Multiplex Detection of 10 SNPs Located in the Coding Region of the Mitochondrial Genome

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Single nucleotide polymorphisms (SNPs) represent an important class of DNA variation in which sequence differences between individuals are examined. A number of different SNP detection and typing technologies exist. This work focuses on the use of a SNP typing approach that works on a multi-color fluorescence capillary electrophoresis platform. Fluorescent SNP detection is accomplished (through a mini-sequencing assay) by using the commercially available SNaPshot multiplex kit.

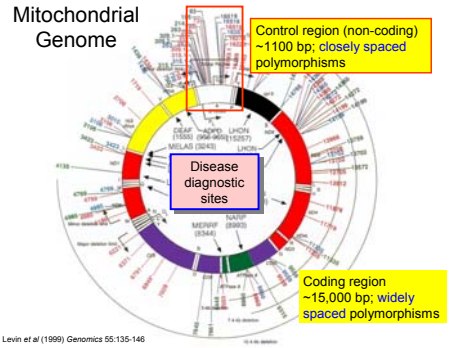
Mitochondrial DNA (mtDNA), which is maternally inherited, can play an important role in many aspects of human identity testing due to the fact that it is more resilient to environmental degradation. A desire to gain more information than can be provided by the hypervariable regions of the mtDNA control region has led to a search for informative sites outside the control region around the remaining 15,000 base pairs of the mtDNA genome. A set of 10 highly informative sites from around the mtDNA genome has been combined into a multiplex PCR and SNP detection assay that can be detected in high-throughput fashion using multi-color fluorescence and multi-capillary instrumentation.

Experimental conditions for the multiplex amplification of nine regions in the mitochondrial genome containing ten SNP sites have been optimized. Using the multiplex generated PCR amplicons as templates ten different SNP sites are probed simultaneously in the same tube using tailed extension primers and reagents contained the ABI SNaPshot multiplex SNP kit. The products of the fluorescently labeled primer extension reactions are separated and detected on the ABI 3100 16 capillary electrophoresis instrument.

Mitochondrial DNA

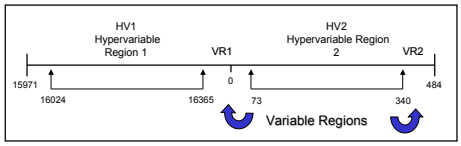
- The mitochondrial genome contains ~16569 bps
- Maternally inherited
- ~1000's of copies per cell (tends to survive under adverse environmental conditions)
- Polymorphic control region (D-loop) (~1100 base pairs) is typically used for human identification purposes (less than 7% of total mt genome)

Mitochondrial Genome



Levin *et al* (1999) *Genomics* 55:135-146

Current Amplification & Sequencing Strategies Focus on the Hypervariable Regions of the Mitochondrial Genome (HV1 and HV2)



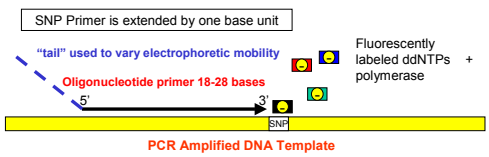
The principal limitation in forensic mtDNA testing (using solely HV1 and HV2) is the low power of discrimination that is obtained when common "mtDNA types" are involved in a case.

Coding Region Polymorphisms

- Sequence data from mtDNA genome coding region reveals numerous SNPs that can help distinguish Caucasians sharing common HV types
- 10 SNP sites are being evaluated to resolve individuals having the most common HV type (Haplogroup H-CRS)

- We are using the fluorescent primer extension assay SNaPshot for multiplex probing of coding region SNPs
- The primer extension assay allows for flexibility in designing custom multiplex assays
- Assay is run on a capillary electrophoresis platform (ABI 310, 3100, 3700) common to most forensic laboratories

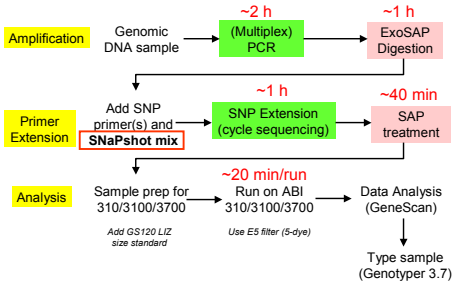
Primer Extension with SNaPshot™



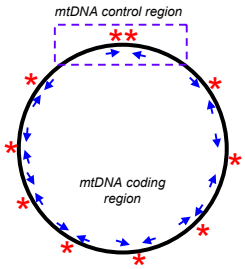
ddNTP	Dye label	Color
A	dR6G	Green
C	dTAMRA	Black
G	dR110	Blue
T	dROX	Red

25 Cycles
96°C 10s
50°C 5s
60°C 30s

SNaPshot™ "Kit" Protocol



Coding Region mtSNP 10-plex Assay



In a SNaPshot (primer extension) assay the researcher is responsible for designing, ordering, and optimizing the multiplex PCR and primer extension reactions

10 mtSNP sites are present in 9 PCR amplicons

PCR product sizes kept under 200 bp to enable success with degraded DNA samples

Multiplex PCR used to co-amplify all regions of interest at once

Locus	PCR Primer Sequence	Length	tm	PCR prod size (bp)
3010-F	GCGCAATCCTATTCTAGAGTCC	22	59.4	124
3010-R	TCACGTAGGACTTTAATCGTTGA	23	58.8	
4580-F	TCTTTGCAGGCACACTCATC	20	60.0	130
4580-F	GCAGCTTCTGTGGAACGAG	19	59.7	
4793-F	CAACCGCATCCATAATCCTT	20	59.8	186
4793-R	ATGTCAGAGGGGTGCCTTG	19	61.1	
5004-F	TCCATCATAGCAGGCAGTTG	20	59.8	124
5004-R	TGGTTATGTTAGGGTTGTACGG	22	58.8	
7028-F	GGCCTGACTGGCATTGTATT	20	60.0	125
7028-R	AAGCCTCCTATGATGGCAAA	20	59.7	
7202-F	ACGCCAAAATCCATTTCACT	20	59.4	126
7202-R	TTCATGTGGTGTATGCATCG	20	58.9	
10211-F	ACCACAACTCAACGGCTACA	20	59.2	143
10211-R	GGAGGGCAATTTCTAGATCAAA	22	59.6	
12858-F	ATGATACGCCCGAGCAGA	18	60.3	126
12858-R	TGTGGGTCTCATGAGTTGGA	20	60.1	
16472-F	ACCACCATCCTCCGTGAAAT	20	61.6	183
16519-R	AGACCTGTGATCCATCGTGA	20	59.1	

PCR primers were selected using the web based Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The set of 9 primer pairs were screened for primer-dimer interactions using in house software.

Multiplex PCR Protocol

•Typical reaction volume = 15 uL
- 1-2 ng of genomic DNA
- 1 Unit Taq Gold polymerase
- 5 mM Mg^{++}
- 1x Taq Gold buffer
- 1 μM of each f/r PCR primer
- 0.16 mg/mL BSA
- 250 μM dNTPs

•General thermal cycling conditions (T_a = annealing temperature)
- Initial T_a = 50 °C for 3 cycles
- Increasing T_a + 0.2 °C for 19 cycles
- T_a = 55 °C for 9 cycles

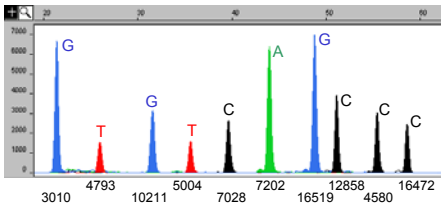
Multiplexing is Achieved Through the Use of "Tailed" SNP primers

Sequences for 10 SNP primers

3010-F	G/A	TCAGAAGTGAAAGGGGGC	18/na
4793-R	A/G	TTTTTTTTGTTGGATCAGGACATCCC	19/26
10211-R	C/T	TTTTTTTTTTACTAAGAAGAATTTTATGGA	20/30
5004-F	T/C	TTTTTTTTTTTTTTAGAGCCCAGCTACGCAAAATC	20/34
7028-F	C/T	TTTTTTTTTTTTTTTTTGACACGTACTACGTTGTAGC	20/38
7202-F	A/G	TTTTTTTTTTTTTTTTTTTTTTTCCACAACACTTTCTCGGCCT	20/42
16519-R	T/C	TTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGGGCTATTTAGGCTTTATG	22/46
12858-F	C/T	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCAGCCATTCAAGCAATCCTATA	23/50
4580-R	G/A	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGTTAGAACTGGAATAAAAGCTAG	25/54
16472-R	G/A	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGGATACAGTTCACTTTAGCTACC	24/58

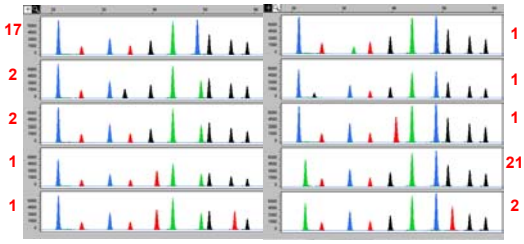
Template binding sequence – black
Tailed sequence for fragment separation – red
SNP primer sequences were selected using an in house program

Result of 10 plex Primer Extension Assay



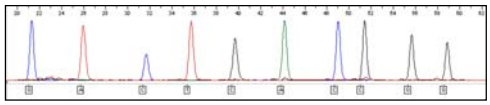
SNaPshot assay conditions were performed according to the ABI protocol. SNP primer concentrations varied from 0.5 – 10.0 μM (balanced empirically). CE runs were performed on the ABI 3100 in POP6 polymer; 36 cm capillary.

10 Unique Types Present in 49 Samples (Haplogroup H-CRS)



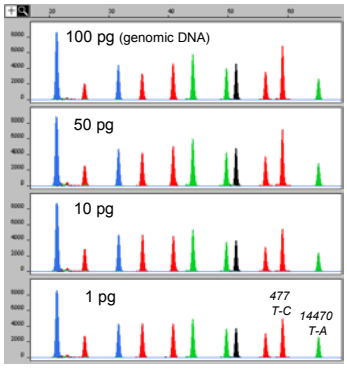
The frequency of a specific type is indicated in red, N = 49

Genotyper Macro for SNaPshot



Macros for automated typing of mtSNP assays have been developed

Sensitivity Study on New mtSNP 11 plex Assay



Note: 16472 was removed due to lack of variation in haplogroup H-CRS; mtSNP 477 and 14470 were added to the assay.

Future Goals

- SNaPshot assay development for informative sites in other common HV types
- Further testing of mtSNP 11 plex (mixtures, data basing, sensitivity)
- Testing and optimizing Genotyper Macros
- Decreasing electrophoresis run time (shorter capillary, different polymers)
- Increasing the number of loci probed (12- 15 plex)

Acknowledgements

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