Multiplexed Assays for Probing Y Chromosome and Mitochondrial Markers

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Outline of Presentation

- Multiplexing
- Assays and Instrumentation
- Y Chromosome and Mitochondrial DNA
- Primer design strategy
- Results
  - mtSNP 10 plex
  - Y SNP 5 plex
  - Y STR multiplexes

Multiplexing

Probing multiple loci/markers simultaneously

Multiple strands of short DNA bind to template DNA and chemistry occurs (PCR, primer extension, hybridization, etc)

What are the Advantages of Multiplexing?

Obtain more information per unit time

Reduce the amount of limited forensic sample used

Save on reagents; enzyme, buffers, DNA oligomers

Reduces labor

Streamlines data analysis

For certain markers it is essential (SNPs, YSTRs)

Coincides with high capacity instrumentation

What are the Challenges of Multiplexing?

Only guidelines exist for designing multiplexes

More markers = increased complexity

Testing a robust multiplex

Inclusion of useful markers in the multiplex

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

What Assays are we Multiplexing?

Polymerase chain reaction (PCR)

- Amplification of specific region of the human genome
- Typically used for STR and SNP
- Use **Capillary Electrophoresis** for detection

Primer Extension reaction (minisequencing)

- Typically used for SNP markers
- Use **Capillary Electrophoresis** and **Mass Spectrometry** for detection

NIST Goals for Multiplex Assays

Working with collaborators who have markers of forensic interest

By using our multiplex assays collaborators can type markers and evaluate forensic utility

Further understanding of multiplex assays

Publish assay details for others to evaluate (commercial and research)

Multiplexing

Assays and Instrumentation

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Primer design strategy

Results

- mtSNP 10 plex
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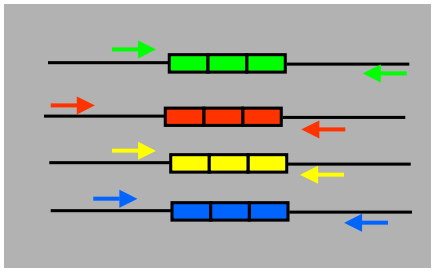
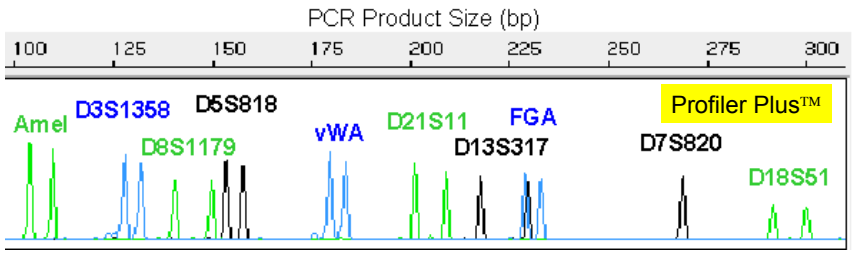
Multiplex PCR



Multiple primer pairs target more than one specific site on the DNA strand

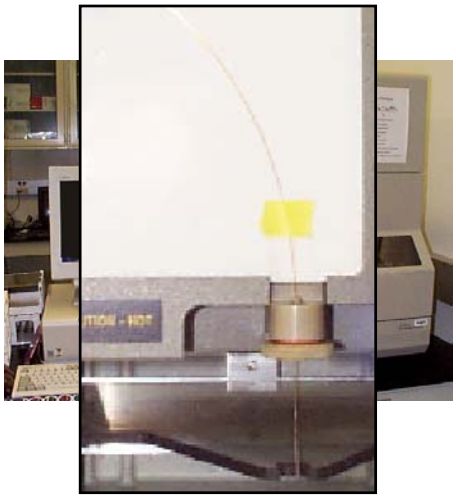
Compatible primers are the key to successful multiplex PCR

Commercial kits are available for targeting and simultaneously amplifying 16 markers



Capillary Electrophoresis Instrumentation

ABI 310
single capillary



ABI 3100
16-capillary array

ABI PRISM® SNaPshot™ Multiplex System

Primer extension assay that utilizes fluorescently labeled ddNTPs

Analysis of fragment size and fluorescent label identity by CE allows typing of multiple SNPs

Multiplexed amplicons or pooled singleplex PCR amplicons can be used as templates

Primer design must be done by user!

Primer Extension with SNaPshot™

SNP Primer is extended by one base unit

"tail" used to vary electrophoretic mobility

Oligonucleotide primer 18-28 bases

5' 3'

SNP

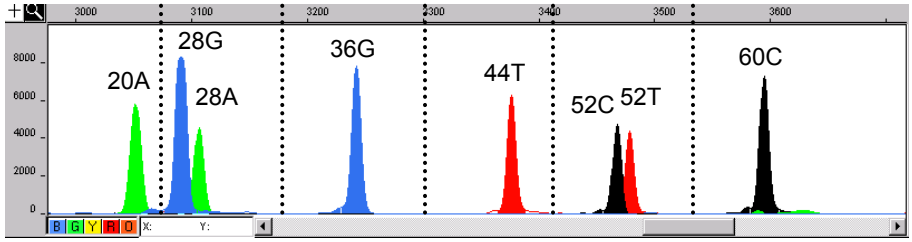
Fluorescently labeled ddNTPs + polymerase

PCR Amplified DNA Template

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

Detection of SNPs with ABI 310/3100



SNaPshot™ CEPH Control Reaction

20 nucleotides ddA

36 nucleotides ddG

44 nucleotides ddT

60 nucleotides ddC

Multiplexing possible by use of different length primers

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

Priming site

Primer Extension for MALDI TOF MS Analysis

SNP Primer is extended by one base unit

Natural non-labeled ddNTPs + polymerase

Oligonucleotide primer 18-28 bases

5' 3'

SNP

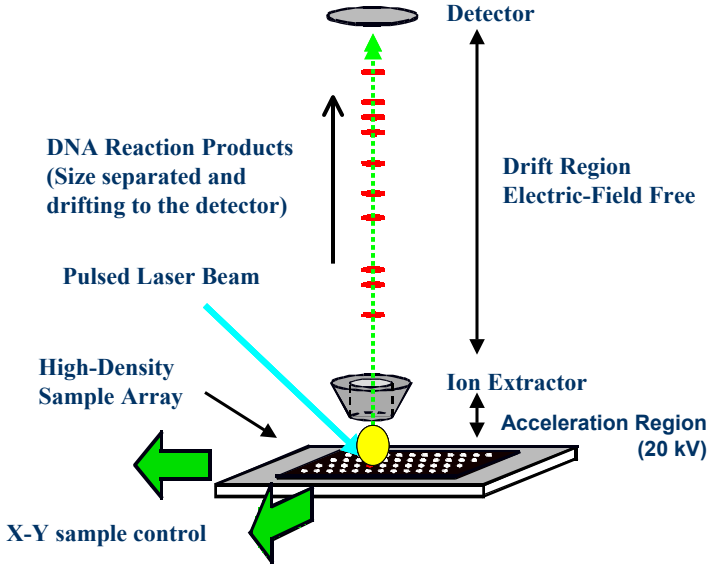
PCR Amplified DNA Template

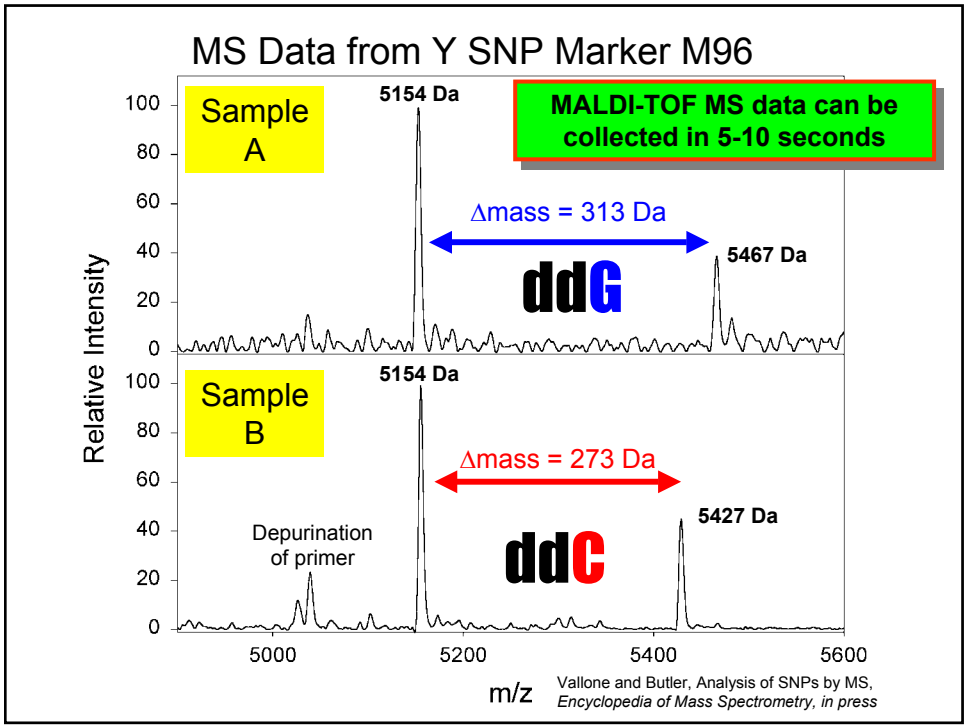
ddNTP	Mass (Da)
A	297
C	273
G	313
T	288

40 Cycles
96°C 10s
50°C 20s
72°C 30s

Mass difference between SNP primer and single base extension product provides genotype

Time-of-Flight Mass Spectrometry (TOF-MS)





Multiplexing

Assays and Instrumentation

Y Chromosome and Mitochondrial DNA

Primer design strategy

Results

- mtSNP 10 plex
- Y SNP 5 plex
- Y STR multiplexes

Markers of Interest

- Mitochondrial DNA (mtDNA)
  - maternally inherited
  - polymorphic control region (D-loop)
  - ~1000's of copies per cell
- Y chromosome
  - paternally inherited
  - variety of Y STR and Y SNP markers
  - ***haplotype rather than genotype***

Require large databases because recombination does not occur

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Multiplex PCR Primer Selection

Identify markers of interest (collaborations, literature, research)

Obtain reference sequences containing the sites of interest (Genbank) with approximately 500 bases of sequence information upstream and downstream of the marker

Decide upon a desired 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

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 fine for the screen viewing or printing but not for organizing in spreadsheets

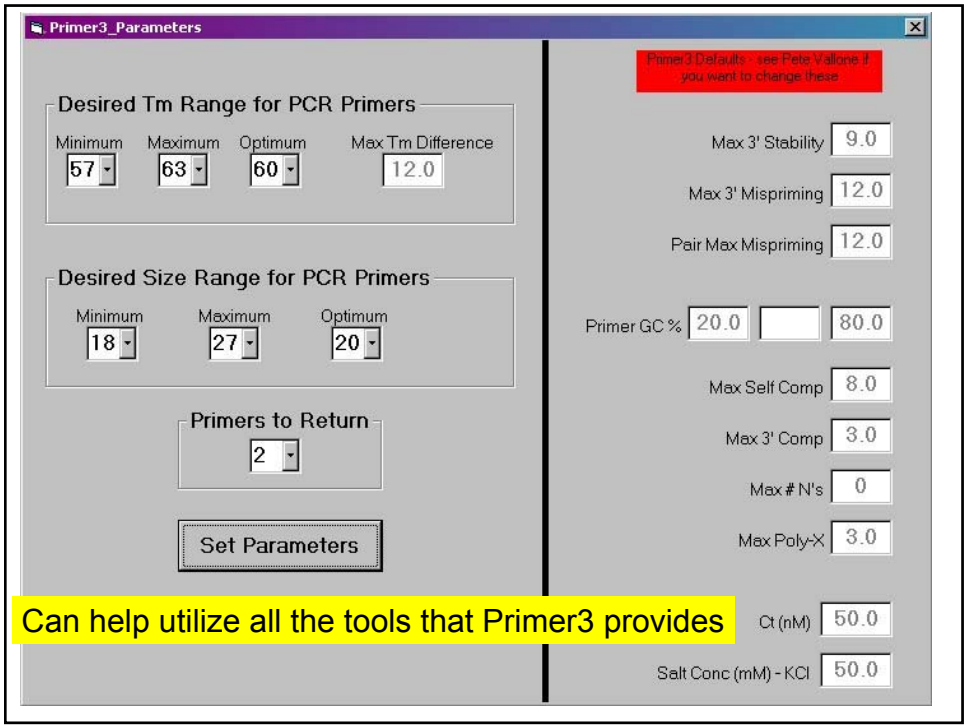
Primer3 is publicly available and can be run (in batch!) on a Unix, Linux, or Mac (OSX) computer

Developed a program that formats files for Primer3 input

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

Example input format for Primer3

```
PRIMER_SEQUENCE_ID=M9
SEQUENCE=GCAGCATATAAAACTTTCAGGACCCTGAAATACAGAACTG
CAAAGAAACGGCCTAAGATGGTTGAATNCTCTTTATTTTTCTTTAATTTAG
ACATGTTCAAACGTTCAATGTCTTACATACTTAGTTATGTAAGTAAGGTAG
CGCTTACTTCATTATGCATTTCAATACTCAAAAAAAATTCCTTTGTGAAAT
GTTGAAATATTTTTCTAATCTGTTTCACGAGCTTCAAAAATGAGGAAAAAA
GATTCAGTTTACATTTCAGCAAAATGCCTCTTTTTAATCGGATTTATGTTT
ACTTAACATTTACAGTACATTTACGCTTGAGCAAAGTTAGGTTTT
PRIMER_COMMENT=(340 bp); G to C at position 68
PRIMER_MISPRIMING_LIBRARY=/Users/vallone/Desktop/primer3/misprM9
PRIMER_MAX_MISPRIMING=8
PRIMER_PAIR_MAX_MISPRIMING=20
EXCLUDED_REGION=38,60
PRIMER_PRODUCT_SIZE_RANGE=90-150
PRIMER_PRODUCT_OPT_SIZE=105
PRIMER_MIN_SIZE=18
PRIMER_MAX_SIZE=27
PRIMER_OPT_SIZE=20
PRIMER_OPT_TM=60
PRIMER_MIN_TM=57
PRIMER_MAX_TM=63
PRIMER_NUM_RETURN=1
PRIMER_EXPLAIN_FLAG=1
PRIMER_LIBERAL_BASE=1
=
PRIMER_SEQUENCE_ID=M42
SEQUENCE=AAAGCGAGAGATTCAATCCAGGATGACAGAATGCGTTCAC
CTTTAAAGGGATTAAAAGAAGTATAATACAGTCTGTATTATTAGATCACCC
AGAGACACACAAAACAAGAACCGTGAATTGAATTAGTGGTATACTAATAG
```



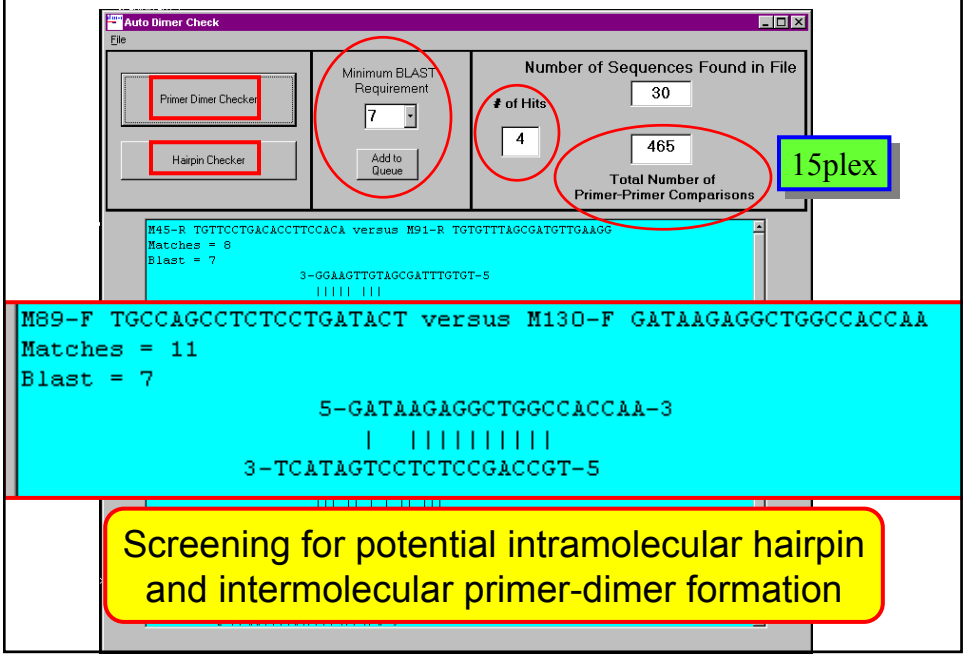
Non-Specific Interactions

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**

AutoDimer Check



Screening for potential intramolecular hairpin and intermolecular primer-dimer formation

PCR Assay Design

If primer pairs meet criteria

Obtain primer pairs and test singleplex PCR
(QC all primers with MS/CE/HPLC)

Begin initial testing of multiplex PCR
Start with a PCR mix containing
1.0 μM of each primer pair

Evaluate amplicon yields, presence and balance

Vary primer pair concentrations, [polymerase], number of cycles, [Mg^{++}], [dNTPs], BSA

Redesign and retest failing loci

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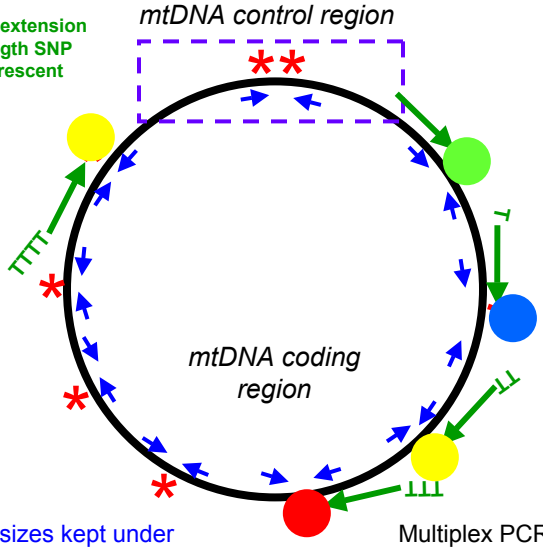
Coding Region mtSNP 10-plex Assay

Multiplex primer extension with different length SNP primers and fluorescent ddNTPs

3010
4580
4745
5004
7028
7202
10211
12858
16472
16519



Collaboration with Tom Parsons (AFDIL) provided sequence variation sites located in the coding region

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

Tailed SNP primers allows for multiplexing in the SNaPshot assay

Sequences for 10 SNP primers

TCAGAAGTGAAAGGGGGC	18/na
TTTTTTTGTTGGATCAGGACATCCC	19/26
TTTTTTTTTTACTAAGAAGAATTTTATGGA	20/30
TTTTTTTTTTTTTTAGACCCAGCTACGCAAAATC	20/34
TTTTTTTTTTTTTTTTTTGACACGTACTACGTTGTAGC	20/38
TTTTTTTTTTTTTTTTTTTTTTCCACAACACTTTCTCGGCCT	20/42
TTTTTTTTTTTTTTTTTTTTTTTTTTGTGGGCTATTTAGGCTTTATG	22/46
TTTTTTTTTTTTTTTTTTTTTTTTTTTTGCAGCCATTCAAGCAATCCTATA	23/50
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGTTAGAACTGGAATAAAAGCTAG	25/54
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAACCATAACCAATACTACCAATCA	25/58

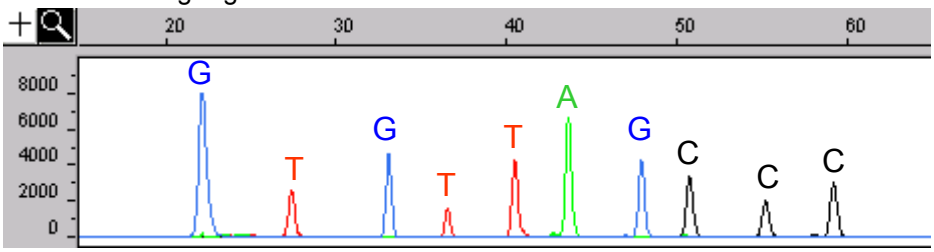
Template binding sequence – black
Tailed sequence for fragment separation - red

mtSNP 10-plex run on ABI 3100

(SNapShot™ assay)

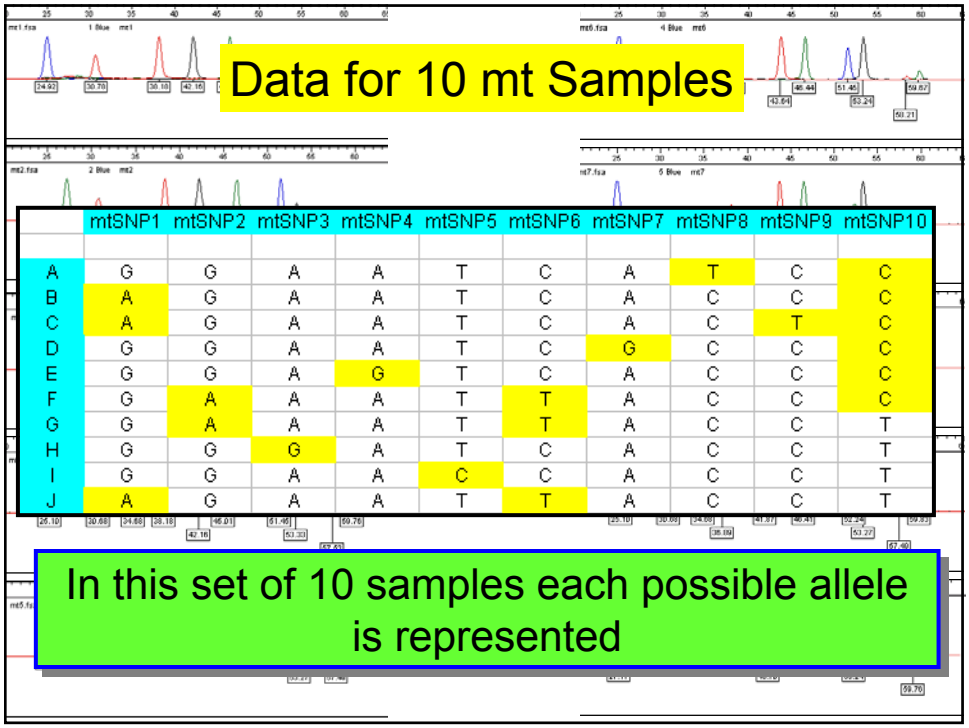
Multiplex PCR and Multiplex SNP Detection

mtDNA coding region SNPs

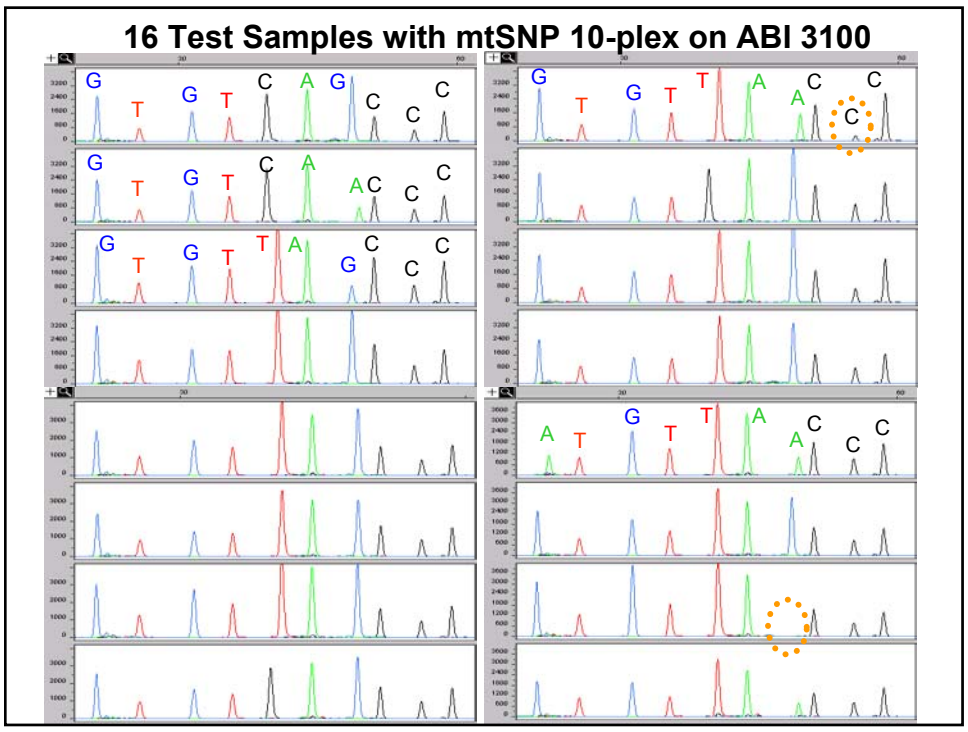


SNP types

Sizing performed by comparison to GS120 LIZ internal size standard (not shown)



	mtSNP1	mtSNP2	mtSNP3	mtSNP4	mtSNP5	mtSNP6	mtSNP7	mtSNP8	mtSNP9	mtSNP10
A	G	G	A	A	T	C	A	T	C	C
B	A	G	A	A	T	C	A	C	C	C
C	A	G	A	A	T	C	A	C	T	C
D	G	G	A	A	T	C	G	C	C	C
E	G	G	A	G	T	C	A	C	C	C
F	G	A	A	A	T	T	A	C	C	C
G	G	A	A	A	T	T	A	C	C	T
H	G	G	G	A	T	C	A	C	C	T
I	G	G	A	A	C	C	A	C	C	T
J	A	G	A	A	T	T	A	C	C	T



Multiplexing

Assays and Instrumentation

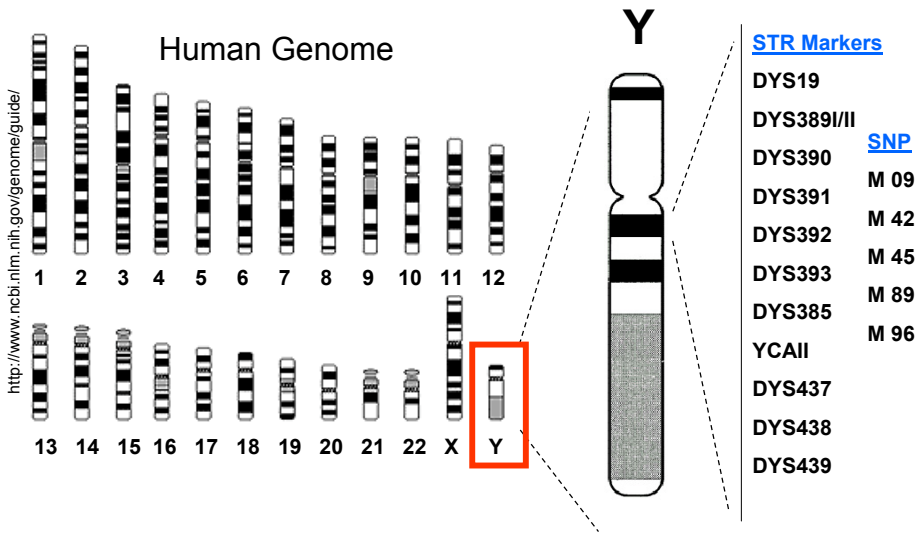
Y Chromosome and Mitochondrial DNA

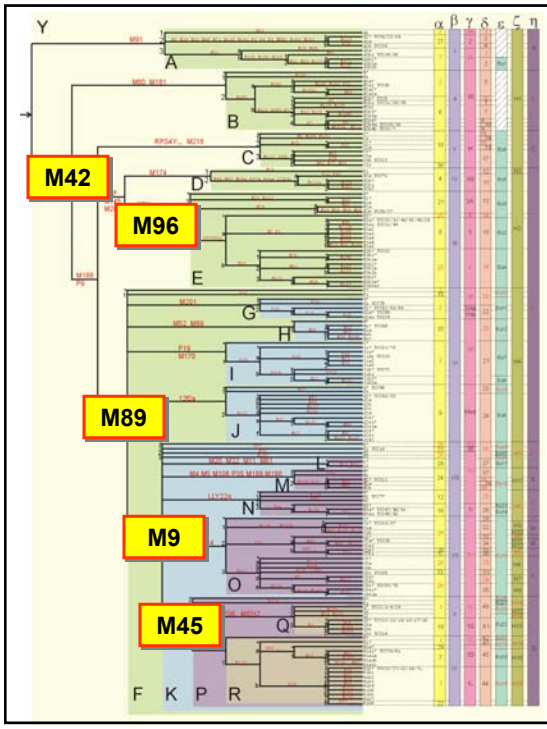
Primer design strategy

Results

- mtSNP 10 plex
- Y SNP 5 plex
- Y STR multiplexes

There is a growing interest in the Y-chromosome to aid forensic, paternity, and missing persons testing…





The Y Chromosome Consortium (2002) *Genome Res.* 12: 339-348

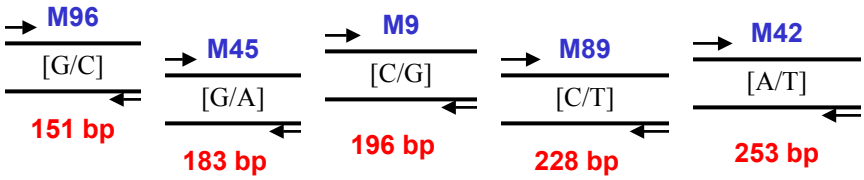
245 Y SNPs typed
74 males (YCC cell lines)
153 haplogroups observed

This paper unifies previous haplogroup nomenclatures

Primers and other information for all 245 markers are included in supplementary material

5 Y SNPs typed define major haplogroups

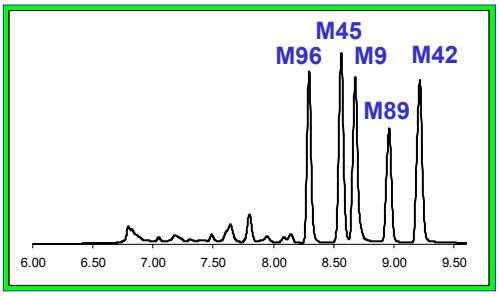
Multiplex PCR with Y-Chromosome SNP Markers



5-plex PCR

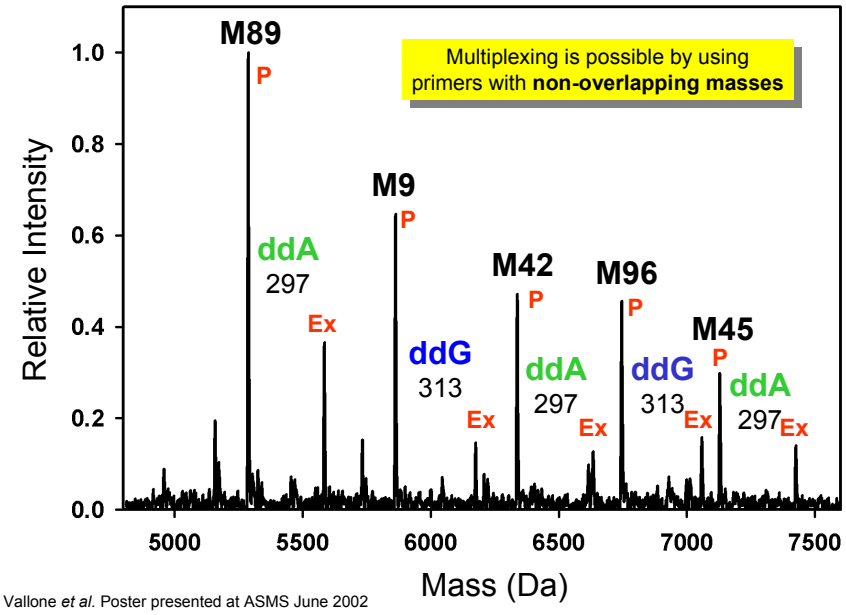
Rapid CE Separation and Quantitation of Multiplex PCR Products

Intercalating dye used to fluorescently label amplicons



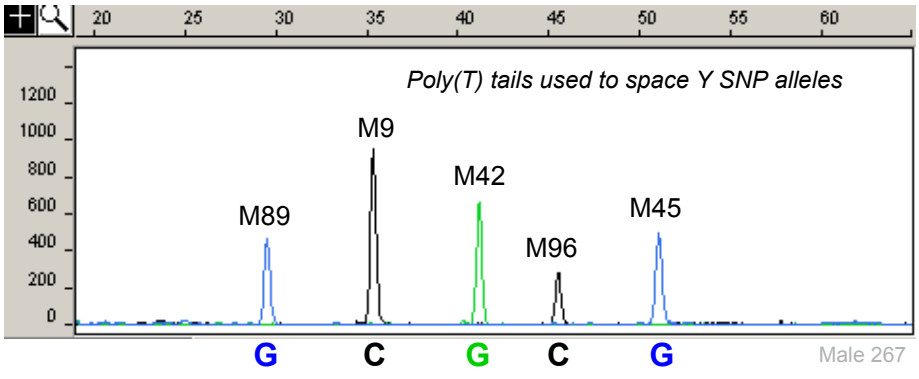
Butler *et al. (2001) Fresenius J. Anal. Chem.* 369: 200-205

Multiplexed Y SNPs (5-plex) Analyzed by TOF-MS



Vallone *et al.* Poster presented at ASMS June 2002

Y SNP Results with SNaPshot Assay



Room for additional Y SNP markers

Data obtained by Gordon Spangler (graduate student at American University)

Y SNP Haplotypes for 16 Test Samples

Sample ID	M9 (C/G)	M42 (A/T)	M45 (G/A)	M89 (C/T)	M96 (G/C)
Male 1	G	T	A	T	C
Male 2	G	T	A	T	C
Male 3	G	T	A	T	C
Male 4	G	T	A	T	C
Male 5	G	T	A	T	C
Male 6	G	T	A	T	C
Male 7	G	T	A	T	C
Male 8	C	T	G	C	G
Male 9	C	T	G	C	G
Male 10	C	T	G	C	G
Male 11	C	T	G	C	G
Male 12	C	T	G	C	G
Male 13	C	T	G	C	G
Male 14	G	T	G	T	C
Male 15	C	T	G	T	C
Female	-	-	-	-	-

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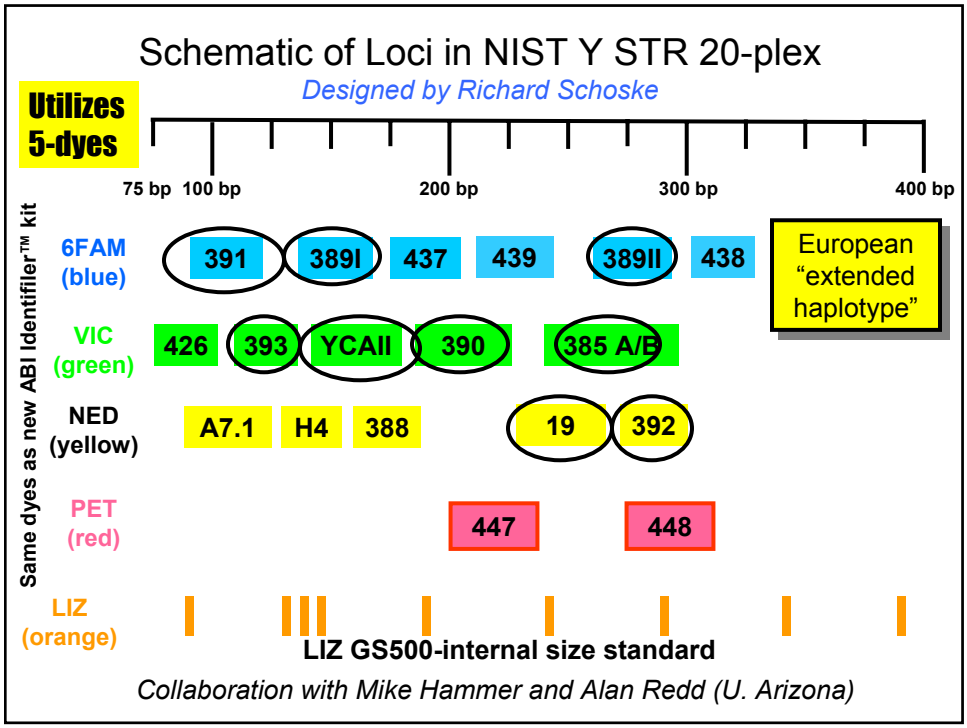
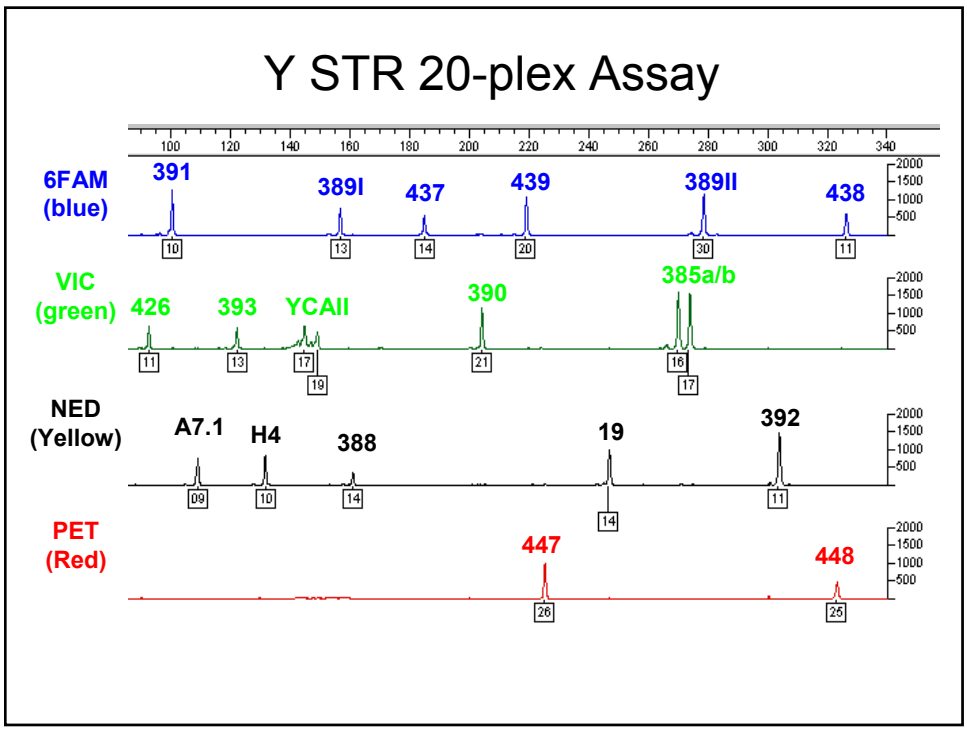
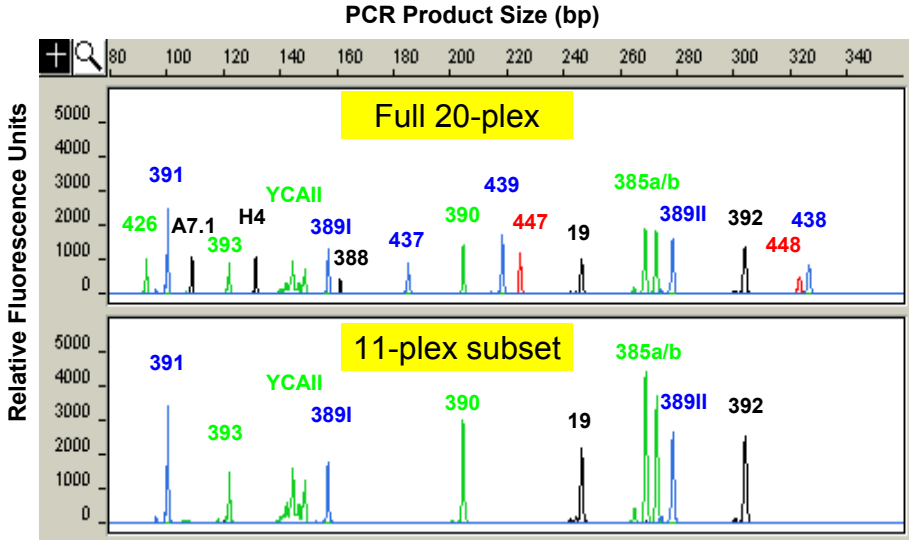

Schematic of Loci in NIST Y STR 20-plex
Designed by Richard Schoske
Utilizes 5-dyes
75 bp
100 bp
200 bp
300 bp
400 bp
Same dyes as new ABI Identifiler™ kit
6FAM (blue)
391
389I
437
439
389II
438
European "extended haplotype"
VIC (green)
426
393
YCAII
390
385 A/B
NED (yellow)
A7.1
H4
388
19
392
PET (red)
447
448
LIZ (orange)
LIZ GS500-internal size standard
Collaboration with Mike Hammer and Alan Redd (U. Arizona)

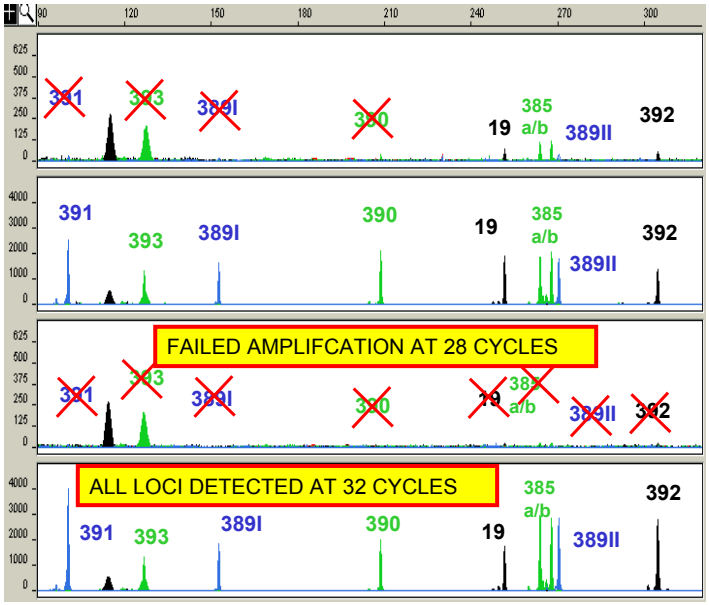

Y STR 20-plex Assay
6FAM (blue)
391
389I
437
439
389II
438
VIC (green)
426
393
YCAII
390
385a/b
NED (Yellow)
A7.1
H4
388
19
392
PET (Red)
447
448

Y STR 20-plex & Y STR 11-plex



Butler et al. (2002) Forensic Sci. Int., in press

Y STR 9-plex – Sensitivity Study



High-throughput Y STR Typing on the ABI 3100 (16-capillary array)

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Interagency Agreement between NIJ and NIST Office of Law Enforcement Standards

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Rich Schoske

Margaret Kline

Gordon Spangler

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Thomas Parsons and Mike Coble (AFDIL)
Mike Hammer and Alan Redd (U. of Arizona)

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