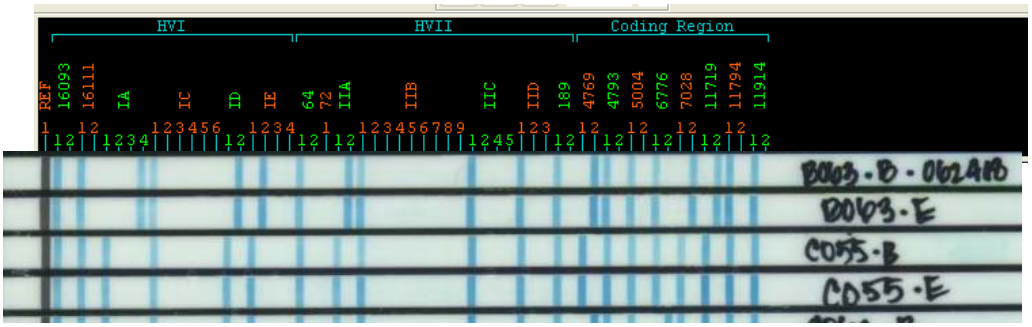
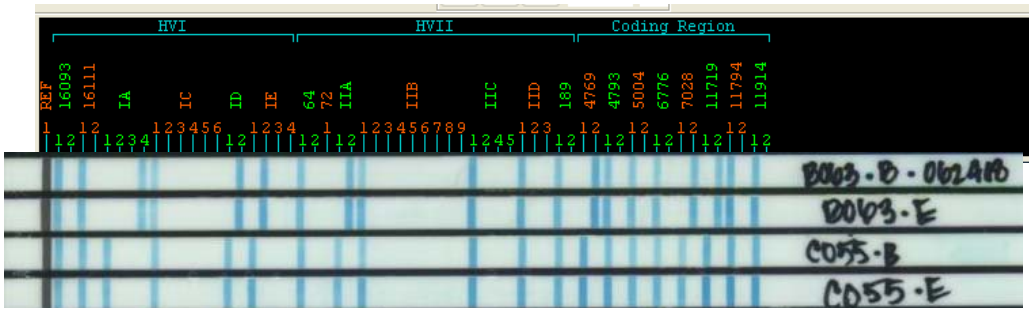


Figure 7b. 10-plex Probe Panel Typing Results



c. Generation of a Population Database Using the Final 5-plex and 10-plex System

A population database was generated by amplifying and typing 674 samples from four population groups (194 African American, 197 US Caucasian, 197 US Hispanic and 86 Japanese) with the final 5-plex and 10-plex PCR and probe panels. The genetic diversity values ($h = (1-\sum freq^2) * (n/1-n)$) were calculated for each of the population groups and were compared to the values obtained with the HVI/HVII linear array assay. The h values are reported in the Table 4 below for each population group for both the HVI/HVII assay and the final HV+ assay. The discrimination power was greatly improved for the Japanese, US Hispanic, and US Caucasian populations with a slight increase for the African American population for the new HV+ assay compared to the

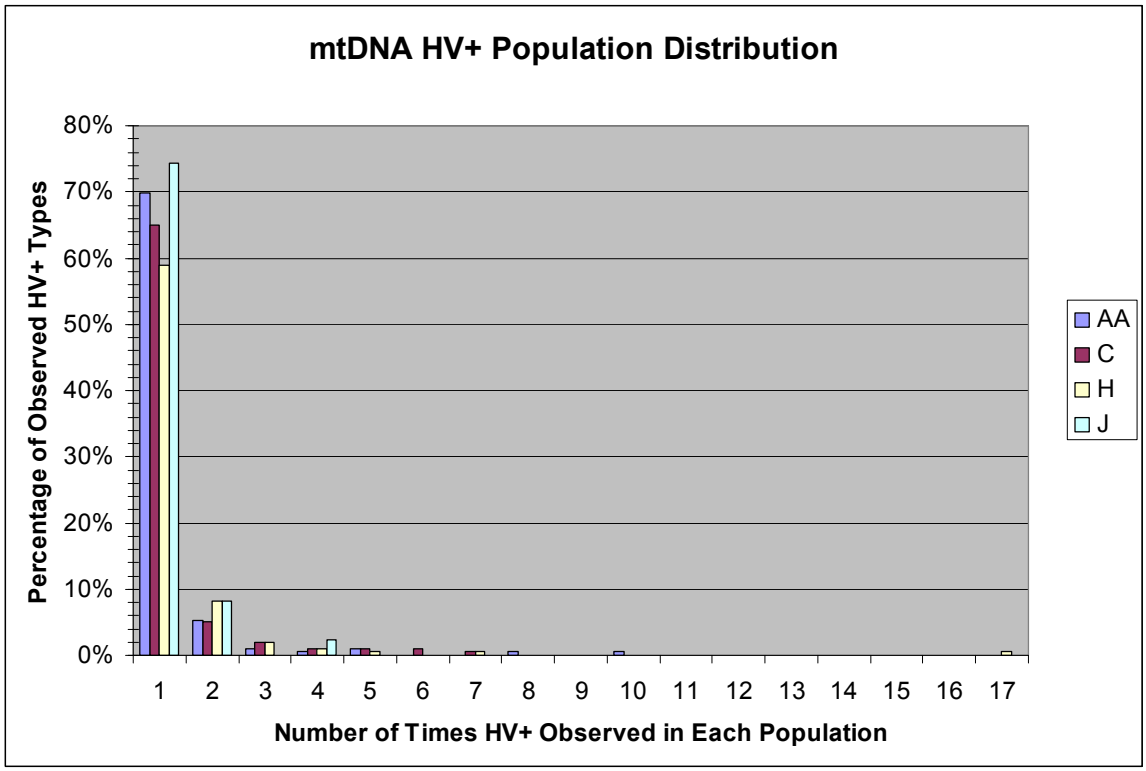
HVI/HVII assay. The discrimination power for the US Caucasian population was increased from 0.9768 with the HVI/HVII assay to 0.9946 with the HV+ assay. The discrimination power was increased from 0.9449 to 0.9893 for the US Hispanic population using the HV+ assay. The number of observed and unique mitotypes increased for all populations as well, with the greatest increases for the US Hispanic and US Caucasian populations; there was an ~35% increase in the number of observed types and an ~43% increase in the number of unique types for both population groups (Table 4).

Table 4. Increased Genetic Diversity Values and Unique Types for US Populations

Population Group (n)	# observed mitotypes			# unique mitotypes			h value	
	HVI/ HVII	HV+	% increase	HVI/ HVII	HV+	% increase	HVI/ HVII	HV+
African American (194)	137	**153**	10.5%	111	**136**	18.4%	0.993	**0.9938**
U.S. Caucasian (197)	99	**149**	33.6%	73	**128**	43.0%	0.9768	**0.9946**
U.S. Hispanic (197)	91	**141**	35.5%	67	**116**	42.2%	0.9449	**0.9893**
Japanese (86)	58	**73**	20.5%	48	**64**	25.0%	0.9806	**0.9948**

The distribution of HV+ mitotypes was also determined for each population group (See Table 5 below). The percentage of observed HV+ types occurring one to 17 times is reported for each of the four population groups. Approximately 60-75% of the HV+ types were unique (occurring only one time in the specified population), ~5-8% of the HV+ types occurred twice and less than 2% of the HV+ types occurred three or more times.

Table 5. Distribution of HV+ Mitotypes for Each Population Group



A few common types were observed within each population group. In the US Hispanic population, the most common type was observed 17 times. Although 17 US Hispanic individuals still shared the same HV+ type, over half of these individuals had identical full mtDNA sequences based on preliminary Affymetrix mtDNA sequencing data collected at the California Department of Justice on these samples. The most common two types in the African American population were observed 10 and eight times and the most common type in the US Caucasian population was observed seven times (See Table 5 above). All types occurring two or more times within a population are reported for each population group in Tables 6a-6c below.

Table 6a. African American Types Occurring 2 or More Times

African American common HV+ Types

HVI						HVII							Coding Region								VRII			Coding Region												VRI	
16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519	#
1	1	1	4	1	2	1	0	2	4	2	1	1	2	1	1	1	2	2	1	2	1	1	1	1	1	2	1	1	1	1	1	1	4	2	1	2	**10**
1	1	2	3	1	4	1	0	2	3	0	2	0	2	1	1	1	2	2	1	1	1	1	3	3	1	2	1	1	1	1	1	1	4	0	1	2	**8**
1	1	1	1	2	2	1	0	2	0	2	1	1	2	1	1	1	2	2	1	2	1	1	1	1	1	2	1	1	1	1	1	1	4	2	1	1	**5**
1	0	3	1	2	2	1	0	2	6	4	1	0	0	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	4	2	1	1	**5**
1	1	2	3	1	4	1	0	2	3	2	2	0	2	1	1	1	2	2	1	1	1	1	3	3	1	2	1	1	1	1	1	1	4	0	1	2	**4**
1	1	1	4	0	2	1	0	2	4	w2	1	1	2	1	1	1	2	2	1	2	1	1	2	1	1	2	1	1	1	1	1	1	4	2	1	2	**3**
1	1	1	1	1	1	1	0	2	5	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	1	1	1	1	1	1	1	4	2	1	2	**3**
1	1	1	6	1	1	1	0	2	6	2	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	4	2	1	2	**2**
1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2	**2**
1	1	1	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	4	2	1	2	**2**
1	1	4	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	4	2	1	2	**2**
1	1	4	1	2	2	1	0	2	1	2	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	4	2	1	2	**2**
1	1	1	1	1	w1	1	0	2	5	0	1	2	2	1	1	1	2	2	1	1	1	1	3	1	1	1	1	1	1	1	1	1	4	2	1	2	**2**
1	1	1	1	1	w1	1	0	2	5	2	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	1	1	1	1	1	1	1	4	2	1	2	**2**
0	1	1	5	1	2	1	0	2	5	w1	1	w1	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	1	1	1	5	2	1	5	**2**
1	1	1	6	1	1	1	0	2	5	2	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	4	2	1	2	**2**
2	1	1	1	1	2	1	0	2	7	4	1	0	2	1	1	1	2	2	1	1	1	1	3	2	1	2	1	1	1	1	1	1	4	2	1	1	**2**

Table 6b. US Caucasian Types Occurring Two or More Times

Caucasian Common HV+ types

HVI						HVII							Coding Region								VRII			Coding Region												VRI	
16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519	#
1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	2	1	1	1	1	1	2	*7*
1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	2	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2	*6*
1	1	2	2	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	1	2	1	2	1	1	2	1	1	1	1	2	1	2	*6*
1	1	2	1	1	1	1	0	2	1	1w	1	0	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1	*5*
1	1	1	1	1	1	1	1	1w	1	1	1	1	2	1	1	1	2	1	1	1	1	1	1	1	1	2	1	1	1	1	2	1	1	1	1	1	*5*
1	1	1	1	1	1	1	0	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	2	1	1	1	1	1	2	*4*
1	1	1	1	2	1	1	0	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	*4*
1	1	2	1	1	1	1	0	2	1	0	1	0	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	2	*3*
1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	1	1	1	2	1	1	1	2	1	1	1	1	1	2	*3*
2	1	1	3	1	1	1	0	2	1	2	1	1	2	1	1	1	2	2	1	1	1	1w	1w,2,3w	1	1	2	1	2	1	1	1	1	4	2	3	2	*3*
1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1	1	1	1	2	1	2	1	2	1	1	1	1	1	2	3	2	*3*
1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2	*2*
1	1	1	1	1	1	1	0	2	1	0	1	2	2	1	1	1	2	2	1	1	1	1	1	2	1	2	1	1	1	1	1	1	1	2	1	2	*2*
1	1	1	1	1	1	1	0	2	1	2	1	1	2	1	1	1	1	1	1	1	1	1	3	1	1	2	1	1	1	2	1	1	1	1	1	2	*2*
1	1	1	1	1	3	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	2	1	*2*
1	1	2	1	1	1	1	0	2	1	1	1w	1	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	2	1	1	4	2	1	1	*2*
1	1	1	3	1	1	1	0	1	1	2	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	*2*
1	1	1	1	1	1	1	0	1	2	1	1	1	2	1	1	1	1	1	1	1	2	1	1	1	1	2	1	1	1	2	1	1	1	1	1	2	*2*
1	1	1	3	1	1	1	0	2	2	2	1	1	2	1	1	1	2	2	1	1	1	1	2	1	1	2	1	2	1	1	1	1	4	2	3	2	*2*
1	1	1	1	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	2	1	1	2	*2*
1	1	1	1	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	2	1	1	1	1	1	2	*2*

Table 6c. US Hispanic Types Occurring Two or More Times

US Hispanic common HV+ types																																											
HVI						HVII							Coding Region								VRII			Coding Region																		VRI	
16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519	#
1	1	1	1	1	1	1	0	2	1	1	3	1	2	1	1	1	2	2	1	2	1	3	3	1	1	2	1	1	1	1	1	1	5	2	1	2	**17**
1	2	1	5	2	1	2	0	2	8	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	1	**7**
1	1	1	1	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	2	**5**
1	1	1	1	1	1	1	0	2	1	1	3	1	2	1	1	1	2	2	1	2	1	3	3	1	1	2	1	1	1	1	1	1	5	2	1	1	**4**
1	2	1	5	2	1	2	0	2	9	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	1	**4**
1	1	1	1	1	1	1	0	2	1	1	3	1	2	1	1	1	2	2	1	2	1	2	1	1	1	2	1	1	2	1	1	1	5	2	1	1	**3**
2	1	1	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	4	2	1	2	**3**
1	1	1	5	2	1	2	0	2	8	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	2	**3**
1	2	3	5	2	1	2	0	2	8	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	1	**3**
0	1	1	1	1	2	1	0	2	0	1	3	1	2	1	1	1	2	2	1	2	1	3	3	1	1	2	1	1	1	1	1	1	5	2	1	1	**2**
0	1	1	1	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	2	**2**
1	1	1	1	1	1	1	0	2	1	1	3	1	2	1	1	1	2	2	1	2	1	2	1	1	1	2	1	1	2	1	1	1	5	2	1	5	**2**
1	1	1	5	1	1	1	0	2	2	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	2	**2**
1	1	1	5	2	1	1	0	2	2	1	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	1	**2**
0	1	1	1w	1	1	1	0	2	3	1	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	2	**2**
1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1	1	1	1	2	1	2	1	2	1	1	1	1	1	2	3	2	**2**
1	1	1	1	1	1	1	0	1	5	5	1	2	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	4	2	1	1	**2**
1	1	1	6	1	3	1	0	2	5	1	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	1	**2**
1	0	1	1	2	2	1	0	2	6	4	1	0	0	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	4	2	1	1	**2**
1	1	1	5	2	1	1	0	2	8	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	1	**2**
1	1	1	5	2	1	2	0	2	8	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	1	**2**
1	2	1	5	2	0	1	0	2	8	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	2	1	2	1	1	**2**
1	2	1	5	2	1	2	0	2	8	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	2	1	1	1	1	2	1	5	**2**
0	2	1	5	2	1	1	0	2	9	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	1	**2**
1	2	1	5	2	1	2	0	2	9	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	2	**2**

Table 6c. Japanese Types Occurring Two or More Times

Japanese common HV+ types

HVI						HVII							Coding Region								VRII			Coding Region													VRI	
16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519	#
1	1	1	1	2	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	2	1	1	5	2	1	1	**4**
1	1	3	1	2	1	1	0	2	3	1	1	1	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	2	1	1	5	2	1	2	**4**
1	1	1	1	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	1	1	1	5	2	1	1	**2**
1	1	1	1	2	1	1	0	2	1	0	1	0	2	1	1	1	2	2	1	1	1	2	3	1	1	2	1	1	1	2	1	1	5	2	1	2	**2**
1	1	1	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	2	1	2	1	2	1	1	1	1	1	1	5	2	1	2	**2**
2	1	1	1	1	1	1	0	2	1	1	3	1	2	1	1	1	2	2	1	2	1	2	3	1	1	2	1	1	1	1	1	1	5	2	1	1	**2**
2	1	1	1	1	1	1	0	2	1	1	3	1	2	1	1	1	2	2	1	2	1	2	1	1	1	2	1	1	1	1	1	1	5	2	1	1	**2**
1	1	1	5	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1	1	1	3	1	1	2	1	1	1	1	1	1	1	2	1	1	**2**
1	1	3	1	1	1	1	0	2	5	0	1	1w	2	1	1	1	2	2	1	1	1	2	1	1	1	2	1	1	1	1	1	1	5	2	1	1	**2**

IV. Validation Studies

A. Mixture Study

a. Samples and Methods

Nuclear and mtDNA copy number were estimated using a qPCR assay as described (Hudlow *et al.* 2008) in at the California Department of Justice. Two DNA samples (C018 and C004) were mixed at the following 13 different mixture ratios based on the estimated mtDNA copy number:

1. 0% C004 + 100% C018
2. 2.5% C004 + 37.5% C018
3. 5% C004 + 95% C018
4. 10% C004 + 90% C018
5. 20% C004 + 80% C018
6. 35% C004 + 65% C018
7. 50% C004 + 50% C018
8. 65% C004 + 35% C018
9. 80% C004 + 20% C018
10. 90% C004 + 10% C018
11. 95% C004 + 5% C018
12. 97.5% C004 + 2.5% C018
13. 100% C004 + 0% C018

Each DNA mixture was amplified using the final 5 plex and 10 plex primer mix and cycling parameters. The product was run out on a 3% Agarose LE gel in 1X TBE at 125V for ~1 hour, 10 minutes. For each mixture, 15 μL of PCR product was typed with the final 5 and 10 plex probe panels and following the 4mL Linear Array protocol. Images were scanned and typing results were generated using Strip Scan Software.

b. Results

Similar product yields were observed for all mixed DNA samples amplified with the respective 5-plex or 10-plex PCR system. For the 5-plex assay, minor components were detected at 10% level within all mixed probe regions and within a single probe region at 5%

level (Figure 8a and Table 7a). For the 10-plex assay, the minor component was detected at 10% level within all mixed probe regions for 10% C018 and at 5% level within all mixed probe regions for 5% C004 (Figure 8b and Table 7b). A mixture in a single region was detected at 5% level for the C018 minor component and within two regions at 2.5% level for the C004 minor component.

c. Conclusion

Minor components present at 5-10% were detectable with the 5-plex assay and 2.5-5% with the 10-plex, depending on the probe, with some probes being more sensitive than others. A minor component present at ~10% is reliably detected with the 5-plex assay and ~5% with the 10-plex assay.

Figure 8a. 5 Plex Mixture Study Typing Results

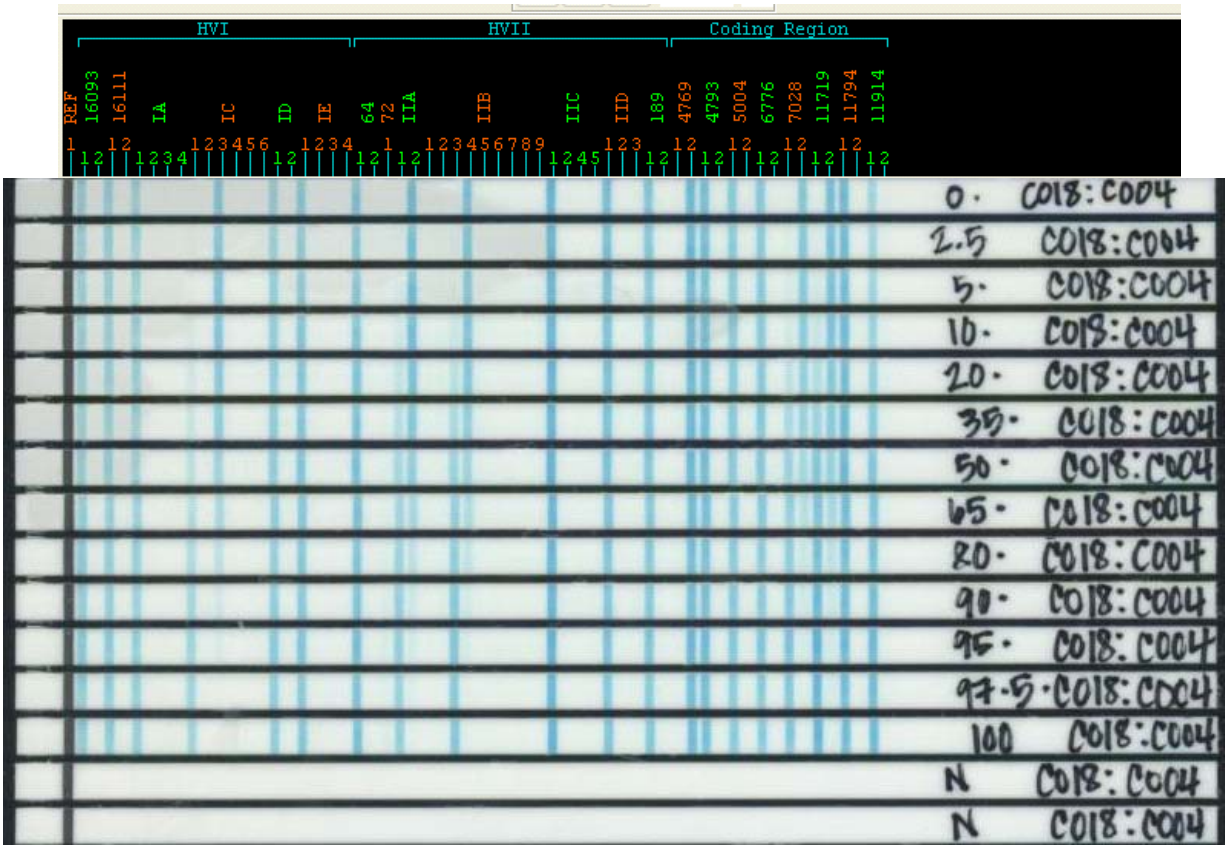


Table 7a. 5 Plex Mixture Study MitoReport

	HVI						HVII						Coding Region									
	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
0	1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1
2.5	1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1
5	1	1	1	3	1	1	1	0	2	4	1	1	1	2	1	1	1	2	2	1	1
10	1	1	1	1w,3	1	1	1	0	1w,2	3w,4	1	1	1	2	1	1	1	1w,2	1w,2	1	1
20	1	1	1	1w,3	1	1	1	0	1w,2	3w,4	1	1	1	2	1	1	1	1w,2	1w,2	1	1
35	1	1	1	1,3	1	1	1	0	1,2	3,4	1	1	1	2	1	1	1	1,2	1,2	1	1
50	1	1	1	1,3	1	1	1	0	1,2	3,4	1	1	1	2	1	1	1	1,2	1,2	1	1
65	1	1	1	1,3	1	1	1	0	1,2	3,4	1	1	1	2	1	1	1	1,2	1,2	1	1
80	1	1	1	1,3w	1	1	1	0	1,2w	3,4w	1	1	1	2	1	1	1	1,2w	1,2w	1	1
90	1	1	1	1,3w	1	1	1	0	1,2w	3,4w	1	1	1	2	1	1	1	1,2w	1,2w	1	1
95	1	1	1	1,3w	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1
97.5	1	1	1	1	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1
100	1	1	1	1	1	1	1	0	1	3	1	1	1	2	1	1	1	1	1	1	1
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 8b. 10 Plex Mixture Study Typing Results

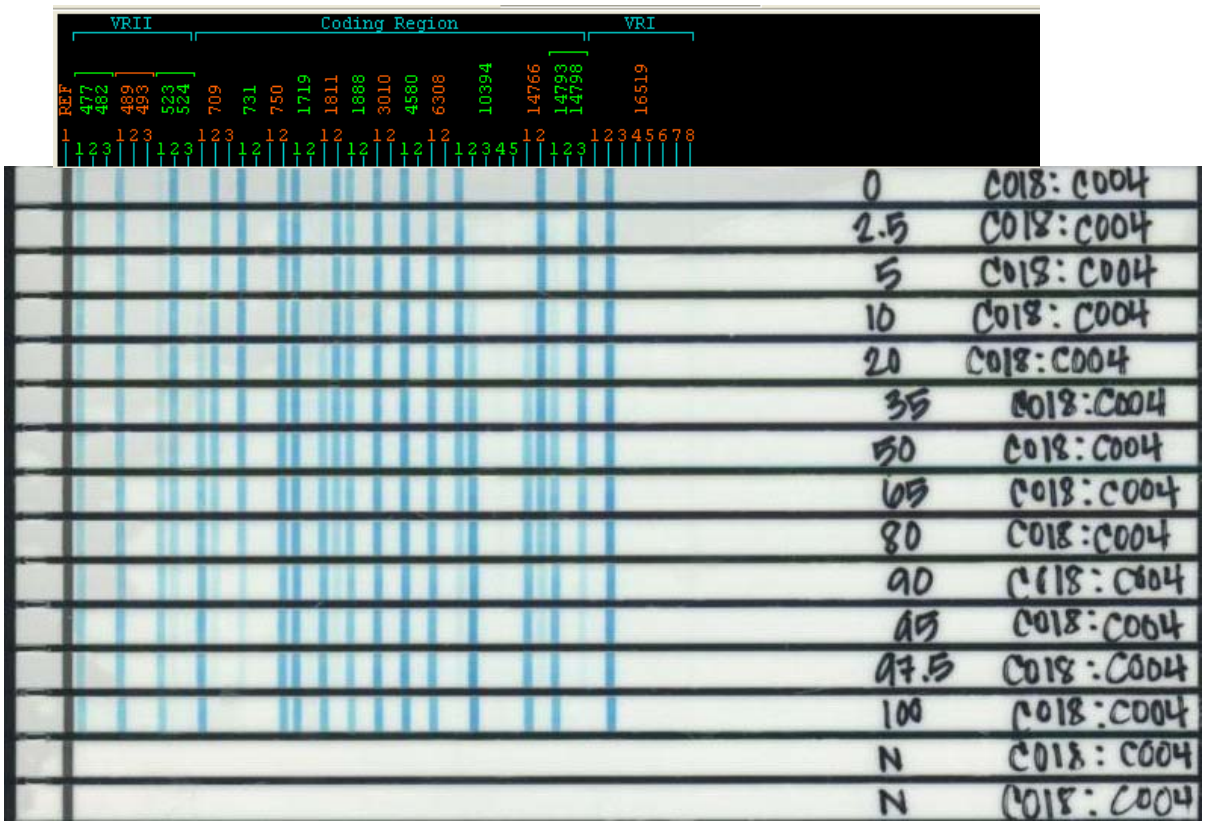


Table 7b. 10-Plex Mixture Study MitoReport

	VRII		Coding Region												VRI	
	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
0	1	1	1w,2	2	1	2	1	2	1	1	1	1	1	2	3	2
2.5	1	1	2	2	1	2	1	2	1	1	1	1	1	2	3	2
5	1	1	2	1w,2	1	2	1	2	1	1	1	1	1	2	3	2
10	1	1	1w,2	1w,2	1	2	1	1w,2	1	1	1	1	1w,2	1w,2	1w,3	2
20	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2
35	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2
50	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2
65	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2
80	1	1	1,2	1,2	1	2	1	1,2	1	1	1	1	1,2	1,2	1,3	2
90	1	1	1,2w	1,2w	1w	2	1	1,2w	1	1	1	1	1w,2	1,2w	1,3w	2
95	1	1	1,2w	1,2w	1w	2	1	1, 2w	1	1	1	1	1w,2	1,2w	1,3w	2
97.5	1	1	1	1	1w	2	1	1	1	1	1	1	2	1	1,3w	2
100	1	1	1	1	0	2	1	1	1	1	1	1	2	1	1	2
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

B. Sensitivity Study

a. Samples and Methods

Two Samples (C004 and C018) were amplified at 34, 36 and 38 cycles in duplicate at 8 different DNA inputs (1ng, 500pg, 100pg, 50pg, 10pg, 5pg, 1pg, 0.5pg) using the final 5 plex and 10 plex PCR assays and cycling parameters. The PCR products were run on a 3% Agarose LE gel in 1X TBE at 125V for ~1hour, 10minutes.

b. Results

Typeable results were observed for 5 pg at 34 cycles (~730 mtDNA copies) (Figures 9a), 1 pg at 36 cycles (~150 mtDNA copies) and 0.5 pg at 38 cycles (~75 mtDNA copies) (Figure 9b) for the 5-plex assay. For the 10-plex assay, typeable results were observed for 1 pg at 34 cycles (~150 mtDNA copies) (Figure 10a), 0.5 pg at 36 cycles and 38 cycles (~75 mtDNA copies) (Figure 10b). mtDNA copy number was estimated using the quantitative PCR assay as described by (Hudlow *et al.* 2008).

c. Conclusion

Both the 5-plex and 10-plex assays are highly sensitive assays, with the 10-plex being more sensitive.

Figure 9a. Sensitivity Study Results: 5-plex 34 cycles

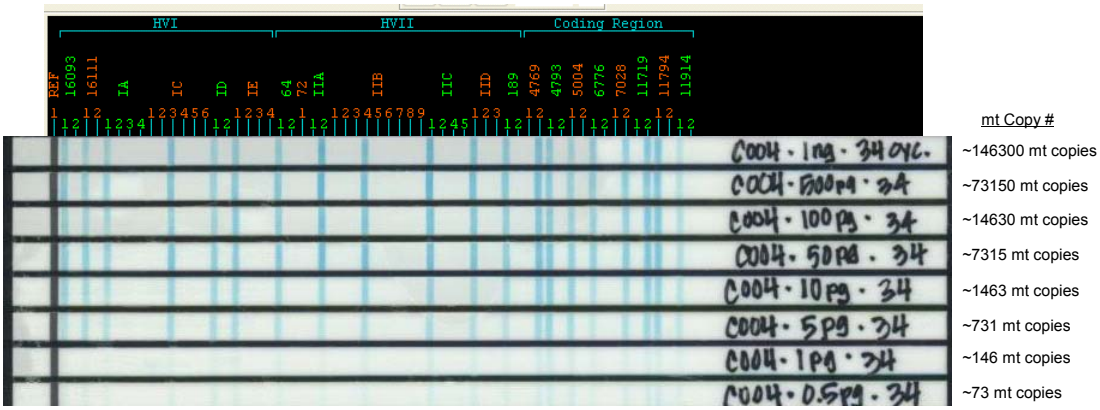


Figure 9b. Sensitivity Study Results: 5-plex 38 cycles

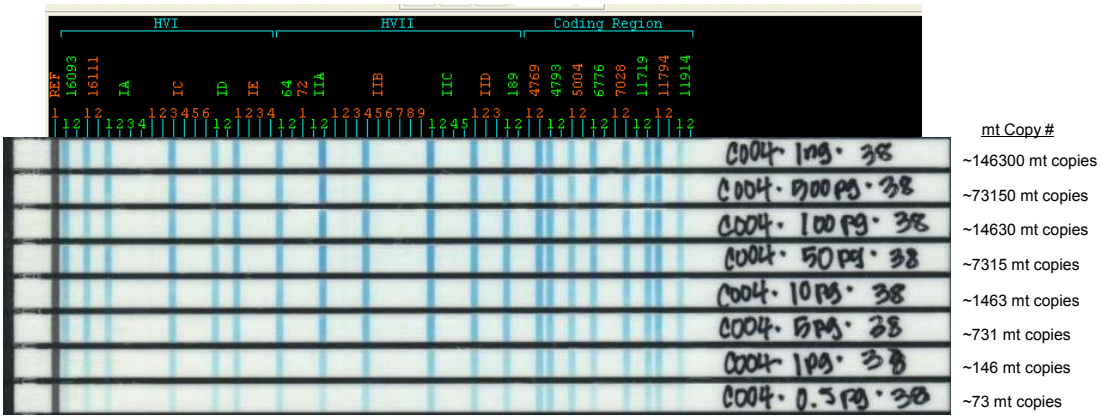


Figure 10a. Sensitivity Study Results: 10-plex 34 cycles

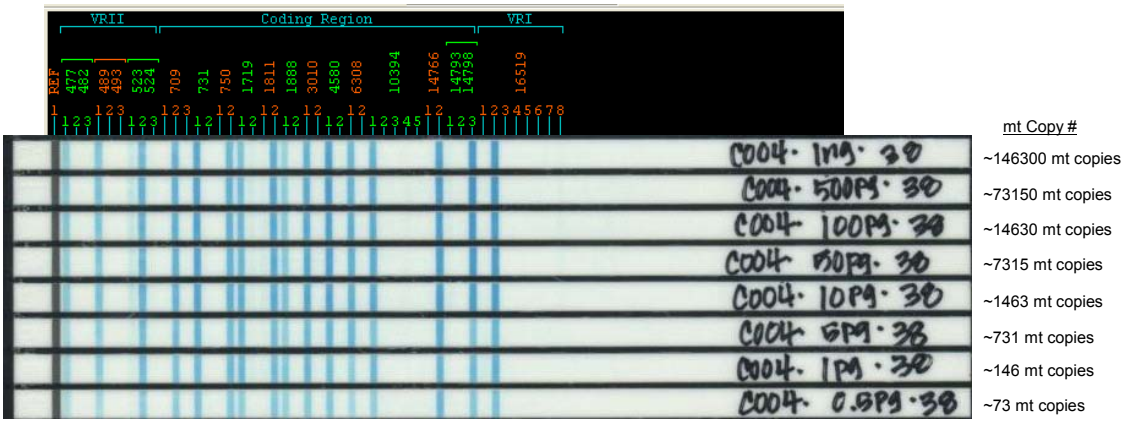
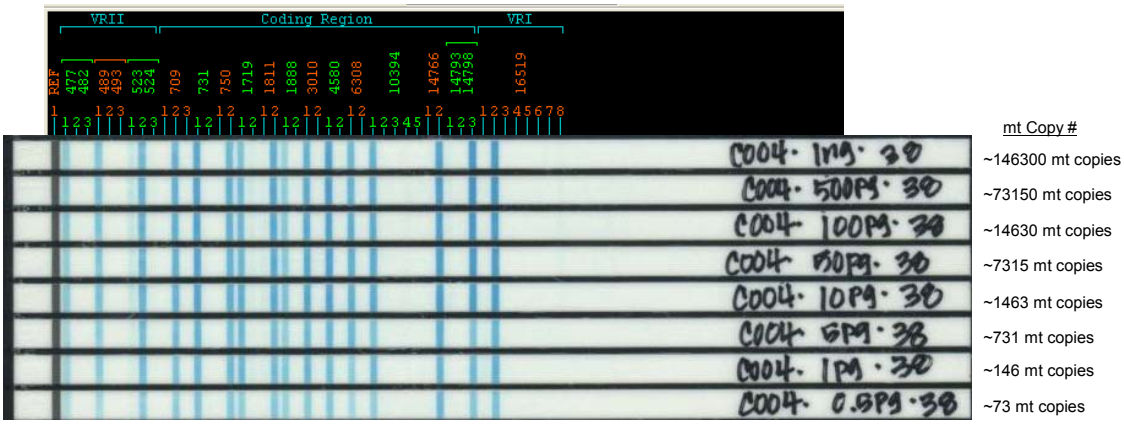


Figure 10b. Sensitivity Study Results: 10-plex 38 cycles



C. Testing of Single Primer Pairs of the 5-plex and 10-plex PCR System

a. Methods

1. 5 Plex Individual Primer Study

One hundred picograms of template DNA from two samples was amplified in duplicate with each of the single primer pairs (HVI, HVII, 6776/7028, 4769/5004, and 11719/11914) of the 5 plex primer mix as well as with the 5 plex primer mix. All reactions were amplified following the standard cycling parameters for 34 cycles. The generated PCR products were run on a 3% Nusieve + 1% SeaKem agarose gel in 1X TAE at 120V for ~1 hour, 15 minutes. The gel was imaged using a UV box and AlphaImager camera.

2. 10 Plex Individual Primer Study

One hundred picograms of template DNA from two samples was amplified in duplicate with each of the single primer pairs (16519, 709, 477, 1811/1888, 14700, 4580, 3010, 10394, 1719, 6308) of the 10 plex primer mix as well as with the 10 plex primer mix. All reactions were amplified following the standard LINEAR ARRAY cycling parameters for 34 cycles. The generated PCR product was run on a 3% Nusieve + 1% SeaKem agarose gel in 1X TBE at 120V for ~1 hour, 15 minutes. The gel was imaged using a UV box and AlphaImager camera.

b. Results

All targets of the 5 plex were successfully amplified with single primer pairs and the yields are comparable (Figure 11a). Results for one sample are shown in the gel image below. The amplicon sizes for the 5 plex targets range from 314bp-444bp. In the 5 plex control, the HVI (444bp) and HVII (415bp) bands appear to be slightly lower in amplification yield. All other single primer pair bands are comparable in intensity to the bands in the 5 plex control.

All targets of the 10 plex were amplified successfully with the single primer pairs and the yields are comparable (Figure 11b). The amplicon sizes for the 10 plex targets range from 102bp-183bp. In the 10 plex control, the 477, 1811/1888 and 1719 bands appear to be lower in intensity compared to other bands in the 10 plex. All other single primer pair bands are comparable in yield to the bands in the 10 plex control. No non-specific products were visible and none to very little primer dimer was observed.

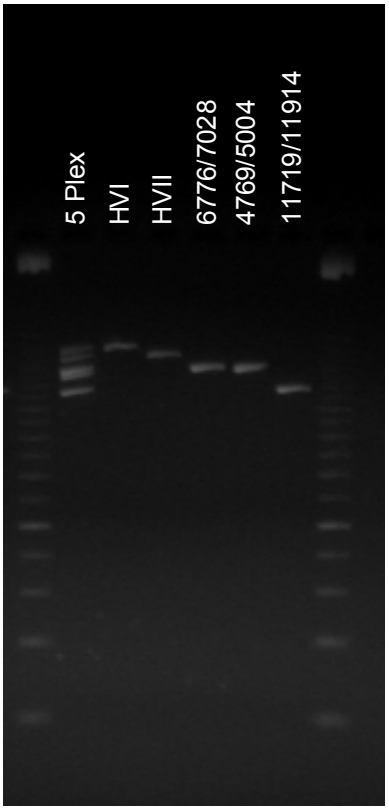
c. Conclusion

In the 5 plex control, the amplification yield of the HVI (444bp) and HVII (415bp) PCR products is slightly lower than the other three products. The somewhat reduced efficiency is likely due to the larger amplicon size of the HVI/HVII products compared to the other three products. However, probe signal intensity is still balanced across the 5-plex probe panel as the probe concentrations were adjusted accordingly in optimization of the assay to account for this difference in amplification efficiency.

The amplification yield of the 477, 1811/1888 and 1719 PCR products is somewhat lower than the other seven target products in the 10 plex. The lower amplification yield of these three products in the 10 plex is a result of amplification of two larger products which are formed with the forward and reverse primers of two target regions which are close in proximity. In one case, the 1719F and the 1811/1888R primers are forming a larger 266 bp product which can be seen on the gel image. Also, in some cases, the 477F and 709R primers amplify a larger 395bp product which is slightly visible in this gel image for the sample shown. Although the PCR efficiency of the 477 target is slightly affected by the amplification of the larger target, the 709

target is not noticeably affected. Only the 477, 1811/1888 and 1719 bands are lower in yield than other products in the 10 plex. Although a lower yield for these three (or sometimes four) products may be observed with the 10 plex PCR, the probe signals across the 10-plex probe panel are still balanced as the probe concentrations of these probes were adjusted accordingly during optimization. All other individually amplified bands are comparable in amplification yield to the appropriate 5 or 10 plex control. Overall, the 5-plex and 10-plex PCRs are very specific with little to no primer dimer formation and amplify the expected target.

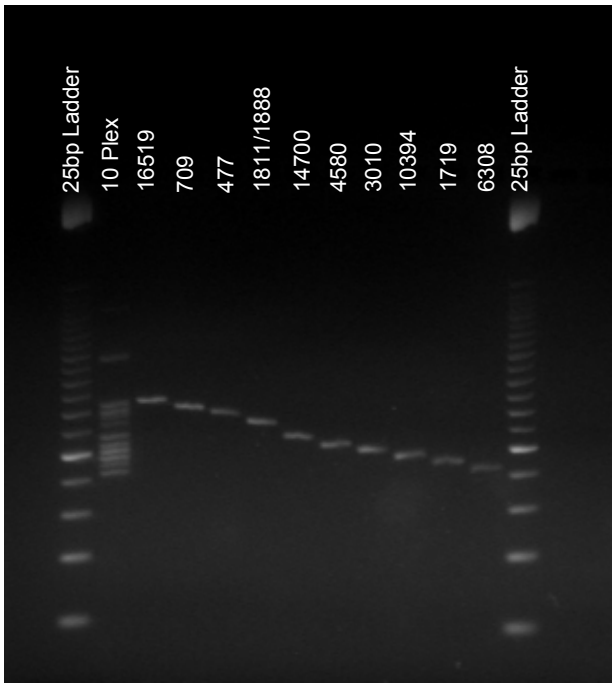
Figure 11a. Agarose Gel of Individual Primer Pair Testing of the 5-plex PCR System



5 Plex

Target	Size
HVI	444 bp
HVII	415 bp
6776/7028	375 bp
4769/5004	362 bp
11719/11914	314 bp

Figure 11b. Agarose Gel of Individual Primer Pairs of the 10-plex PCR System



10 Plex

Target	Size
477F+709R *	395 bp
1719F+1811/1888R *	266 bp
16519	183 bp
709	173 bp
477	163 bp
1811/1888	156 bp
14700	141 bp
4580	130 bp
3010	124 bp
10394	117 bp
1719	110 bp
6308	102 bp

* Products formed by F an R primer of targets in close proximity

V. Summary of Results from Developmental Validation Studies Conducted at NCFS for the 5-plex and 10-plex PCR and Typing Assay

A subset of the studies required to satisfy the developmental validation requirement for a new PCR based assay as outlined in the SWGDAM guidelines were completed at NCFS. Validation studies completed by NCFS include species specificity, reproducibility, analysis of case type samples, precision and accuracy and environmental studies to assess stability of DNA

For all studies conducted at NCFS, samples were extracted, amplified using the 5-plex and 10-plex PCR assay and amplified products were verified by gel electrophoresis. All gel images were provided to CHORI for analysis. Fifteen microliters of PCR product for each sample was typed with the 5-plex and 10-plex probe panels following the four mL Linear Array procedure. After color development, all probe panels were aligned on a black tray and scanned using an Epson scanner at 150 dpi and saved as jpg images by NCFS. All scanned images were provided to CHORI for analysis with the Mitotyper StripScan software and mitoreports were generated for all samples at CHORI. All collected data were analyzed and interpreted by CHORI and the results are summarized below.

A. Species Specificity

a. Animal Study

1. Samples and Methods

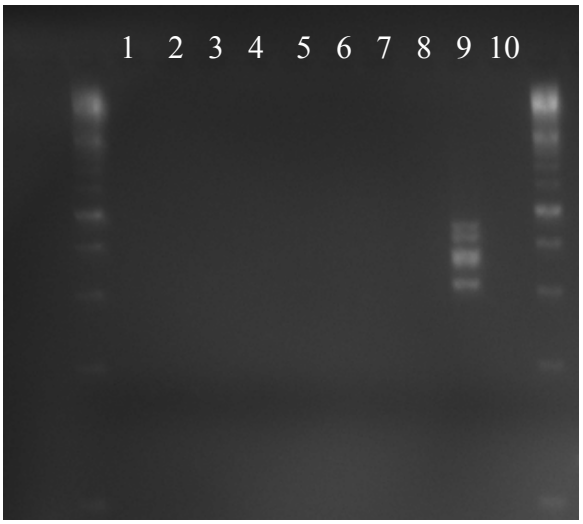
Animal Blood was obtained from commercial sources or by donation from zoos.

Pig (Yorkshire)	Bioreclamation, Inc (Long Island, NY)
Dog	Bioreclamation, Inc (Long Island, NY)
Rabbit (New Zealand White)	Bioreclamation, Inc (Long Island, NY)
Goat	Bioreclamation, Inc (Long Island, NY)
Mouse (Strain CD-1)	Bioreclamation, Inc (Long Island, NY)
Cow	Hemostat Laboratories (Dixen, CA)
Tortoise	Central Florida Zoo (Sanford, FL)
Ferret	Marshall Farms (North Rose, New York)

2. Results

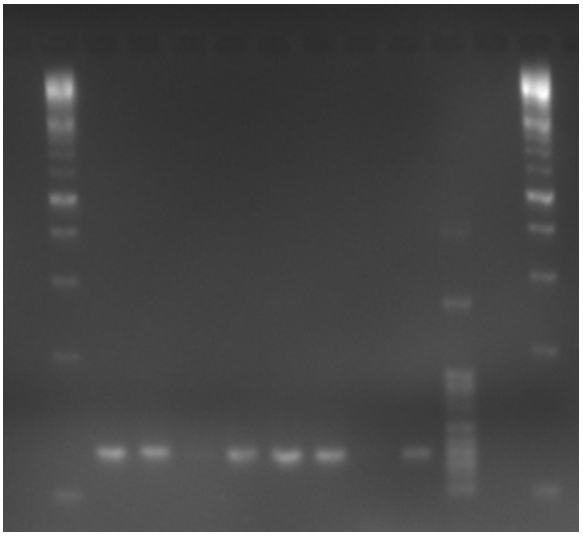
No amplified product was visible by gel electrophoresis for any of the eight animal DNAs amplified with the 5-plex PCR (Figure 12a). For six of the eight animal DNAs tested (except the Tortoise and Mouse), a region of the 16s rRNA (primers targeting site 3010) amplified with the 10-plex PCR (see gel image Figure 12b below). No probe signals were observed with the 5-plex or 10-plex probe panels for any of the animal DNAs tested, including the 3010 probe region for which amplified product was observed for the dog, ferret, cow, rabbit, goat, and pig (see figures 12c and 12d below).

Figure 12a. 5-plex Agarose Gel Results for Animal Study



1. Dog
2. Ferret
3. Tortoise
4. Cow
5. Rabbit
6. Goat
7. Mouse
8. Pig
9. HL60
10. AB

Figure 12b. 10-plex Agarose Gel Results for Animal Study



1. Dog
2. Ferret
3. Tortoise
4. Cow
5. Rabbit
6. Goat
7. Mouse
8. Pig
9. HL60
10. AB

Figure 12c. Animal Study: 5-plex Probe Panel Typing Results

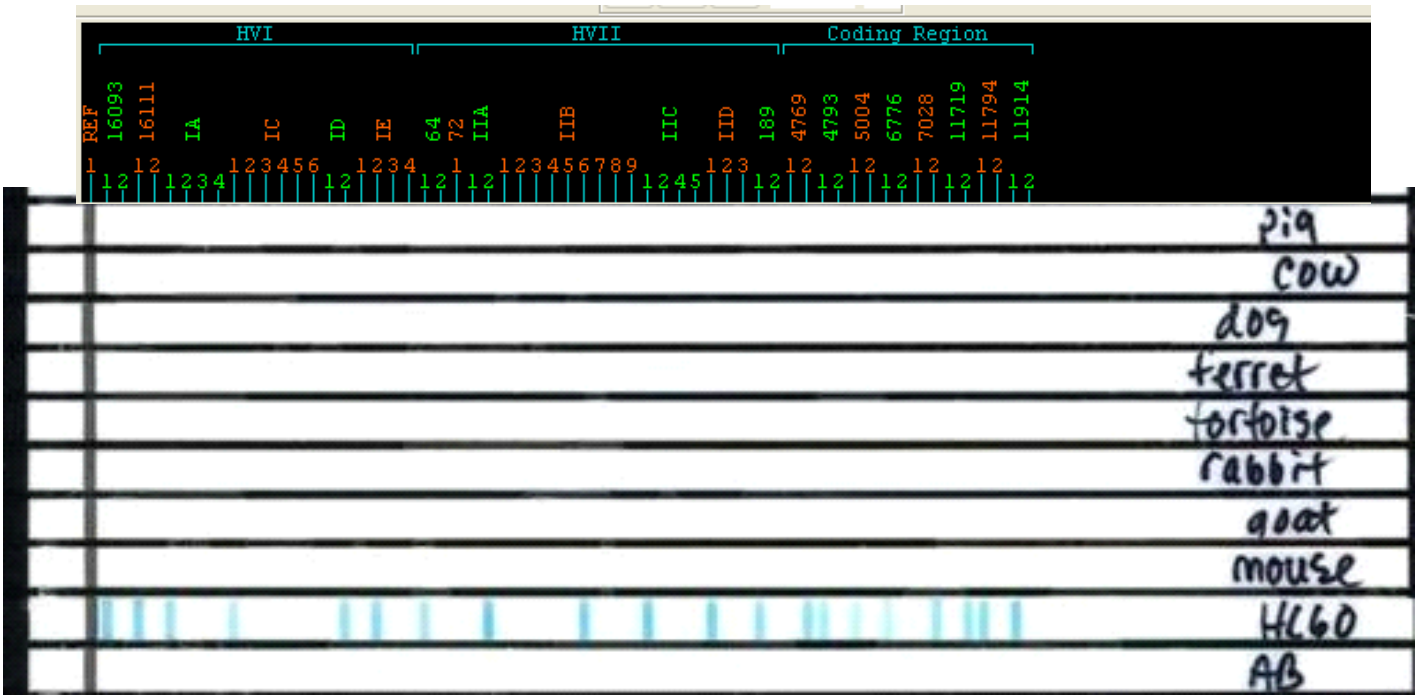
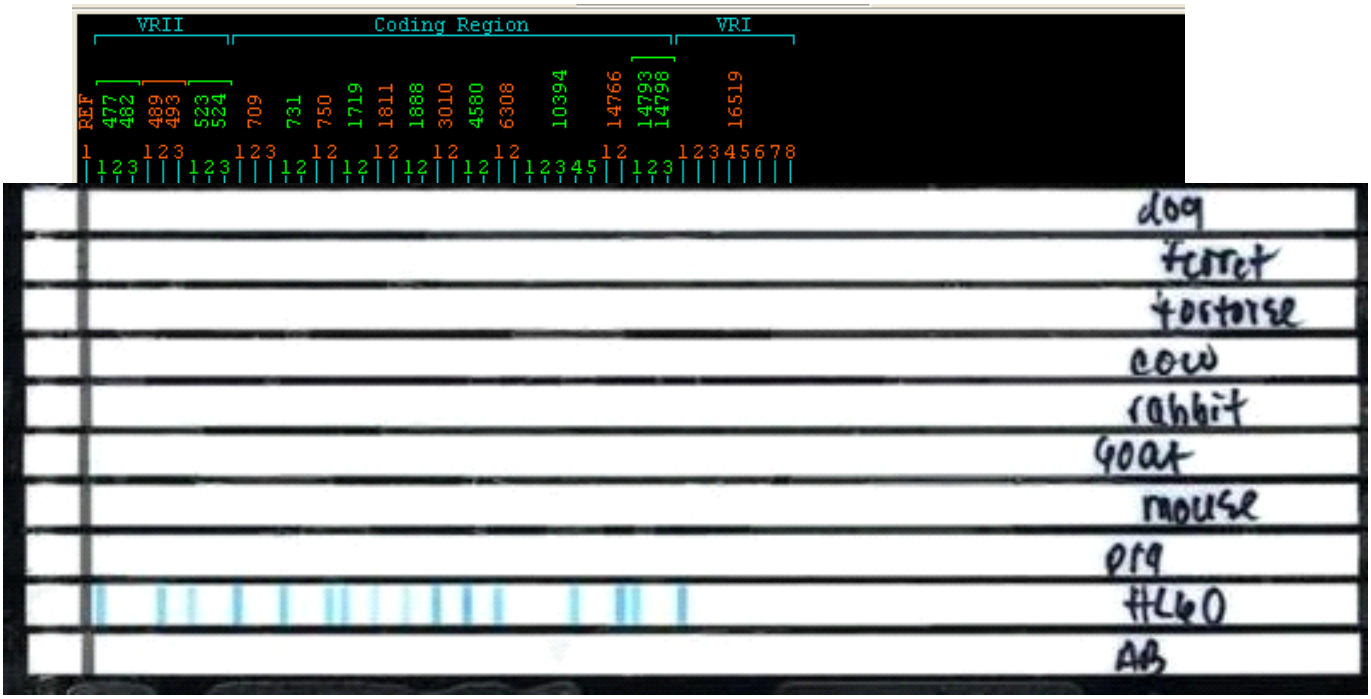


Figure 12d. Animal Study: 10-plex Probe Panel Typing Results



b. Primate Study

1. Samples and Methods

Nineteen primate DNA samples were evaluated, including the following: Sumantran orangutan (male), Western Lowland gorilla (male), De Brazza guenon (male), Siamang (male), Mandrill (male), Spider monkey (male), African Green monkey (male), Cynomolgous monkey (male), Baboon (male), Chimpanzee (male), Black howler monkey, Spot-nosed guenon (female), Pig-tailed macaque (male), Rhesus macaque (female), Red-chested mustached tamari (6 year old female), Common woolly monkey (17 year old female), Ring-tailed lemur (11 year old female), and Bonobo (8 year old male). Primate DNA was obtained from commercial sources (lemur, common woolly monkey, tamari, bonobo – Primate DNA panel, Coriell Institute, Camden, NJ; orangutan, gorilla, De Brazza guenon, siamang, mandrill – San Diego Conservation Research). Primate blood samples were obtained from commercial sources (African Green monkey, Cynomologous monkey, baboon, chimpanzee – Bioreclamation, Inc, New York) or by donation from local zoos (howler monkey, spot nosed guenon, macaques, spider monkey – Central Florida Zoo, Sanford). All primate blood samples were extracted using the Qiagen QIAmp® DNA Investigator kit on the QIACube according to manufacturer's protocols or using a standard organic extraction (yield gel quantitation).

2. Results

At least one region amplified using the 5-plex PCR for 12 out of the 18 primate DNAs (excluding human) (see Figure 13a). One or more regions amplified using the 10-plex PCR for all primates tested; a region of the 16s rRNA which includes site 3010 amplified in all primate DNAs tested (see gel image figure 13b). One or more probe signals were observed for the Gorilla, Chimp and Bonobo using the 5-plex probe panel as well as both Macaques. No probe

signals were observed for the Orangutan, De Brazza Guenin, Siamang, African Green Monkey, Cynomologous monkey, Baboon, or the Spot nosed Guenon using the 5-plex probe panel although PCR product was observed for one or more regions by gel electrophoresis (See figure 13c). Positive probe signals were observed in one or more regions of the 10-plex probe panels for the gorilla, Siamang, Spider monkey, chimpanzee, and bonobo. Both the pig-tailed and Rhesus Macaque showed similar homology with the human (HL60 sample) and contamination could not be ruled out for these two samples (see figure 13d).

3. Conclusion

Based on the species specificity results for the animal and primate studies, we concluded that the probes for both the 5-plex and 10-plex probe panels are primate specific. Additionally, the primers for the 5-plex and 10-plex PCRs, with the exception of the pair targeting the 16s rRNA region which includes site 3010, are also primate specific.

Figure 13a. 5-plex Product Gel for Primate Study

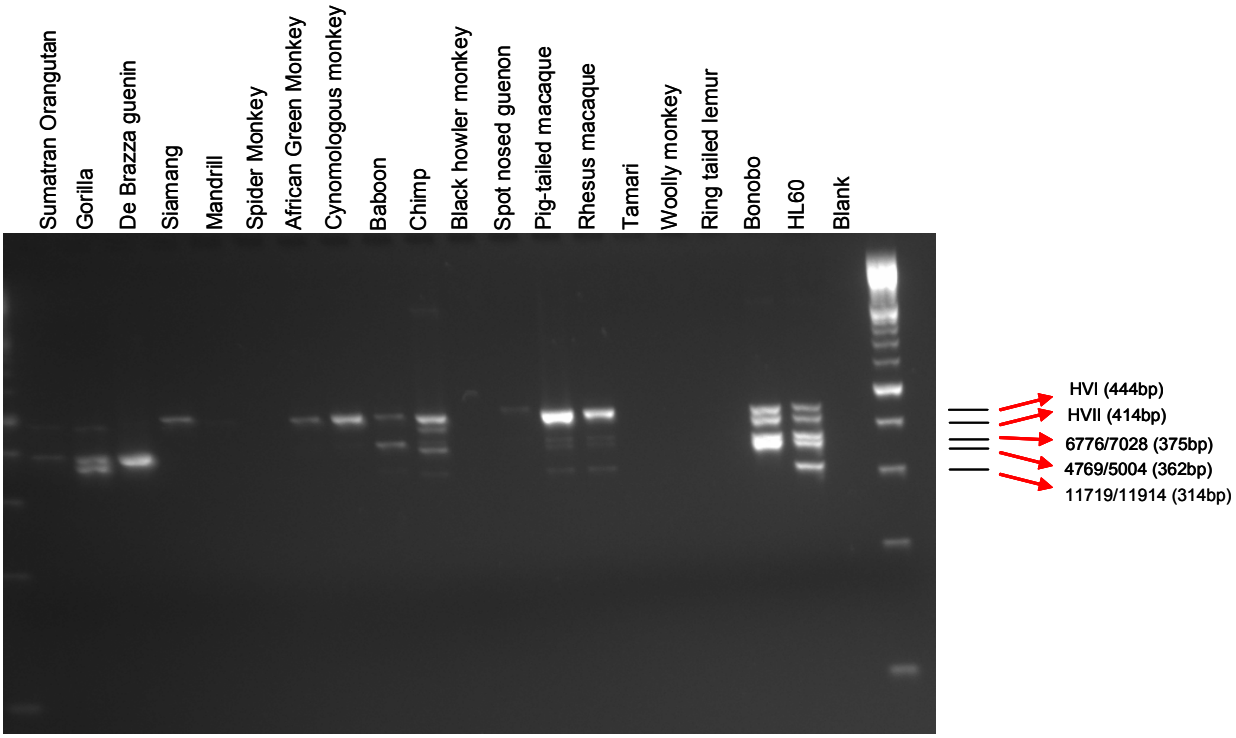


Figure 13b. 10-plex Product Gel for Primate Study

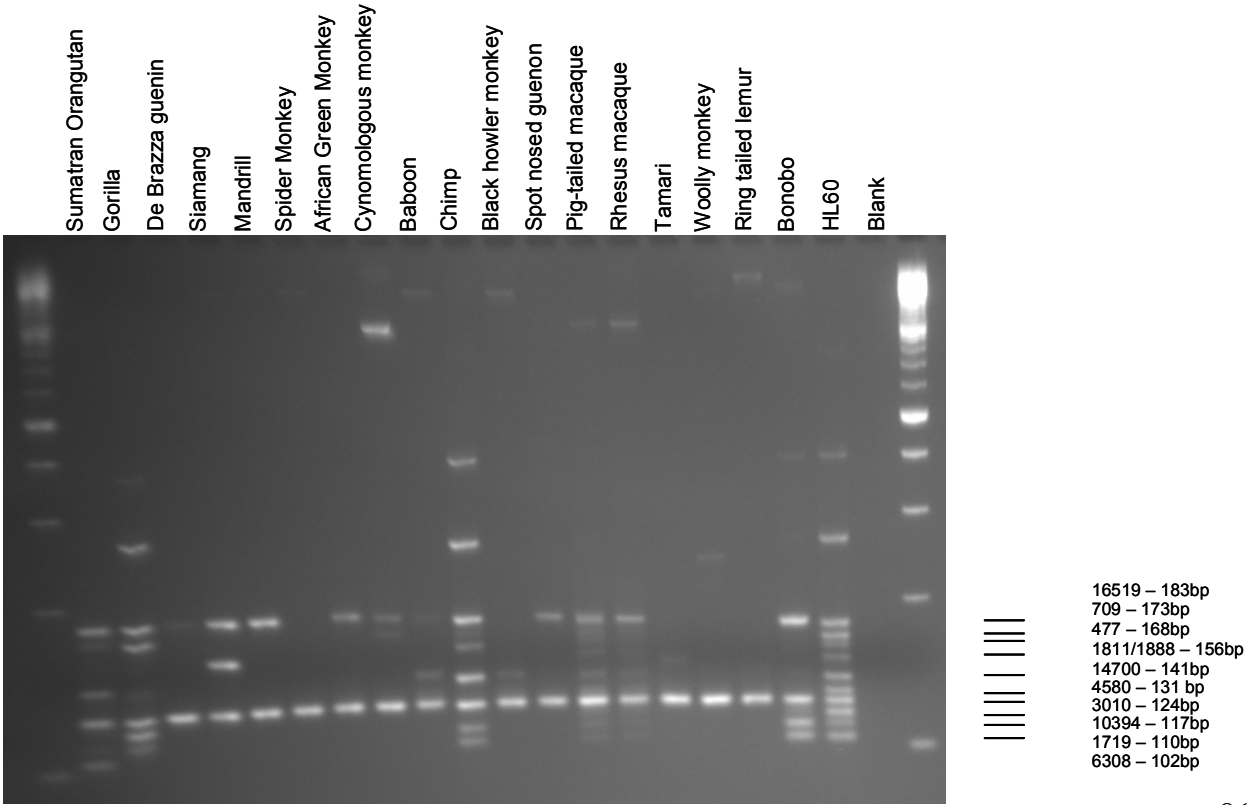
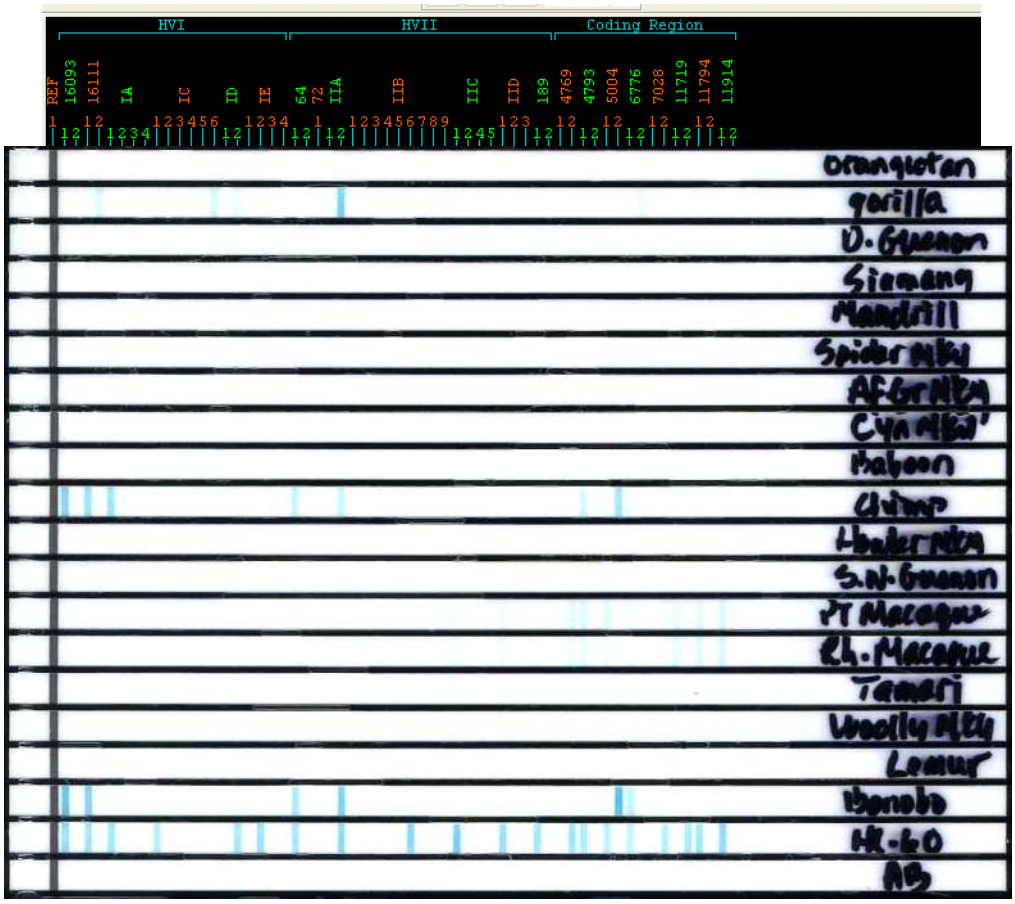
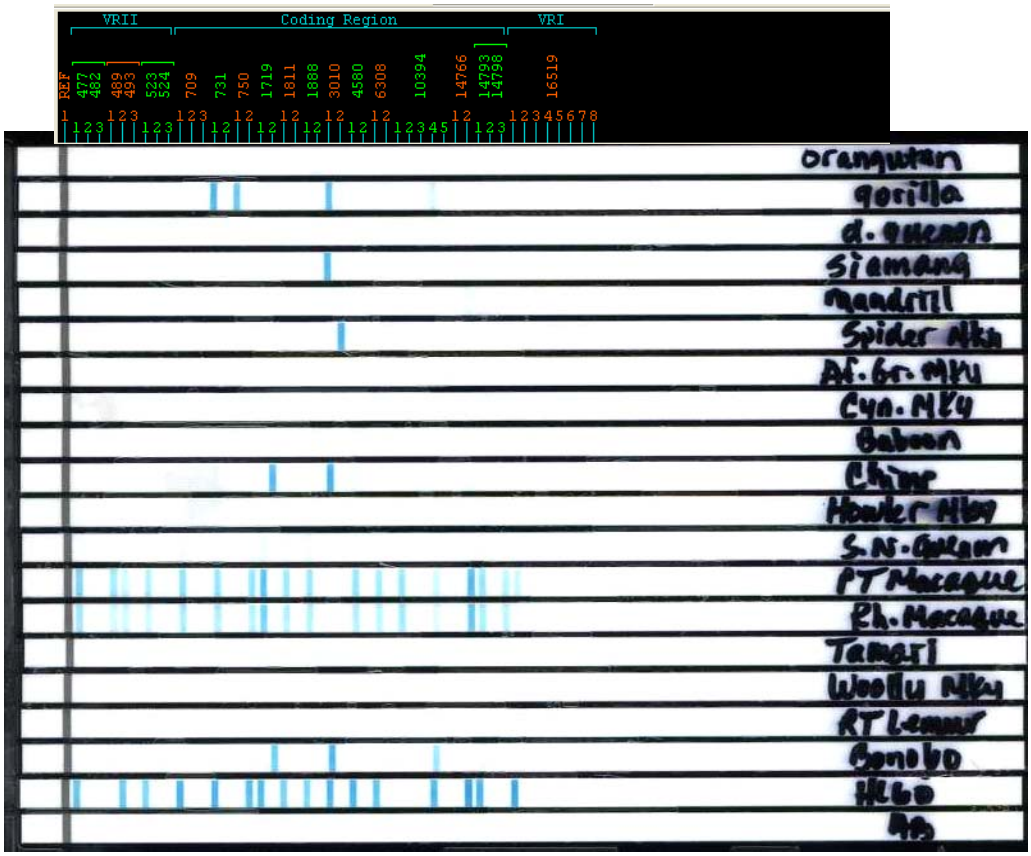


Figure 13c. 5-plex Probe Panel Typing Results



	HVI						VRI		HVII					Coding Region							
Primate Study	####	####	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	####	####	####
Orangutan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gorilla	0	2	0	6	2w	0	0	0	2	0	0	0	0	0	0	0	2	0	0	0	0
D. guenin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Siamang	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mandrill	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spider Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Af. Gr. Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyn. Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Baboon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chimp	1	1	1	0	0	0	1	0	2	0	0	0	0	0	1	2	0	0	0	0	0
Howler Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.N. Guenon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P.T. Macaque	0	0	0	0	0	0	1w	0	0	0	0	1w	0	2	1	1	1w	0	1w	1	1
Rh. Macaque	0	0	0	0	0	0	0	0	0	0	0	1w	0	2w	1w	1w	0	0	1w	1	1
Tamari	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Woolly Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R.T. Lemur	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bonobo	1	1	0	0	0	0	1	0	2	0	0	0	0	0	0	2	1	0	0	0	0
HL60	1	1	1	1	2	2	1	0	2	6	1	1	1	2	1	1	1	2	2	1	1
AB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 13d. 10-plex Probe Panel Typing Results



	VRII			Coding Region												VRI
Primate Samples	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
Orangutan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gorilla	0	0	0	0	1	1	0	0	0	1	0	0	4	0	0	0
D. guenin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Siamang	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Mandrill	0	0	0	0	0	0	0	0	0	0	0	0	0	2w	0	0
Spider Monkey	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
Af. Gr. Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyn. Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Baboon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chimp	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0	0
Howler Monkey	0	0	0	2w	0	0	0	0	0	0	0	0	0	0	0	0
S.N. Guenon	0	0	0	1w	0	0	1w	0	1w	0	0	0	0	0	0	0
P.T. Macaque	1	1,2	1	1	1	2	1	1	1	0	1	1	1,4	2	1,3	1
Rh. Macaque	1	1,2	1	1	1	2	1	1	1	0	1	1	1,4	2	1,3	0
Tamari	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Woolly Monkey	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R.T. Lemur	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bonobo	0	0	0	0	0	0	2	0	0	1	0	0	4	0	0	0
HL60	1	2	1	1	1	2	1	1	1	1	1	1	4	2	1	1
AB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

B. Reproducibility Study

a. Samples and Methods

A blind interlaboratory reproducibility study was conducted whereby both laboratories amplified and typed DNA from five control samples plus a negative and positive control using the 5-plex and 10-plex PCR and typing assays. Pooled PCR product was generated at CHORI by amplifying 100 picograms of DNA from the five samples in replicate (x4) using both the 5-plex and 10-plex PCR systems. A 3% agarose LE gel was run for ~1 hour at 125 volts to verify amplification. Fifteen µL of PCR product from each pooled sample was typed at CHORI with the 5 and 10 plex probe panels following the 4mL linear array assay. The strips were scanned and mitoreports were generated using StripScan Software at CHORI. An aliquot (52ul) of the pooled PCR product and template DNA (40ul) was provided to NCFS for each of the 5 samples listed below. The concentrations of the DNA template are indicated.

Cor37 (10pg/ul)
Cor73 (10pg/ul)
Cor79 (10pg/ul)
Cor140 (10pg/ul)
Cor184 (10pg/ul)

One hundred picograms of each template DNA was amplified with both the 5-plex and 10-plex PCR systems at NCFS. The NCFS generated PCR products and CHORI provided PCR products were run side-by-side on a 3:1 Nusive: SeaKem agarose gel. The generated PCR products (N) and provided PCR products (P) were typed with the 5-plex and 10-plex probe panels following the provided 4mL Linear Array assay procedure. The image was scanned at 150dpi and sent to CHORI for analysis and comparison.

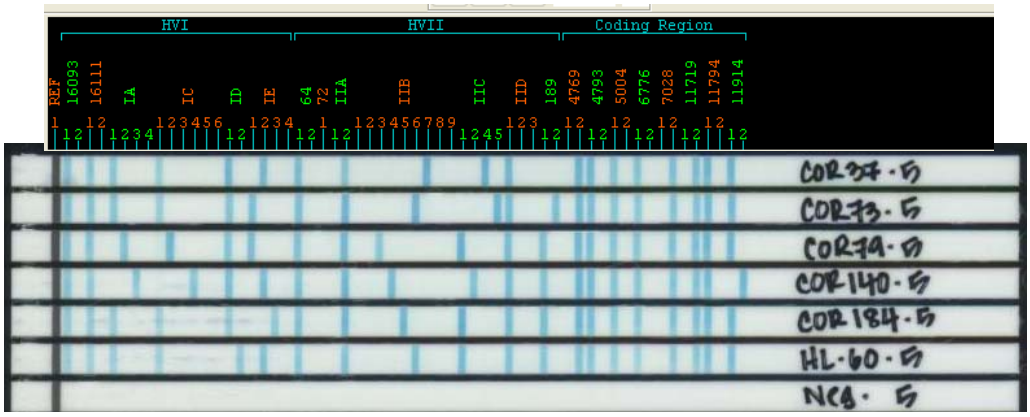
b. Results

For each sample, similar amplification yields were observed between labs which can be seen in the gel image with alternating PCR products generated by CHORI and NCFS. The expected probe signals were observed for all regions for each of the samples typed at both labs. Additionally, similar probe signal intensities were observed for the PCR products generated at CHORI and at NCFS and typed at NCFS (see figure 14a and 14b). Typing results were concordant between labs. In conclusion, amplification and typing is reproducible between laboratories.

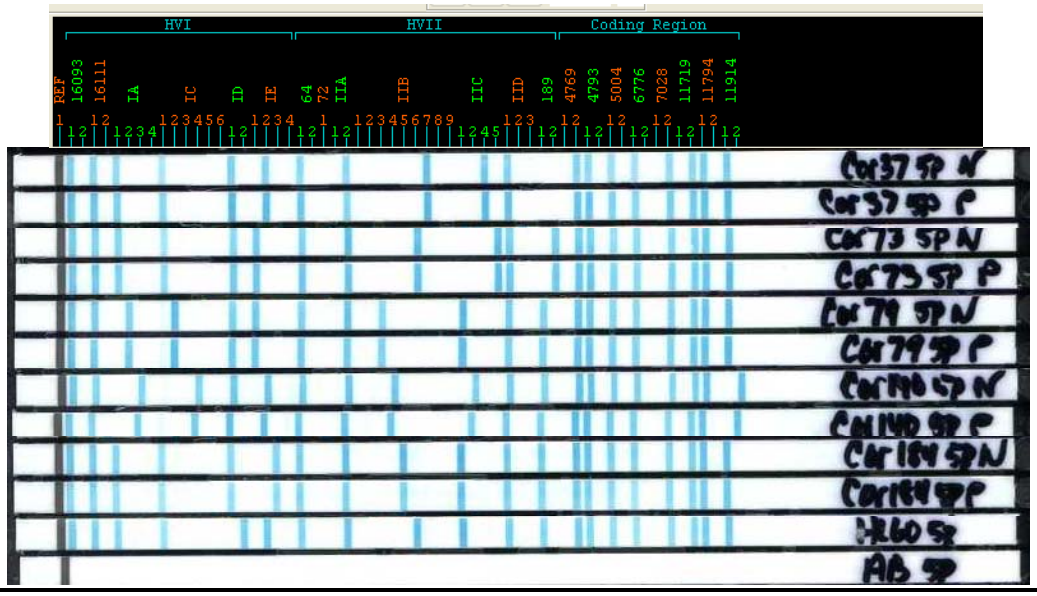
Figure 14a. CHORI VS NCFS Reproducibility Typing Results

5-Plex Probe Panel

CHORI



NCFS

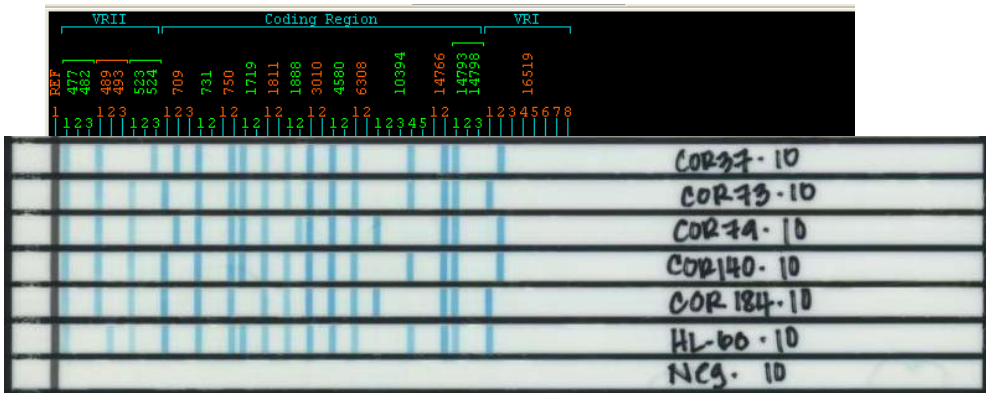


	HVI						VRI		HVII					Coding Region							
CHORI/NCFS	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
Cor37	1	1	1	1	1	2	1	0	2	7	4	1	0	2	1	1	1	2	2	1	1
Cor73	1	1	1	1	1	1	1	0	2	6	5	1	2	2	1	1	1	2	2	1	1
Cor79	1	1	2	2	1	1	1	0	2	3	1	1	1	2	1	1	1	2	2	1	1
Cor140	1	1	3	4	1	2	1	0	2	4	2	1	1	2	1	1	1	2	2	1	2
Cor184	1	1	1	1	1	3	1	0	2	5	1	1	1	2	1	1	1	2	2	1	1
HL60	1	1	1	1	2	2	1	0	2	6	1	1	1	2	1	1	1	2	2	1	1
Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

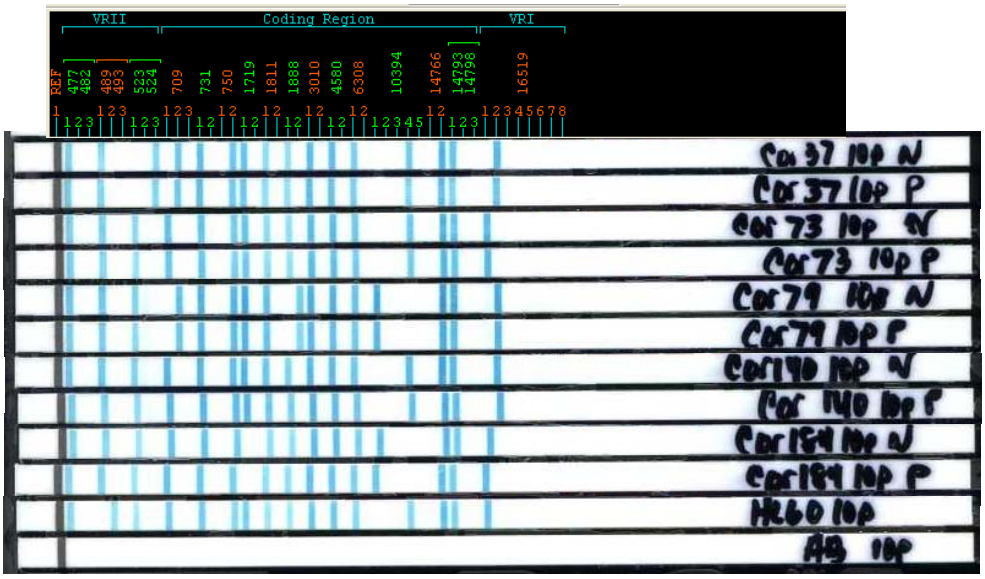
Figure 14b. CHORI VS NCFS Reproducibility Typing Results

10-Plex Probe Panel

CHORI



NCFS



	VRII			Coding Region												VRI
CHORI/NCFS	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
Cor37	1	1	3	2	1	2	1	1	1	1	1	1	4	2	1	2
Cor73	1	1	1	1	1	2	1	1	1	1	1	1	4	2	1	1
Cor79	1	1	1	2	1	2	1	1	2	1	1	1	1	2	1	2
Cor140	1	1	1	1	1	2	1	1	1	1	1	1	4	2	1	2
Cor184	1	1	1	1	1	2	0	1	1	1	1	1	1	2	1	1
HL60	1	2	1	1	1	2	1	1	1	1	1	1	4	2	1	1
Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

C. Casework Samples

a. Sample Collection

1. Semen from a vasectomized male:

Semen from a vasectomized male was dried onto sterile cotton swabs. The semen swab was then extracted using the Qiagen DNA Investigator kit (QIACube protocol, DTT add, 60 µl elution) and quantified using the ABI Quantifiler Human Real Time PCR kit.

2. Swab of beverage container lids:

A sterile cotton swab moistened with sterile Millipore water was used to swab the lid of two separate beverage container lids (coffee cup, tea cup) used by two different individuals. The samples were extracted using the Qiagen DNA Investigator kit (QIACube protocol, 60 µl elution) and quantified using the ABI Quantifiler Human Real Time PCR kit.

3. Skin:

Swabs of human skin were collected from two donors, one male and one female. A sterile cotton swab was moistened with sterile Millipore water and was used to swab the skin surface. A swab of the palm of the hand, the index finger and the chest (just below the collar bone) was collected from the female donor and a swab of the arm and face was collected from the male donor. The samples were extracted using the Qiagen DNA Investigator kit (QIACube protocol, 60 µl elution) and quantified using the ABI Quantifiler Human Real Time PCR kit.

4. Fingerprints:

A 50 mL conical tube was cleaned with 70% ethanol and placed under UV light for 10 minutes to remove any surface contamination. A male volunteer was asked to hold the tube for approximately 30 seconds. The area of the tube where the volunteer's thumb was placed was swabbed using a cotton swab moistened with sterile Millipore water. The swab was extracted using the Qiagen DNA Investigator kit (QIACube protocol, 60 µl elution) and quantified using the ABI Quantifiler Human Real Time-PCR kit.

5. Cigarette Butt:

A used cigarette butt was collected from a volunteer. The cigarette butt was stored at -40^{o}C until analysis was performed. The outer paper portion of the end of the cigarette was removed and extracted using the Qiagen DNA Investigator kit (QIACube protocol, 60 µl elution) and quantified using the ABI Quantifiler Human Real Time PCR kit.

6. Menstrual Blood:

Menstrual blood swabs were collected from two female donors through vaginal swabbing during the second day of menstruation. The samples were extracted using the Qiagen DNA Investigator kit (QIACube protocol, 60 µl elution) and quantified using the ABI Quantifiler Human Real Time PCR kit.

7. Post Coital Samples:

A female volunteer was asked to provide post coital cervico-vaginal samples collected 26 hours and 65 hours after separate acts of unprotected sexual intercourse. Prior to the collection of

samples, the volunteer was asked to abstain from sexual intercourse for seven days. Sperm and non-sperm cells were separated using a standard differential lysis protocol, with minor modifications. Post-coital cervicovaginal swabs were incubated overnight at 37°C in 400 µl of DNA extraction buffer (100mM NaCl, 10 mM Tris-HCl, 25mM EDTA, 0.5% SDS) and 0.1mg/mL Proteinase K. Swab remnants were removed to a Spin-ease basket, the basket inserted back into the original tube, and centrifuged at 14,000g for 5 min. The resulting supernatant, containing the non-sperm DNA fraction, was removed into a separate tube for further analysis. The sperm pellet was washed using 1 mL DNA extraction buffer and then re-suspended in 400 µl of DNA extraction buffer, 0.1mg/mL Proteinase K, and 40 µl of 0.39M DTT and incubated for 1 h at 56°C. A volume of phenol/chloroform/isoamyl alcohol equal to the volume of the crude extract was added and vigorously intermixed by shaking. The aqueous layer, containing the DNA, was removed. Precipitation of the DNA was accomplished by the addition of cold absolute ethanol (two and a half times the volume of the aqueous layer extract) and allowed to progress overnight at –20°C. The DNA was pelleted by centrifugation, washed once using 70% ethanol and re-solubilized with 100 µl of TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) overnight at 56°C. All sample extracts were quantified using the ABI Quantifiler Duo Real Time PCR kit.

b. Results

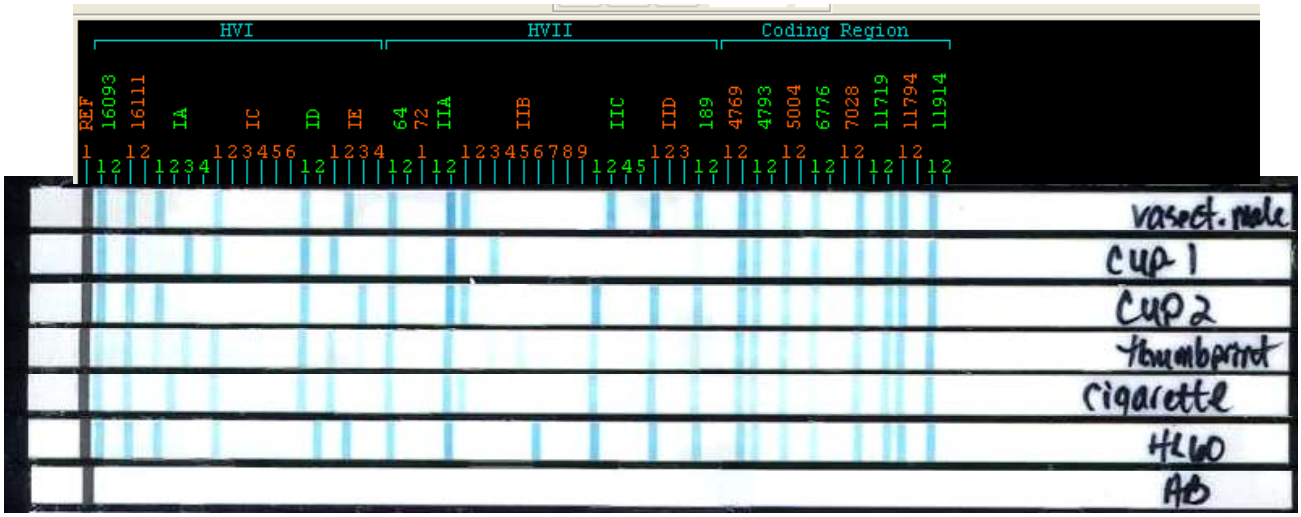
Visible PCR products for each of the 15 targets were observed by gel electrophoresis for all casework samples amplified using the 5-plex and 10-plex PCR assays, including DNA from thumbprints, swabbed from cups, skin cells, semen from the vasectomized male, and menstrual blood. Typeable results were obtained for all casework samples using both the 5-plex and 10-plex probe panels. Strip images and typing results for each of the casework samples typed are

shown in Figures 15-19. The sperm DNA fraction and non-sperm DNA fraction from the post-coital samples collected at 26 and 65 hours are consistent with the female reference sample (data not shown). These results are consistent with previous findings that mtDNA typing results from the sperm fraction separated using a differential DNA extraction method are consistent with the female type and not the male type. Presumably, DNA from female epithelial cells are not completely separated from the sperm during the extraction procedure, resulting in a female type. Additionally, mtDNA are found in the midpiece of the sperm which may be lost with the sperm tails during the extraction procedure and the number of mtDNA copies found in epithelial cells is much greater than that of sperm.

c. Conclusions

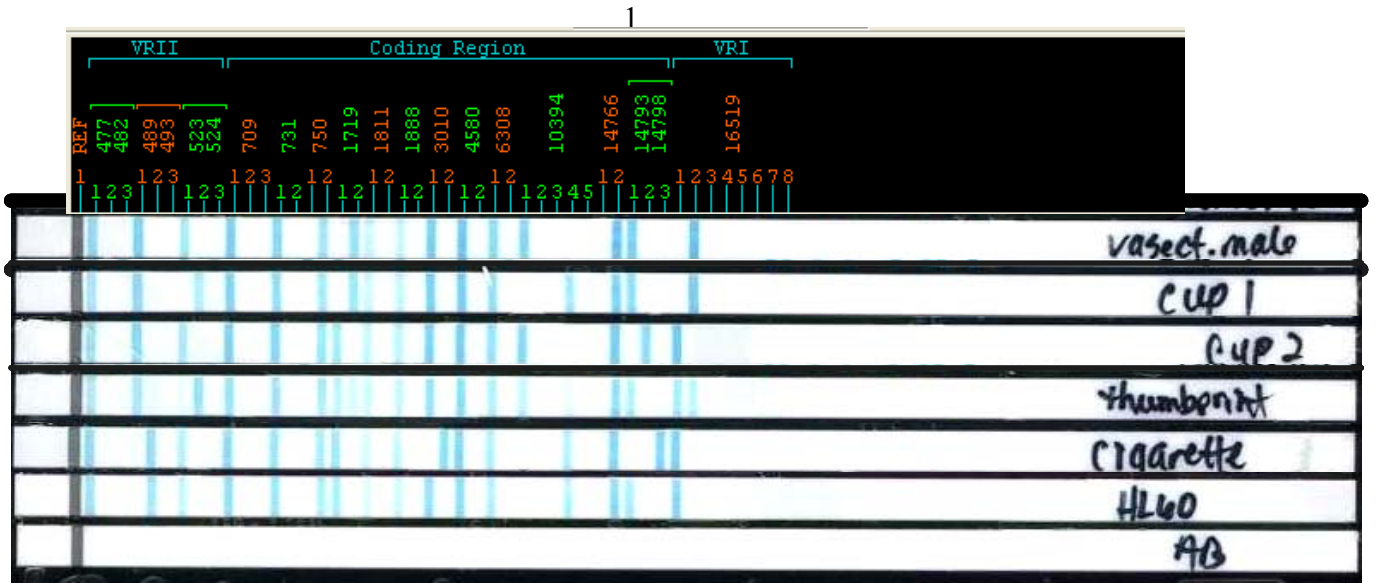
Amplification and typing using the 5-plex and 10-plex PCR and typing assay was successful for many types of casework samples, including low copy number samples such as DNA swabbed from cups and thumbprints. mtDNA analysis is not useful for the analysis of sperm fractions from post-coital samples when collected using the differential extraction method.

Figure 15. 5-plex Probe Panel Results for Contact Samples, Vasectomized Male



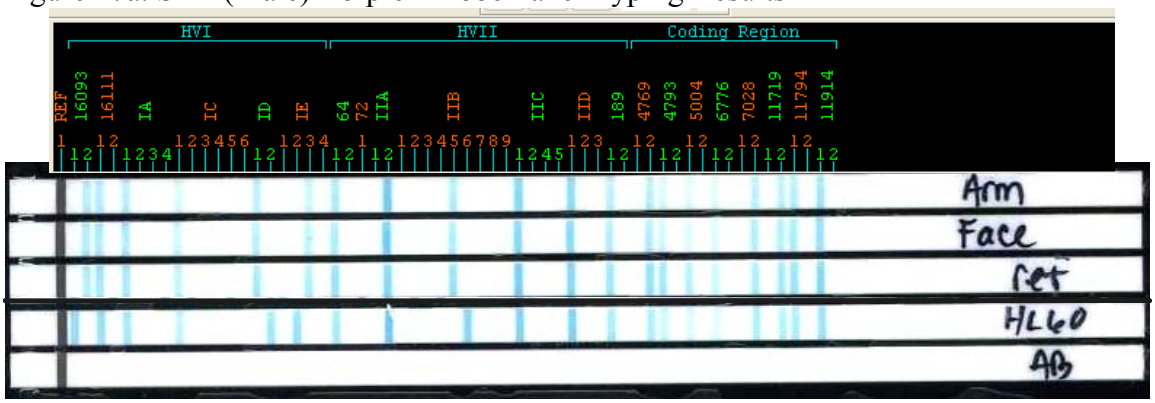
	HVI						VRI		HVII					Coding Region							
Vasect. Male, Contact Samples	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
Vasect. Male	1	1	1	1	1	2	1	0	2	1	2	1	1	2	1	1	1	2	2	1	1
Cup 1	1	1	3	1	1	1	1	0	2	3	0	0	1w	2	1	1	1	2	2	1	1
Cup 2	1	1	1	0	1	3	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
Thumbprint	1	1	1,3	1	1	1,3w	1	0	2	3w,5w	1	1	1	2	1	1	1	2	2	1	1
Cigarette	1	1	2	1	1	1	1	0	2	1	1	1	1	2	1	1	1w	2	2	1	1
HL60	1	1	1	1	2	2	1	0	2	6	1	1	1	2	1	1	1	2	2	1	1
AB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 16. 10-plex Probe Panel Results for Contact Samples, Vasectomized Male



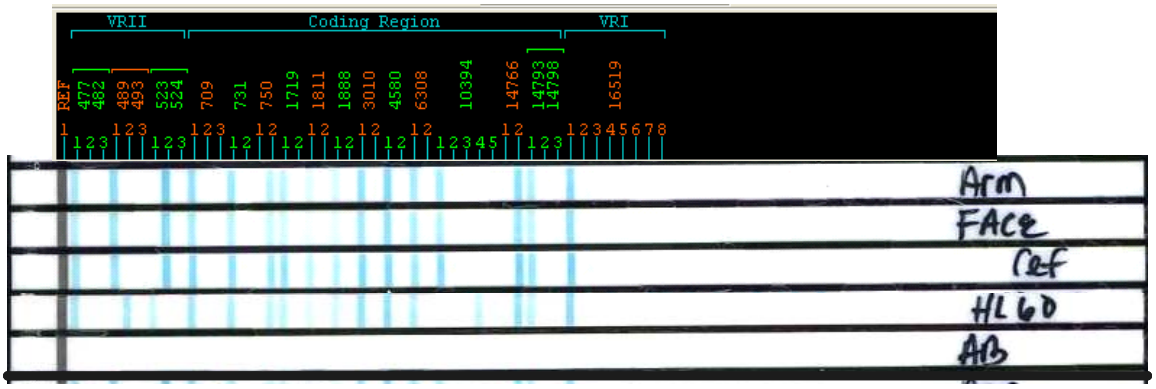
	VRII			Coding Region												VRI
Vasect. Male, Contact Samples	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
Vasect. Male	1	1	1	1	1	2	2	1	1	1	1	1	1	2	1	2
Cup 1	1	1	2	1	1	2	2	1	1	1	1	1	4	2	1	2
Cup 2	1	1	1	1	1	2	1	1	1	1	1	1	1	2	2	1
Thumbprint	1	1,2w	1w,2	1	1	2	1,2	1	1	1	1	1	1,4w	2	1	1,2
Cigarette	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1
hl60	1	2	1	1	1	2	1	1	1	1	1	1	4	2	1	1

Figure 17a. Skin (Male) – 5 plex Probe Panel Typing Results



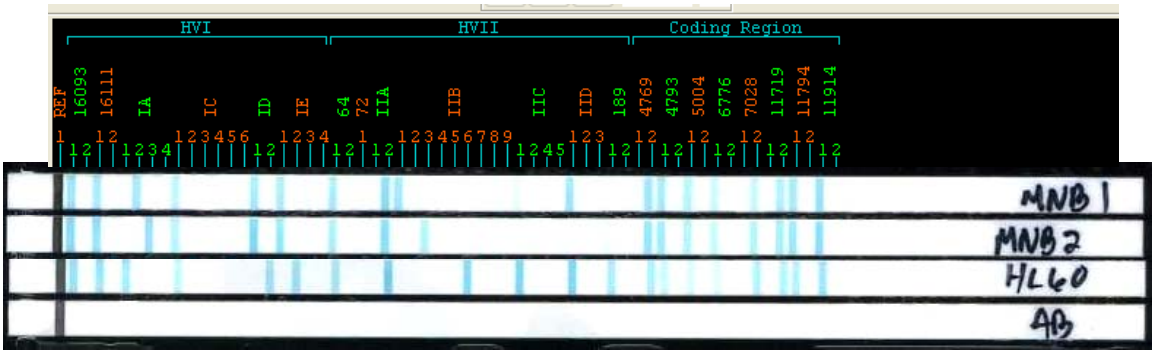
	HVI						VRI		HVII					Coding Region							
Skin (male) Samples	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
arm	2	1	1	1	1	3	1	0	2	5	1	1	1	2	1	1	1	2	2	1	1
face	2	1	1	1	1	3	1	0	2	5	1	1	1	2	1	1	1	2	2	1	1
ref	2	1	1	1	1	3	1	0	2	5	1	1	1	2	1	1	1	2	2	1	1
HL60	1	1	1	1	2	2	1	0	2	6	1	1	1	2	1	1	1	2	2	1	1
AB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 17b. Skin (Male) – 10 plex Probe Panel Typing Results



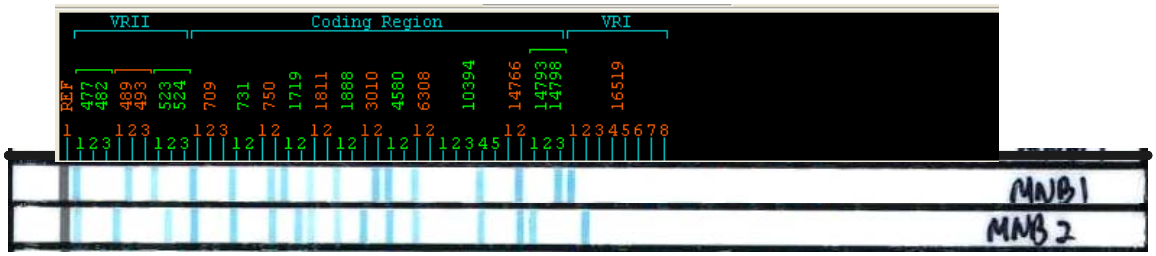
	VRII			Coding Region												VRI
Skin (male) Samples	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
arm	1	1	2	1	1	2	1	1	1	1	1	1	1	2	1	1
face	1	1	2	1	1	2	1	1	1	1	1	1	1	2	1	1
ref	1	1	2	1	1	2	1	1	1	1	1	1	1	2	1	1
HL60	1	2	1	1	1	2	1	1	1	1	1	1	4	2	1	1

Figure 18a. Menstrual Blood – 5 plex Probe Panel Typing Results



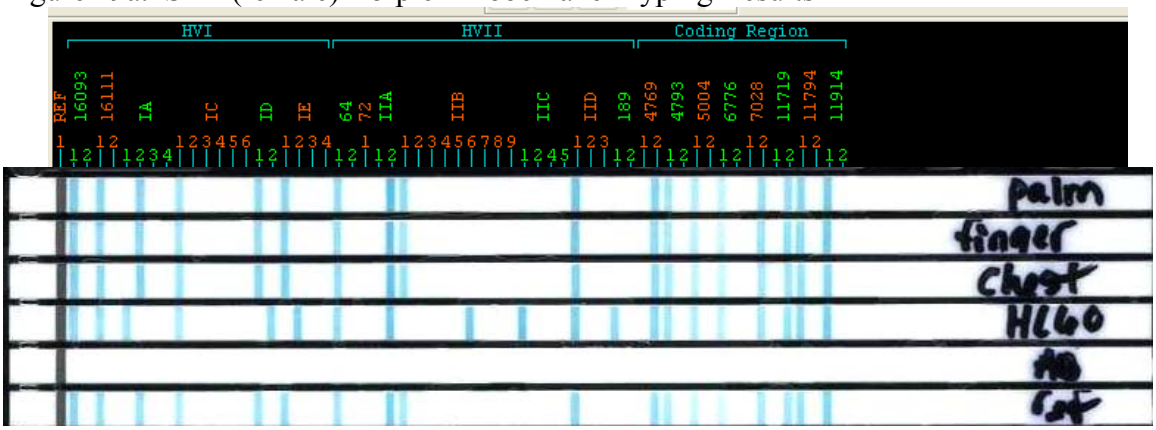
	HVI						VRI		HVII					Coding Region							
Menstrual Blood Samples	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
MNB 1	1	1	2	1	1	1	1	0	2	1	1w	1	0	2	1	1	1	2	2	1	1
MNB 2	1	1	3	1	1	1	1	0	2	3	0	0	1w	2	1	1	1	2	2	1	1
HL60	1	1	1	1	2	2	1	0	2	6	1	1	1	2	1	1	1	2	2	1	1
AB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 18b. Menstrual Blood – 10 plex Probe Panel Typing Results



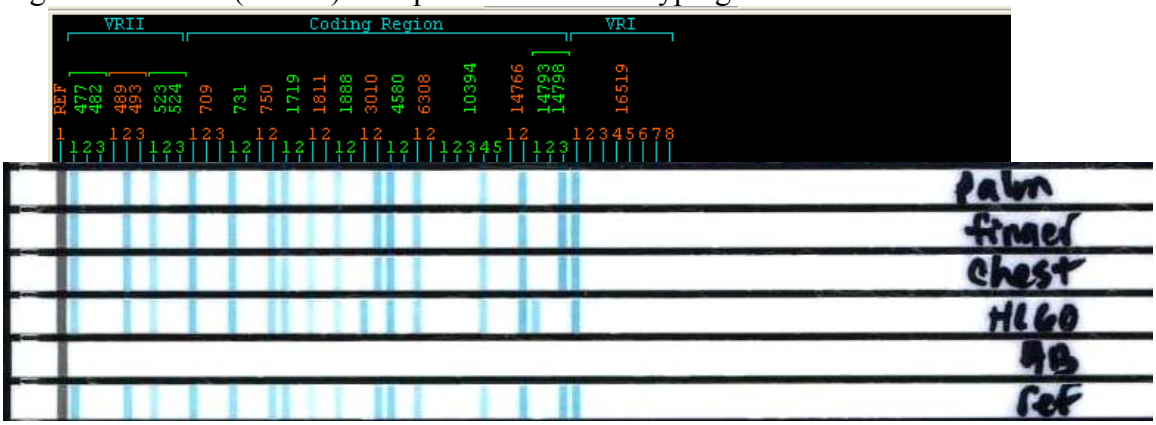
	VRII			Coding Region												VRI
Menstrual Blood Samples	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
MNB 1	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1
MNB 2	1	1	2	1	1	2	2	1	1	1	1	1	4	2	1	2
AB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 19a. Skin (female) – 5 plex Probe Panel Typing Results



	HVI						VRI		HVII					Coding Region							
Skin (female) Samples	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
palm	1	1	2	1	1	1	1	0	2	1	0	1	0	2	1	1	1	2	2	1	1
finger	1	1	2	1	1	1	1	0	2	1	0	1	0	2	1	1	1	2	2	1	1
chest	1	1	2	1	1	1	1	0	2	1	0	1	0	2	1	1	1	2	2	1	1
Ref	1	1	2	1	1	1	1	0	2	1	0	1	0	2	1	1	1	2	2	1	1

Figure 19b. Skin (female) – 10 plex Probe Panel Typing Results



	VRII			Coding Region											VRI
Skin (female) Samples	477/482	489/493	523/524	709	731	750	1719	1811	1888	3010	4580	6308	10394	14766	14793/14798	16519
palm	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1
finger	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1
chest	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1
Ref	1	2	1	1	1	2	1	1	1	2	1	1	4	2	3	1
HL60	1	2	1	1	1	2	1	1	1	1	1	1	4	2	1	1

D. Precision/Accuracy Study

a. Samples and Methods

Buccal swabs were collected from four volunteers using sterile cotton swabs. The swabs were dried overnight and then extracted using the Qiagen QIAmp® DNA Investigator kit on the QIACube (Surface and buccal swabs program, 60 µl elution) according to manufacturer's protocols. Extracts were quantified using the Quantifiler® Human Real Time PCR kit according to manufacturer's protocols (Applied Biosystems). The positive control from the AmpFlSTR® Yfiler® PCR Amplification kit (Applied Biosystems), 007 male DNA, was used as the fifth sample. One hundred picograms of each DNA extract was used for amplification. Each sample was amplified five times for the Linear Array 5 plex and 10 plex. Fifteen microliters of PCR product was typed with the 5-plex and 10-plex probe panels. Gel images and scanned strip images were sent to CHORI for analysis and interpretation

b. Results

All 15 targets amplified using the 5-plex and 10-plex PCR assays observed by gel electrophoresis for all 5 replicates from the 5 different individuals. Typeable results were obtained using the 5-plex and 10-plex assays for all samples. Typing results were consistent for each of the replicates for all five samples.

VI. High-Throughput Alternatives: Automate Typing and modify Interpretation Software

We have explored several options for automating the typing steps of the linear array procedure and found that our manual typing procedure was most easily adapted for use with the Tecan ProfiBlot T24 or T48. This instrument allows for automation of linear array typing. All solutions required for typing are prepared and placed on the instrument. Linear array probe panels are placed in tray wells by the user prior to starting the run. The wash buffer is then dispensed by the instrument into the appropriate wells and then the user adds the denatured PCR product to the wells when prompted. The run then continues through all typing steps. At the end of the approximately 1.5 hour run, the user can then remove the developed linear array panels from the tray and proceed to scan or photograph the results for archival purposes. The optimized procedure is listed in Table 9 below.

Table 9. Tecan T24 Profiblot Procedure for 4mL Linear Array Assay

Step	File	Channel	Volume	Time	Temp
1	Temp				55
2	Disp	4	1000ul		
3	Disp	4	3000ul		
4	Pause				
5	Inc			15 min	
6	Asp				
7	Disp	2	1000ul		
8	Disp	2	3000ul		
9	Asp				
10	Disp	1	1000ul		
11	Disp	1	3000ul		
12	Inc			5 min	
13	Asp				
14	Disp	2	1000ul		
15	Disp	2	3000ul		
16	Asp				
17	Disp	2	1000ul		
18	Disp	2	3000ul		
19	Inc			12 min	
20	Asp				
21	Cool				

Step	File	Channel	Volume	Time	Temp
22	Disp	2	1000ul		
23	Disp	2	3000ul		
24	Asp				
25	Disp	5	1000ul		
26	Disp	5	3000ul		
27	Inc			5 min	
28	Asp				
29	Disp	6	1000ul		
30	Disp	6	3000ul		
31	Inc			15 min	
32	Asp				
33	Disp	3	3000ul		
34	Asp				
35	Disp	3	3000ul		
36	Asp				
37	Disp	3	3000ul		
38	END				

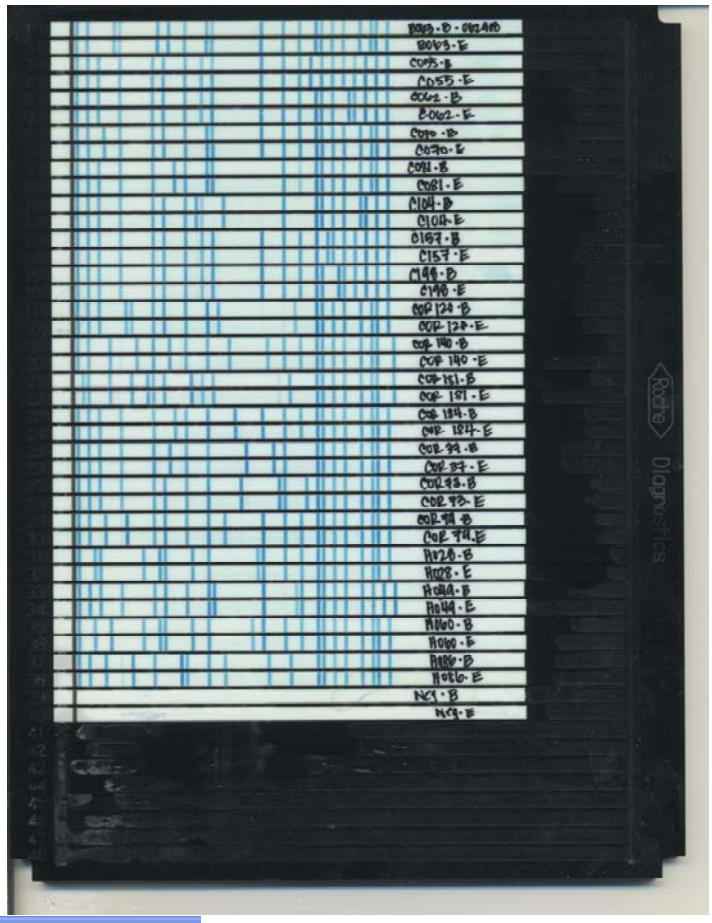
Channel 1 = conjugate solution
Channel 2 = wash buffer
Channel 3 = distillied water
Channel 4 = wash buffer
Channel 5 = Citrate buffer
Channel 6 = Color Development solution

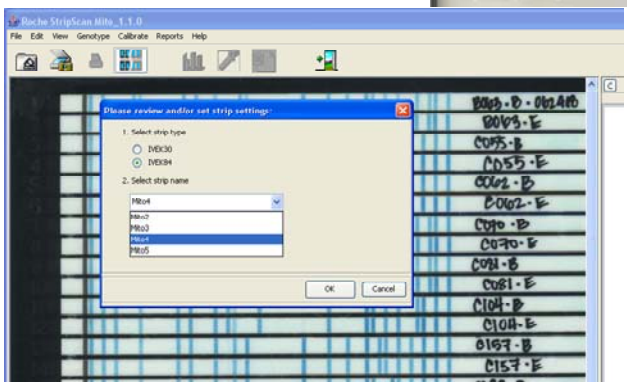
We have also modified existing StripScan software for scanning and interpreting typing results obtained from the 5-plex and 10-plex assays. Several features were added to the software including a modification to allow the user to change the colors of the reference guide and backgrounds. Also, a feature to generate a mitoreport in excel was added. A detailed overview of the StripScan Mitotyper software is provided in the Figure 20 below.

Figure 20. StripScan Mitotyper Detailed Overview

1. **Batches of up to forty-eight strips are assembled on a custom holding plate, and digitally imaged at 150 dpi using a common flatbed scanner.**

2. **Images are saved as jpg files at 150 dpi and can then be imported into the strip scan Mitotyper software program. Alternatively, strips assembled on the custom holding plate can be directly scanned into the strip scan Mitotyper software program by clicking on the scanner icon in the software program.**





3. **Once the image is loaded, the user will be prompted to select the strip type and strip name.**

4. **Select strip type Ivek 30 for HVI/HVII and IVEK 84 for 5-plex and 10-plex assays.**

5. **Select strip name Mito1 for HVI/HVII, Mito4 for 5-plex and Mito5 for 10-plex assays.**

6. **The appropriate grid box overlay will be automatically applied to the strip images starting with the reference.**

7. **Activate the calibration process if calibration is required by selecting the calibration button. Accurate interpretation of probe signals requires that the grid boxes are properly aligned over the probe positions on every strip.**

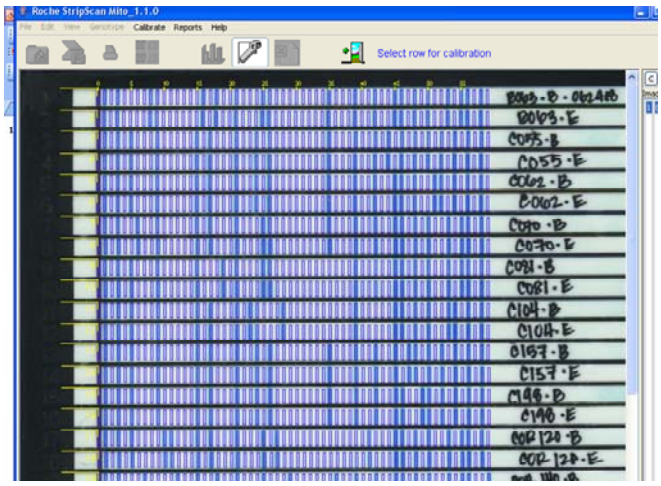
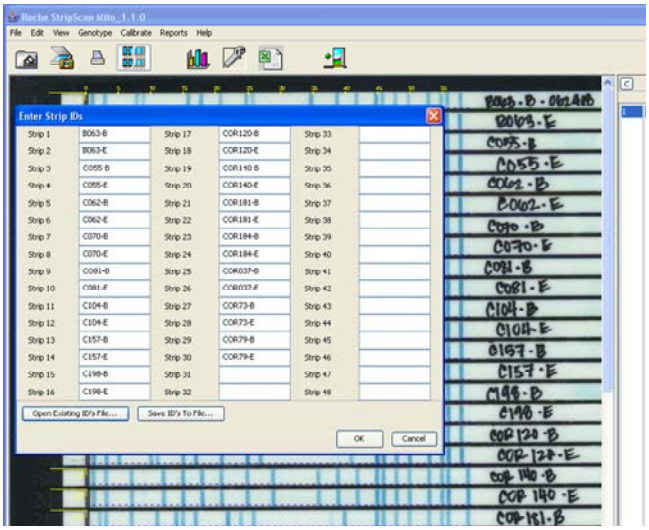
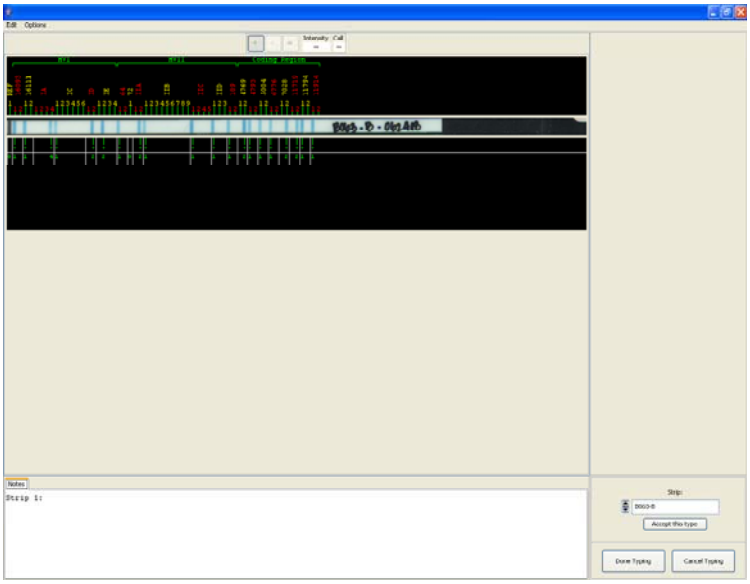


Figure 20. StripScan Mitotyper Detailed Overview (continued)

8. Select row for calibration when prompted.
9. Select grid box for calibration when prompted.
10. Use the arrow keys to adjust grid box over the probe signal.
11. Click the calibration button when grid box is positioned.



12. Strip ID's can be set by selecting Strip ID from the Edit menu.
13. Enter sample identifiers to the corresponding strip number in the strip ID Table. Alternatively, an existing file can be opened by clicking on the corresponding button.
14. Strip ID's can be saved to a file by clicking on the corresponding button.



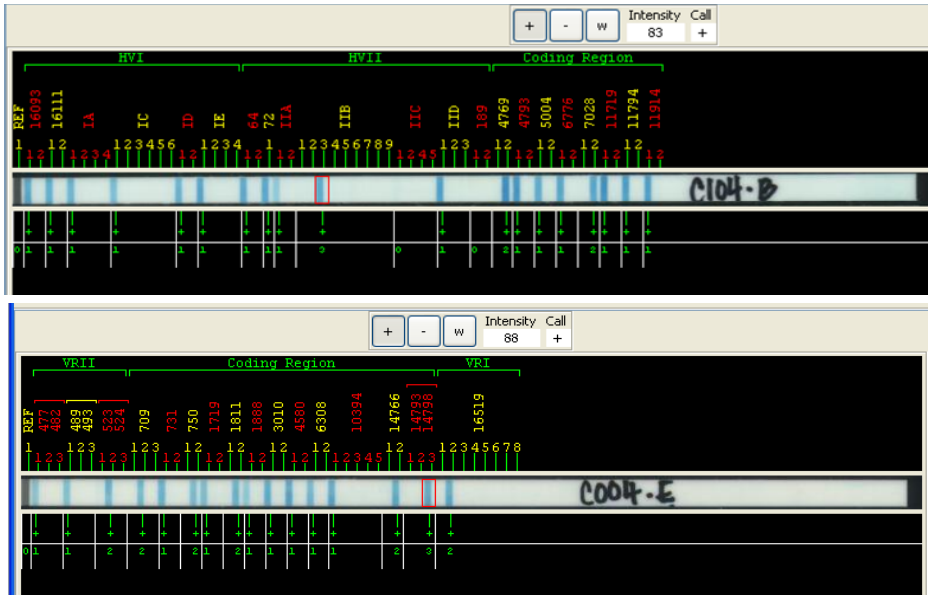
15. StripScan measures the signal intensity for each probe on the strip then interprets the probe signals using genotyping algorithms.
16. Probe calls are indicated under the corresponding probe signals.
17. The Strip ID is shown in the box under 'strip' and the strip number is shown in the notes.

Figure 20. StripScan Mitotyper Detailed Overview (continued)

18. **The probe patterns are reviewed by lab personnel experienced in typing, and can be overridden as needed. The scrolling window can be positioned over each probe signal and the intensity and probe call will be shown in the corresponding boxes. The probe call can be changed by using the '+, -, w' call buttons if necessary.**

19. **Once the typing is completed for the strip, the calls can be accepted by selecting the 'accept the type' button. Once all strips have been typed, the 'done typing' button can be selected.**

20. **Results for the 5-plex and 10-plex probe panels are shown below. The 5-plex and 10-plex assays use 59 and 46 probes respectively to target variation at the most polymorphic sites distributed throughout the mitochondrial genome allowing for increased discrimination compared to HVI/HVII assays.**



21. **After all strips in an image are reviewed, custom designed reports are generated indicating signal intensities (pixel intensity report) and selected probe calls for each strip (mitoreport). A mitoreport can be generated after genotyping by selecting mitoreport under the report menu in excel.**

	HVI									HVII							Coding Region				
	16093	16111	IA	IC	ID	IE	64	72	IIA	IIB	IIC	IID	189	4769	4793	5004	6776	7028	11719	11794	11914
B063-B	1	1	4	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
B063-E	1	1	4	1	2	2	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
C055-B	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
C055-E	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
C062-B	1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	2	1	1	1	1
C062-E	1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1	2	1	1	1	1
C070-B	1	1	2	1	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
C070-E	1	1	2	1	1	1	1	0	2	1	1	1	1	2	1	1	1	2	2	1	1
C081-B	1	1	1	1	1	3	1	0	2	1	0	1	1	2	1	1	1	2	2	1	1
C081-E	1	1	1	1	1	3	1	0	2	1	0	1	1	2	1	1	1	2	2	1	1
C104-B	1	1	1	1	1	1	1	1	1	3	0	1	0	2	1	1	1	2	1	1	1
C104-E	1	1	1	1	1	1	1	1	1	3	0	1	0	2	1	1	1	2	1	1	1
C157-B	1	1	1	1	1	1	1	0	1	1	1	1	1	2	2	1	1	1	1	1	1
C157-E	1	1	1	1	1	1	1	0	1	1	1	1	1	2	2	1	1	1	1	1	1
C198-B	1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	2	1	1	1	1	1
C198-E	1	1	1	1	1	1	1	0	1	1	1	1	1	2	1	2	1	1	1	1	1
COR120-B	1	1	1	2,3	1	1	1	0	2	2	0	3	0	2	1	1	1	2	2	1	1
COR120-E	1	1	1	2,3	1	1	1	0	2	2	0	3	0	2	1	1	1	2	2	1	1
COR140-B	1	1	3	4	1	2	1	0	2	4	2	1	1	2	1	1	1	2	2	1	2
COR140-E	1	1	3	4	1	2	1	0	2	4	2	1	1	2	1	1	1	2	2	1	2
Cor181-B	2	1	3	3,6	1	1	2	0	1	1	5w	2	0	2	1	1	1	2	2	1	2
Cor181-E	2	1	3	3,6	1	1	2	0	1	1	5w	2	0	2	1	1	1	2	2	1	2

VII. Conclusions

A. Discussion of Findings

We successfully identified a highly informative set of polymorphic sites and developed a highly sensitive, easy to use 5-plex and 10-plex PCR and linear array assay for simultaneous analysis of polymorphic regions in the non-coding and coding regions for increased discrimination compared to currently available mtDNA assays. A population study was conducted in order to determine the power of discrimination for the new expanded HV+ array and results show that the informativeness was improved for all population groups tested and a significant increase in the power of discrimination was observed for both the US Caucasian (0.9946 from 0.9768) and the US Hispanic (0.9893 from 0.9449). A sensitivity study was conducted and it was shown that both the 5-plex and 10-plex assays are highly sensitive assays, with the 10-plex being more sensitive. Typeable results were observed with ~5 pg of DNA input for the 5-plex at 34 cycles and ~1pg input for the 10-plex. At 38 cycles, ~0.5 pg of DNA input yielded typeable results for both the 5-plex and 10-plex assays. Results from a mixture study show that a minor component present at ~10% is reliably detected with the 5-plex assay and ~5% with the 10-plex assay. The 5-plex and 10-plex probe panels were shown to be primate specific and all primers were shown to be primate specific except the pair targeting the 16s rRNA region. Various case type samples (including low copy number samples) were also successfully amplified and typed by the NCFS. A procedure was also validated for automating the typing assay and scanning and interpretation software were modified for use with the 5-plex and 10-plex assays. Up to 48 samples can be typed manually or automated in less than 2 hours and the data can now be quickly analyzed using the Strip Scan Mitotyper software.

B. Implications for Policy and Practice

The HV+ multiplex PCR and linear array typing system allows for simultaneous amplification and typing of polymorphic sites within the coding region and the commonly targeted HV regions. This approach targets a set of highly discriminating sites distributed throughout the mitochondrial genome which has advantages over sequencing short segements of the coding region as discussed by Coble et al. (2006). With the improved discrimination power, this assay can serve as a stand alone assay. Alternatively, the assay can be used in conjunction with mtDNA HVI/HVII sequence analysis or with additional haplogroup specific coding region assays (Coble *et al.* 2006). This linear array assay will allow for cost-effective, rapid typing of an increased number of samples and could be used routinely by any forensic laboratory.

C. Implications for Further Research

Further collaborative studies are ongoing including additional population studies, analysis of casework samples, internal validation studies and further validation of the mitotyper software.

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Dissemination of Research Findings

A Highly Informative Multiplex PCR and Linear Array Probe panel Targeting 61 Polymorphic Sites Distributed Throughout the Mitochondrial Genome
Cassandra D. Calloway, Sarah M. Stuart, Jeff Post, and Henry Erlich
19th Annual International Symposium on Human Identification
Los Angeles, California, October 13-16th, 2008
Poster Presentation

Demonstration of Strip Scan Mitotyper Software for Interpreting Linear Array Results
Cassandra D. Calloway, Sarah M. Stuart, Jeff Post, and Henry Erlich
National Institute of Justice Conference
July 21-23, 2008: Crystal Springs, Virginia
Demonstration and poster

The Frequency of Heteroplasmy in the Human Mitochondrial Genome Differs Between Tissues and Across Age Groups
Cassandra Calloway
CHORI SPAC Symposium: Oakland, California
May 23, 2008
Oral presentation

Increased Discrimination Using a Panel of 83 Immobilized SSO Probes Targeting 48 Informative Sites within the Mitochondrial Genome
Sarah M. Stuart, Cassandra Calloway, Henry Erlich
CHORI SPAC Symposium: Oakland, California
May 23, 2008
Poster presentation

Improving the Discrimination of mtDNA Typing Using a Multiplex PCR and Linear Array Probe Panel: HV and Beyond
Sarah M. Stuart, Cassandra D. Calloway, Henry A. Erlich
California Association of Criminalists, DNA Workshop
October 16-19, 2007: Berkeley, CA
Oral presentation

Applications, Methods and Interpretation Issues of the LINEAR ARRAY mtDNA HVI/HVII and beyond
Cassandra D. Calloway
Workshop: International Commission of Missing Persons
September 11, 2007: Sarajevo, Bosnia and Herzegovina

Improving the Discrimination of mtDNA Typing Using a Multiplex PCR and Linear Array Probe Panel: HV and Beyond

Cassandra D. Calloway, Sarah M. Stuart, Henry A. Erlich
Oral Presentation: International Society for Applied Biological Sciences
September 3-7, 2007: Split, Croatia

Increased Discrimination Using a Panel of 83 Immobilized SSO Probes Targeting 48 Polymorphic Sites Within the Mitochondrial Genome
Cassandra D. Calloway, Sarah M. Stuart, Henry A. Erlich
Poster Presentation: International Society of Forensic Genetics
August 21-25, 2007: Copenhagen, Denmark

Increasing Mitochondrial DNA Discrimination Using a Panel of 84 Immobilized SSO Probes Targeting Informative Sites within the Mitochondrial Genome
Cassandra D. Calloway, Sarah M. Stuart, Henry A. Erlich
Poster presentation: National Institute of Justice Grantees meeting
July23rd-25th, 2007: Crystal Springs, Virginia

Detection of Sequence Variation in Caucasian and Hispanic Samples across the mitochondrial Genome Using an 83 Immobilized SSO Probe Panel
Cassandra D. Calloway, Sarah M. Stuart, Henry A. Erlich
Oral Presentation: California Department of Justice DNA User's Meeting
July 11, 2007: Richmond, California

LINEAR ARRAY mtDNA HVI/HVII Region-Sequence Typing Kit: Applications, Methods and Interpretation Issues
California Department of Justice Mitochondrial DNA Analysis and Typing Workshop
April 23-26, 2007
Instructor: Cassandra D. Calloway
Assistant Instructor: Sarah M. Stuart

Increasing Mitochondrial DNA Discrimination Using a Panel of 83 Immobilized SSO Probes Targeting Informative Sites within the Mitochondrial Genome
Cassandra D. Calloway, Sarah M. Stuart, and Henry A. Erlich
American Academy of Forensic Sciences 59th Annual Meeting
February 19-24, 2007
Oral presentation

Detection of Sequence Variation in Caucasian and Hispanic Samples across the Mitochondrial Genome Using an 83 Immobilized SSO Probe Panel
Sarah M. Stuart, Cassandra D. Calloway and Henry A. Erlich
American Academy of Forensic Sciences 59th Annual Meeting
February 19-24, 2007
Poster presentation

Increasing Mitochondrial DNA Discrimination among Caucasian and Hispanic Population Samples Using a Panel of 56 Immobilized SSO probes
Sarah M. Stuart, Cassandra D. Calloway and Henry A. Erlich

Promega 17th International Symposium on Human Identification
October 9-12, 2006
Poster presentation

Development of a Highly Informative Multiplex PCR and LINEAR ARRAY Typing System Targeting Variation in the Mitochondrial Genome
Cassandra Calloway
NIJ DNA Grantee Meeting
June 26-30, 2006
Oral Presentation

LINEAR ARRAY mtDNA HVI/HVII Region-Sequence Typing Kit: Applications, Validation and Interpretation Issues
Instructor: Cassandra Calloway, MS
President's DNA Initiative: NFSTC mtDNA Workshop
March 13-15, 2006

LINEAR ARRAY DNA HVI/HVII Region-Sequence Typing Kit: Applications, Methods and Interpretation Issues
Instructor: Cassandra Calloway, MS
Assistant Instructor: Sarah Stuart, BS
Cal DOJ Mitochondrial DNA Analysis and Typing Workshop
January 23-27; February 4, 6-10, 2006