An Allele-Specific Primer Extension Assay for the Detection of 11 SNPs Located in the Mitochondrial Genome

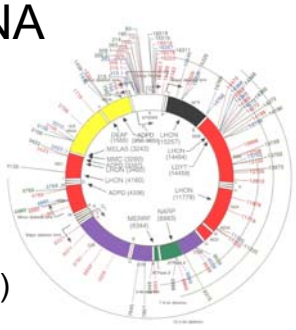
4th Annual Biotechnology Division Retreat

May 15-16th 2003

Peter M. Vallone

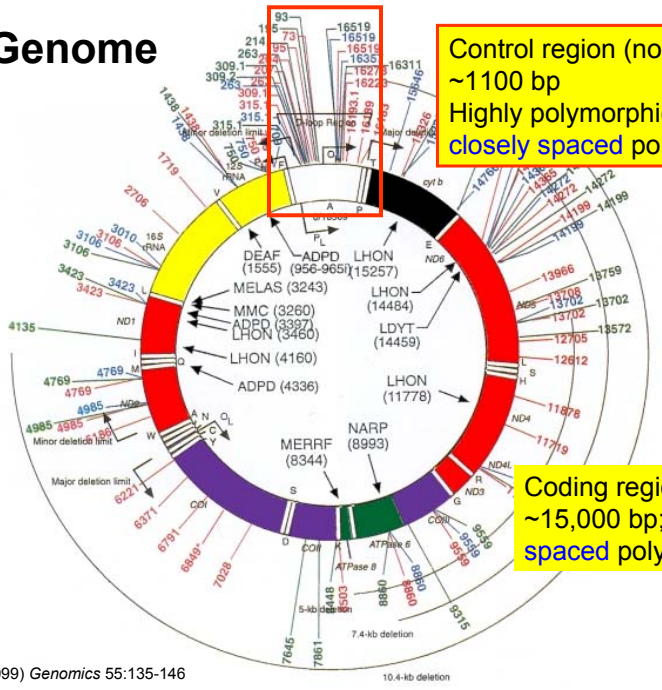
DNA Technologies Group

Mitochondrial DNA



- Mitochondrial DNA (mtDNA)
  - Circular genome ~16,569 bps
  - maternally inherited
  - polymorphic control region (D-loop)
  - coding region
  - 500 - 2000 copies per cell
  - Typed for highly degraded samples
  - mtDNA is a single linked molecule

mtGenome

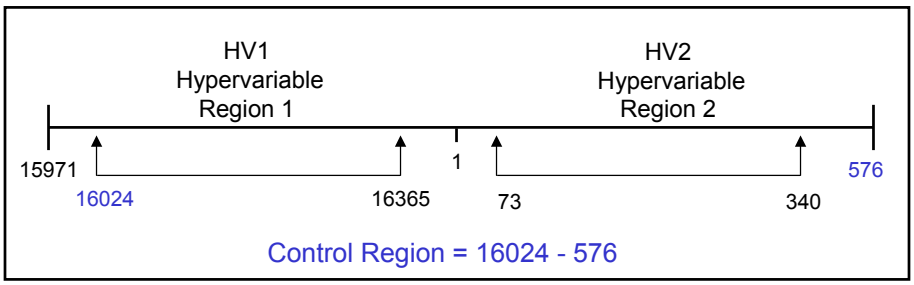


Control region (non-coding)
~1100 bp
Highly polymorphic with
closely spaced polymorphisms

Coding region
~15,000 bp; widely
spaced polymorphisms

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

The Current mtDNA Amplification & Sequencing Strategy Focuses on the Hypervariable Regions of the mitochondrial genome HV1 and HV2



Control Region = 16024 - 576

In Caucasians, approximately 7% of HV1 and HV2 sequences are identical

The Use of Full mtGenome Polymorphisms

- mtGenome sequencing data reveals numerous SNPs that can help distinguish Caucasians sharing common HV types (Tom Parsons and Mike Coble AFDIL)
- 11 SNP sites were selected to help resolve Caucasian individuals having the most common HV1/HV2 type
- mtSNPs: Silent and at third codon positions or fall in the short non-coding regions between genes in the coding region
- Detect in a multiplex assay run on a common forensic instrumental platform

What Type of Genetic Variation?

•Length Variation

short tandem repeats (STRs)

CTAGTCGT**(GATA)(GATA)(GATA)**GCGATCGT

•Sequence Variation

single nucleotide polymorphisms (SNPs)

insertions/deletions

GCTAGTCGATGCTC**(G/A)**GCGTATGCTGTAGC

Allele-Specific Primer Extension

SNP Primer is extended by one base unit

"tail" used to vary electrophoretic mobility

Oligonucleotide primer 18-28 bases

5' 3'

SNP

PCR Amplified DNA Template

ABI PRISM® SNaPshot™ Multiplex System

Fluorescently labeled ddNTPs + polymerase

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

Protocol for ASPE Assay

Amplification

Genomic DNA sample → (Multiplex) PCR → ExoSAP Digestion

Primer Extension

Add SNP primer(s) and **SNaPshot mix** → SNP Extension (cycle sequencing) → SAP treatment

Analysis

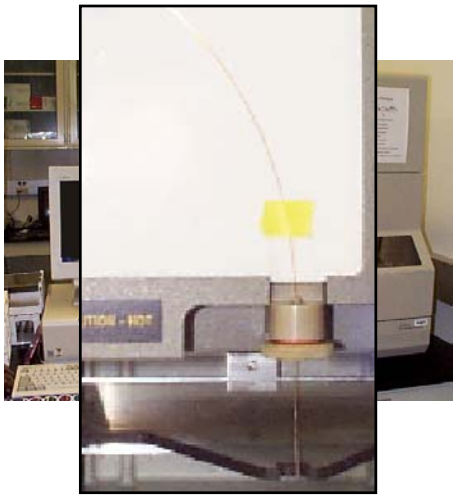
Sample prep for 310/3100 → Run on ABI 310/3100 → Data Analysis (GeneScan) → Type sample (Genotyper 3.7)

Add GS120 LIZ size standard

Use E5 filter (5-dye) and POP4 standard conditions

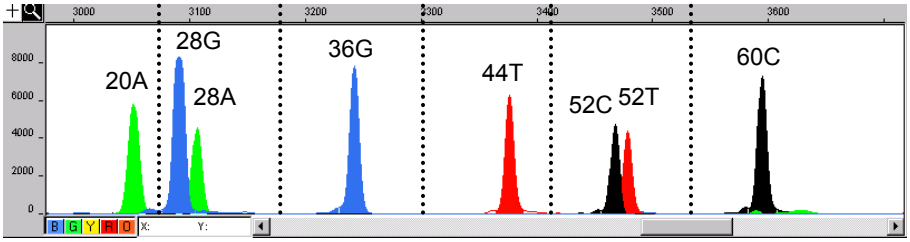
Capillary Electrophoresis Instrumentation

ABI 310
single capillary



ABI 3100
16-capillary array

Detection of SNPs with ABI 310/3100



SNaPshot™ CEPH Control Reaction

20 nucleotides — **ddA**

36 nucleotides — **ddG**

44 nucleotides — **ddT**

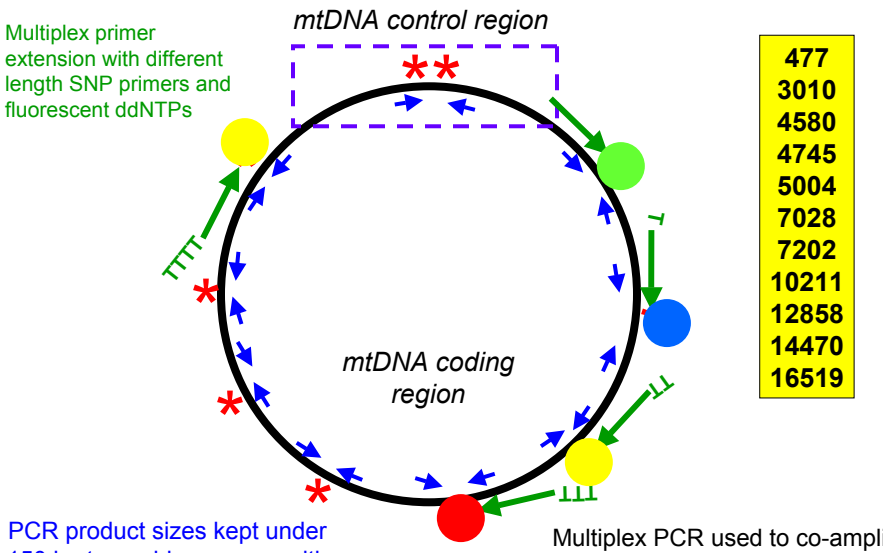
60 nucleotides — **ddC**

Poly(T) tail or non-nucleotide linker to aid separation

Priming site

Multiplexing possible by use of different length primers

mtSNP 11-plex Assay



PCR product sizes kept under 150 bp to enable success with degraded DNA samples

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

Multiplex PCR Primer Selection

Identify markers of interest (collaborations, literature, research)

Organize sequences with ~250 bases of sequence up- and downstream of the SNP

PCR product size
- Short amplicons for degraded samples, SNPs
- Longer amplicons for STRs

Use software for selecting singleplex primer pairs

Primer3
www-genome.wi.mit.edu/genome_software/other/primer3.html

Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ, pp 365-386

Multiplex PCR Design

Select singleplex PCR primers for each amplicon using Primer 3 software

```
OLIGO            start  len      tm      gc%    any    3'   seq
LEFT PRIMER         27   20   60.06    50.00   4.00   2.00 GGGATAACAGCGCAATCCTA
RIGHT PRIMER       174   22   60.31    50.00   8.00   3.00 CGGTCTGAACTCAGATCACGTA
SEQUENCE SIZE: 205
INCLUDED REGION SIZE: 205

PRODUCT SIZE: 148, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 2.00
EXCLUDED REGIONS (start, len)*: 70,65

    1 CTTGACCAACGGAACAAGTTACCCTAGGGATAACAGCGCAATCCTATTCTAGAGTCCATA
                              >>>>>>>>>>>>>>>>>>>>

   61 TCAACAATAGGGTTTACGACCTCGATGTTGGATCAGGACATCCCRATGGTGCAGCCGCTA
               XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

  121 TTAAAGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGATCTGAGTTCAGACCGGAGTAA
      XXXXXXXXXXXXXX                  <<<<<<<<<<<<<<<<<<<<<<<<

  181 TCCAGGTCGGTTTCTATCTACCTTC
```

Stand Alone Primer3

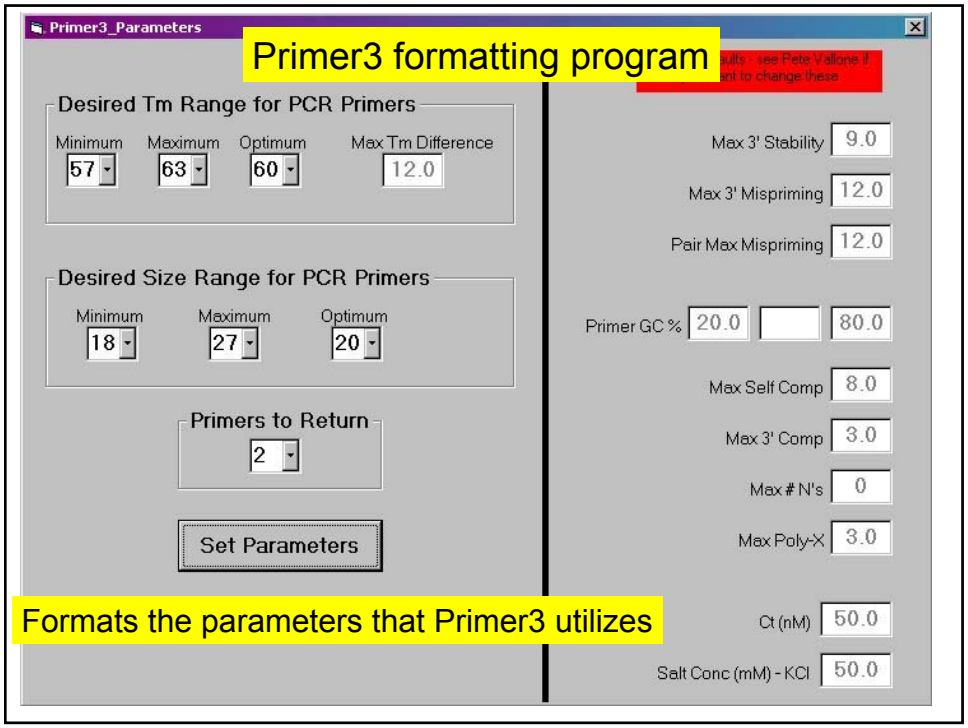
Sending multiple sequences over the web for primer selection can be tedious

The Primer3 web output is acceptable for the screen viewing or printing but not for organizing in spreadsheets

Primer3 is publicly available and can be run on a Unix, PC (Linux), or Mac (OSX) computer

Developed a program that formats files for Primer3 input

Reference sequences that are stored in Excel can be quickly formatted for Primer3

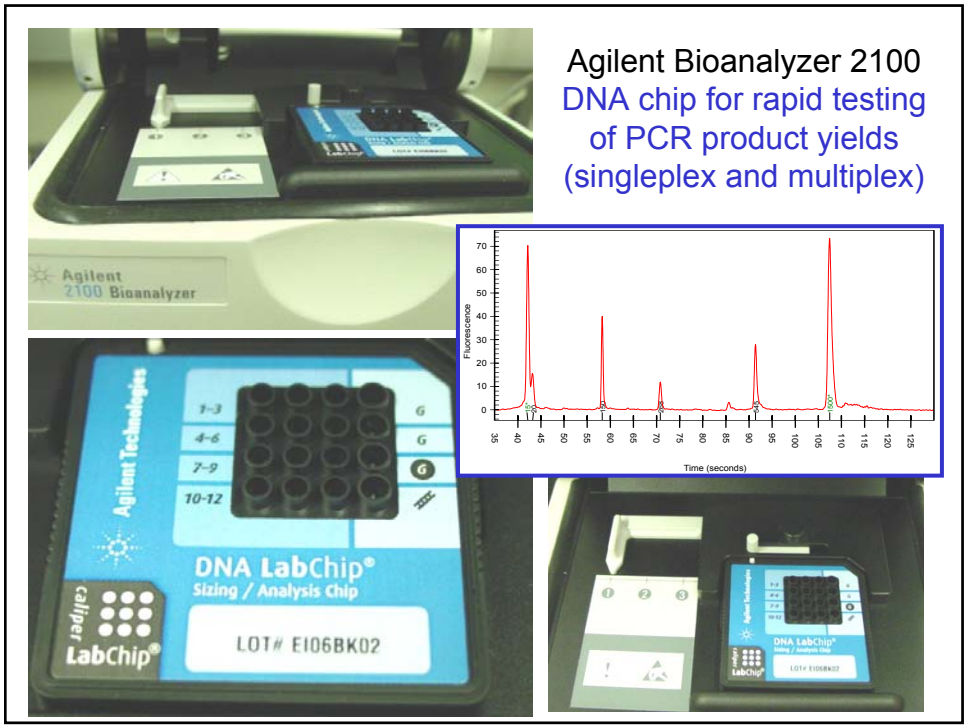


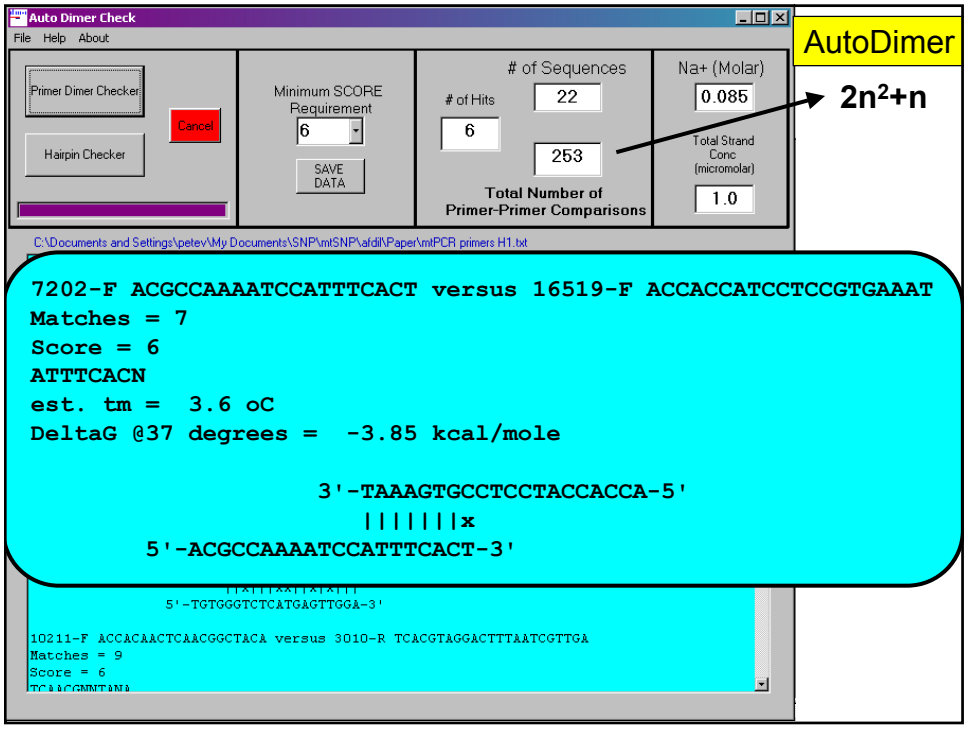
Non-Specific Interactions

Primers that interact with non-specific (undesired) regions of a genome OR with each other can degrade PCR performance

Screening for alternate genomic binding regions can be accomplished using **BLAST** *http://www.ncbi.nlm.nih.gov*

Screening for potential primer-dimer interactions is accomplished using in house software - **AutoDimer**


Agilent Bioanalyzer 2100
DNA chip for rapid testing of PCR product yields (singleplex and multiplex)
Agilent 2100 Bioanalyzer
Agilent Technologies
DNA LabChip®
Sizing / Analysis Chip
LOT# EI06BK02
Fluorescence
Time (seconds)

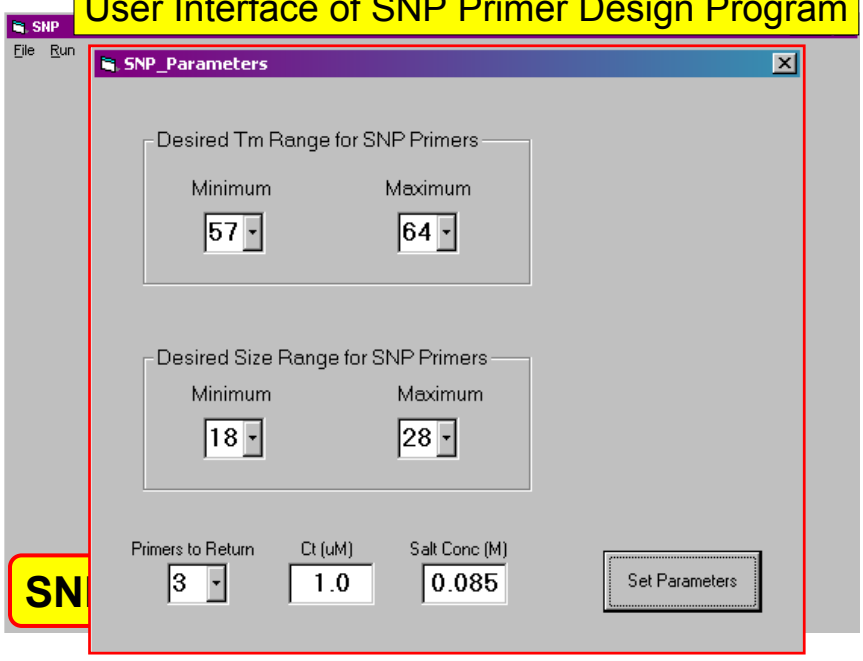

Auto Dimer Check
File Help About
Primer Dimer Checker
Hairpin Checker
Cancel
Minimum SCORE Requirement
6
SAVE DATA
# of Sequences
# of Hits
22
6
253
Total Number of Primer-Primer Comparisons
Na+ (Molar)
0.085
Total Strand Conc (micromolar)
1.0
C:\Documents and Settings\petev\My Documents\SNP\mtSNP\afdil\Paper\mtPCR primers H1.txt
AutoDimer
2n²+n
7202-F ACGCCAAAATCCATTTCACT versus 16519-F ACCACCATCCTCCGTGAAAT
Matches = 7
Score = 6
ATTTCACN
est. tm = 3.6 oC
DeltaG @37 degrees = -3.85 kcal/mole
3'-TAAAGTGCCTCCTACCACCA-5'
|||||||x
5'-ACGCCAAAATCCATTTCACT-3'
5'-TGTGGGTCTCATGAGTTGGA-3'
10211-F ACCACAACTCAACGGCTACA versus 3010-R TCACGTAGGACTTTAATCGTTGA
Matches = 9
Score = 6

Tailed SNP primers allows for multiplexing in the SNaPshot assay

Sequences for 11 extension primers

3010-F	TGTTGGATCAGGACATCCC	19 19
4793-R	$(T)_4$ – TCAGAAGTGAAAGGGGGC	18 22
10211-R	$(T)_{10}$ – ACTAAGAAGAATTTTATGGA	20 30
5004-F	$(T)_{14}$ – AGACCCAGCTACGCAAAATC	20 34
7028-F	$(T)_{18}$ –GACACGTACTACGTTGTAGC	20 38
7202-F	$(T)_{22}$ –CCACAACACTTTCTCGGCCT	20 42
16519-R	$(T)_{24}$ –TGTGGGCTATTTAGGCTTTATG	22 46
12858-F	$(T)_{27}$ –GCAGCCATTCAAGCAATCCTATA	23 50
4580-R	$(T)_{29}$ –TGGTTAGAACTGGAATAAAAGCTAG	25 54
477-F	$(T)_{38}$ –CCCTCCCACTCCCATACTAC	20 58
14470-R	$(T)_{41}$ –GGGAATGATGGTTGTCTTTGG	21 62

User Interface of SNP Primer Design Program



Program Output

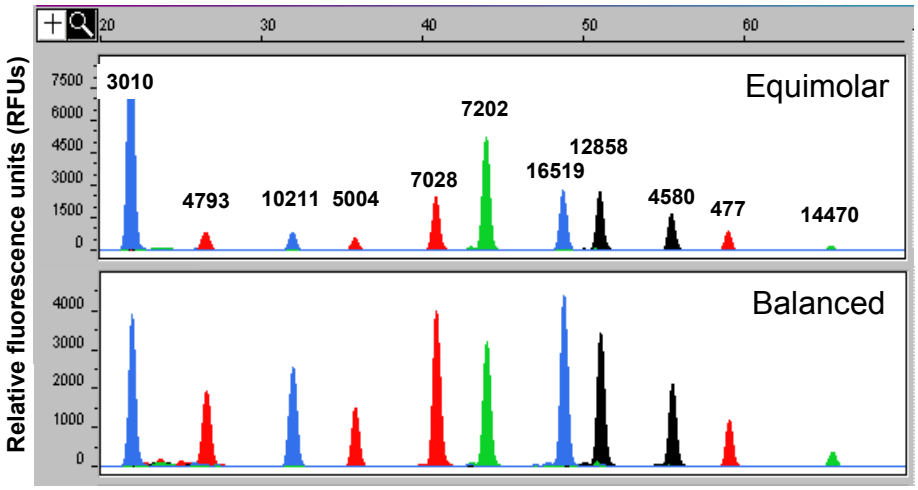
Label	Length	Sequence	Position	Tm
Forward Primers Salt = 0.3Ct = 10				
M42 340 bp (A/T 297 W) AC010889	18	ATTTAGGACACAAAAGCW	280	60.65398
M42 340 bp (A/T 297 W) AC010889	19	GATTTAGGACACAAAAGCW	279	61.96716
M42 340 bp (A/T 297 W) AC010889	20	AGATTTAGGACACAAAAGCW	278	63.67808
Reverse Primers				
M42 340 bp (A/T 297 W) AC010889	23	GCTCTCTTTTTCATTATGTAGTW	319	63.5462
M42 340 bp (A/T 297 W) AC010889	21	TCTCTTTTTCATTATGTAGTW	317	59.28964
M42 340 bp (A/T 297 W) AC010889	20	CTCTTTTTCATTATGTAGTW	316	57.50257

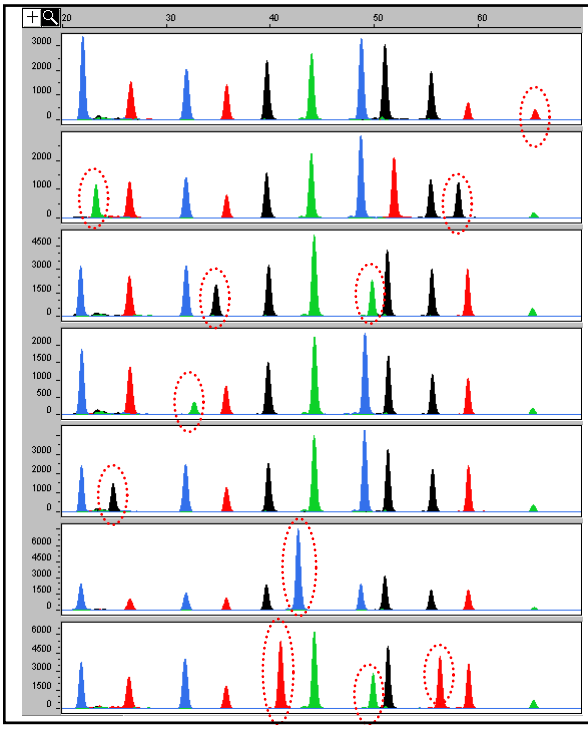
Hairpin	Dimer	Template	Mass	Rank	Mutation	+ddC	+ddT	+ddA	+ddG
4	8	10	5273.48	2.133333	W	N/A	5561.67998	5570.68998	N/A
5	10	10	5602.69	2	W	N/A	5890.889941	5899.899941	N/A
5	10	11	5915.9	2	W	N/A	6204.099902	6213.109902	N/A
4	8	22	6734.42	2.133333	W	N/A	7022.619922	7031.629922	N/A
4	8	20	6116.02	2.133333	W	N/A	6404.22002	6413.23002	N/A
4	8	19	5811.82	2.133333	W	N/A	6100.019824	6109.029824	N/A

mtSNP 11-plex run on ABI 3100

Multiplex PCR and Multiplex SNP Detection

Measured size (nt) (relative to GSLIZ-120 size standard)



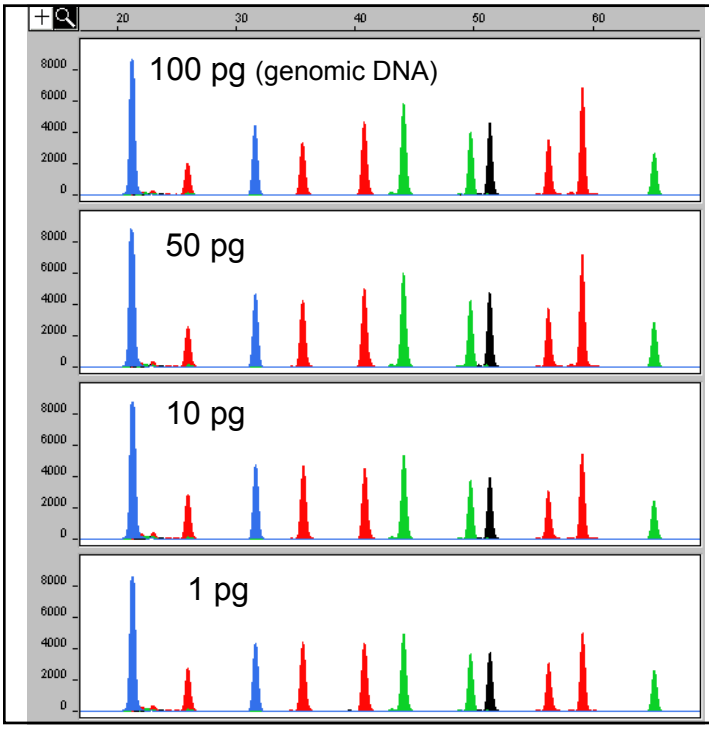


11 plex run on 7 unique samples

All allele variations are represented in these 7 samples

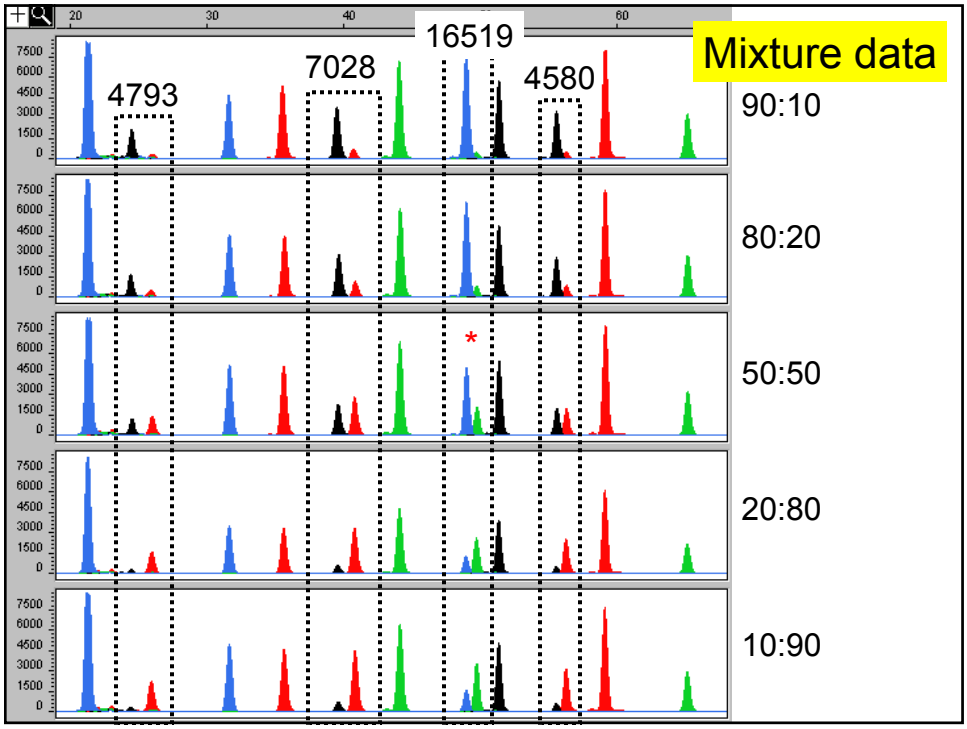
The assay accurately detects each variant

Sizing can be used to develop a macro for automated typing



Sensitivity Study

Assay performs down to 1 pg of genomic DNA



11plex mtSNP assay

Assay is capable of accurately detecting 11 mtSNP in a single assay

The 11plex assay is currently being validated for case work samples at AFDIL

Manuscript is in preparation

Additional multiplex mtSNP assays are being developed for other common HV1/HV2 types in collaboration with AFDIL

Acknowledgments

Funding:

U.S. National Institute of Justice Grant #97-LB-VX-0003

Interagency Agreement between NIJ and NIST Office of Law Enforcement Standards

John Butler

Collaborators

Thomas Parsons, Rebecca Hamm and Mike Coble (AFDIL)