Rapid Genotyping of Y Chromosome SNPs Using a UV Photocleavable Oligonucleotide in MALDI-TOF MS

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The Y Chromosome

60,000kb total size

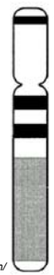
The non recombining region (NRY) consists of 95% of the Y chromosome

We are focusing on sequence variations not insertions/deletions

Genetic information is passed from father to son

Y SNP	Sequence Position
M9	20,934,946
M42	21,071,529
M45	21,072,476
M89	21,122,002
M96	20,934,946

Sequenced so far 22,714,721
http://genome.wustl.edu/projects/human/



Y SNPs

Low mutation rate of SNPs $2e^{-8}$ per base per generation

Over 3,700 SNP found on the Y so far *(http://www.ncbi.nlm.nih.gov)*

245 SNPs validated in population studies (Genome Res. 2002 12:339-348)

Sites discovered using DHPLC (Underhill *et al.*, Nat Genet 2000 26:358-61)

Y SNP validation and nomenclature (Y chromosome consortium, Genome Res. 2002 12:339-348)

Paracchini et. al., have designed 20 multiplexes for typing 118 Y SNPs by MALDI TOF MS (Paracchini *et al.*, Nucleic Acids Res. 2002 30:e27)

NIST Y Chromosome SRM material 2395 will include typing information on 5 Y SNPs (M9, M42, M45, M89, M96)

Forensic Utility of Y Chromosome SNPs

Human identification purposes (criminal, paternity, evolutionary, genealogy)

Y chromosome markers are useful in mixed male - female samples

Simplicity in testing – typically bi-allelic markers (versus length polymorphisms) and haploid (homozygous)

Haplogroups are non-randomly distributed among populations therefore potential exists for predicting population of origin

Improve multiplex assay development (both PCR and SNP detection)

For serious forensic usage parallel high-throughput methods will be required for typing

Phylogeny tree consisting of 116 haplotypes constructed from 167 Y chromosome mutations

The five SNP sites highlighted in yellow are major branch points to be typed

Underhill *et al., Nature Genetics 2000* 26: 358-361

Genotyping single nucleotide polymorphisms (SNPs) by MALDI-TOF MS is a well-established method. Multiplexing will be advantageous to achieve costs acceptable for the high-throughput analysis necessary for unbiased genetic profiling. One approach to increasing the information content of data acquisition is to use smaller analytes. By using an extension primer of regular size with an internal photocleavable linker, the resulting extension product can be shortened prior to data acquisition. The analysis of shorter DNA fragments (~ 5 bases) results in increased sensitivity, greater resolution, and more uniform ionization for multiple fragments when compared to longer DNA oligomers (over 20 bases). The ability to control the mass of the fragment analyzed by MALDI-TOF MS allows for greater freedom in the design of extension primers that must anneal under identical experimental conditions. The incorporation of mass modified nucleosides (e.g. 2'-O-methyladenosine or 2'-O-methylguanosine) can further expand the multiplexing potential of short DNA fragments. Genetic markers located on the non-recombining region of the Y chromosome are useful from a standpoint of evolutionary studies and have potential use as markers for human identification (forensic) purposes. In a project probing the usefulness of Y chromosome SNPs for human identity we are examining 5 Y SNPs (M9, M42, M45, M89, and M96) discovered by denaturing HPLC.

Steps in Primer Extension Assay

Select SNP site of interest and obtain DNA sequence

Design PCR primers and SNP extension primer

Perform PCR

Purify PCR reaction (SAP treat or chromatography)

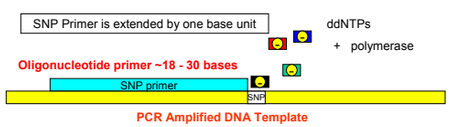
Add SNP reagents and perform single base extension

Purification/Desalting prior to MS analysis

Sample Preparation with 3HPA matrix

Type based on measurement of mass difference

Primer Extension for MALDI Analysis



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

Haff and Smirnov (1997) *Genome Res* 7:378-388

Mass difference between SNP primer and single base extension product provides base present at SNP site

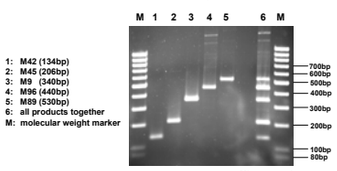
Desalting Primer Extension Reactions

Genopure Beads (single stranded)	Biotin-Strepavdin
8 strip or 96 well format	96 or 384 well format
Coated magnetic beads bind DNA	5' Biotin labeled extension primers are required
Single stranded DNA oligomers are bound and washed free from salts, enzyme, ddNTPs	Primers are bound to a 384 well plate with strepavidin coated wells
Salt free sample is eluted from the bead, spotted on the MALDI target and cleaved by UV light	Salt is washed off as DNA remains anchored to plate surface
	Salt free DNA is cleaved by UV light in solution on the plate and the fragment is spotted onto the MALDI target

Locus	PCR Primer Pair	Amplicon length
M9F	GCAGCATATAAAACTTTCAGG	340
M9R	AAAACCTAACTTTGCTCAAGC	
M42F	AGCTATTGTATTCACCAGTTG	134
M42R	TTTTAGCAAGTTAAGTCACCAGC	
M45F	GCTGGCAAGACACTTCTGAG	206
M45R	GTGACAGTGGCACCAAAGGTC	
M89F	AGAAGCAGATTGATGTCCCACT	530
M89R	TCCAGTTAGGAGATCCCCTCA	
M96F	GTTGCCCTCTCACAGAGCAC	440
M96R	AAGGTCACTGGAAGGATTGC	

Singleplex PCR performed in a 10 μL volume. Components; 5 pmol of each PCR primer, 1x reaction buffer, 1mM $MgCl_2$, 0.5 Units Taq polymerase, 200 μM dNTPs, (10 ng template)
Thermal cycling : 94 °C 4min; 35x {94 °C 10s, 58 °C 40s, 72 °C 40s}; 72 °C 5min

PCR-products



3% agarose gel

Primer Extension Using a UV Photocleavable Analyte

A standard primer extension assay (mini-sequencing) is performed using an extension primer that contains a UV photocleavable linker

After the extension reaction is completed, the linker is cleaved (λ = 366 nm) resulting in a ~5 base oligonucleotide for MALDI TOF MS analysis

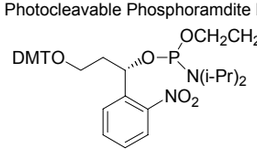
An analyte of reduced mass results in higher sensitivity, resolution, and more uniform ionization for multiplexing

Example M42

5' CCAGCTCTCTTTTTCATTATLTAGT 3' mass = 7492.9

5' TAGT 3' mass = 1268.8

Photocleavable Phosphoramidite Linker



Ordoukhanian, P. and Taylor, J.S., J. Am. Chem. Soc 1995 117:9570-9571

Locus	Extension Primer	Mass
M9R(C/G)	ACATGTCTAAATTAAAGAAAAATAAA^{OMe}GA^{OMe}G	1362.9
M42R(A/T)	CCAGCTCTCTTTTTCATTATGTAGT	1268.8
M45F(G/A)	GCAGTGAAAAATTATAG^{OMe}ATA	1307.8
M89F(C/T)	CTCTTCCTAAGGTTATGTACAAAAATCT	1228.8
M96F(G/C)	AACTTGGAAAACAGGTCTCTCATAATA	1261.8

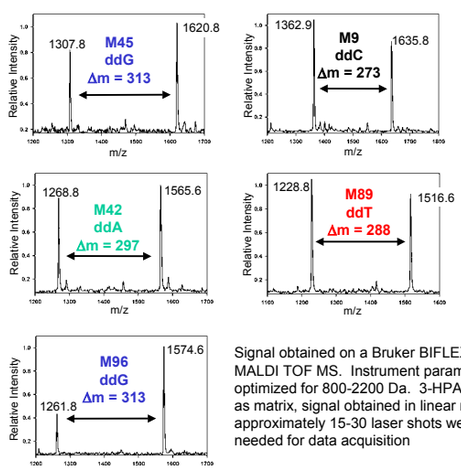
Underlined base = position of UV photocleavable moiety
A^{OMe} and G^{OMe} are 2'-O-methyladenosine and 2'-O-methylguanosine, respectively

Singleplex extension performed in a 10 μL volume. Components; 10 pmol of extension primer, 1x reaction buffer, 2 Units Thermosequenase, 200 μM ddNTPs, 3-5 μL PCR template

Multiplex extension performed in a 20 μL volume. Components; 6.5 – 16.0 pmol of each extension primer, 1x reaction buffer, 2 Units Thermosequenase, 200 μM ddNTPs, 10 μL pooled PCR template

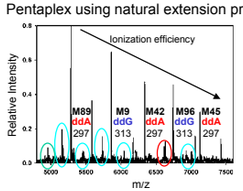
Thermal cycling: 94 °C 5min; 35x {94 °C 10s, 56 °C 60s, 72 °C 40s}; 72 °C 5min

Singleplex Spectra Five Y SNP Markers



Signal obtained on a Bruker BIFLEX III MALDI TOF MS. Instrument parameters optimized for 800-2200 Da. 3-HPA used as matrix, signal obtained in linear mode, approximately 15-30 laser shots were needed for data acquisition

Pentaplex using natural extension primers



Multiplexing issues when using full length extension primers

ionization efficiency

Na+ adducts

depurination

n-1 mer

mass range 5000-7500 Da

Number of laser shots required to obtain quality signal (greater than 50)

Benefits of multiplexing with a UV photocleavable extension primer

Uniform ionization efficiency

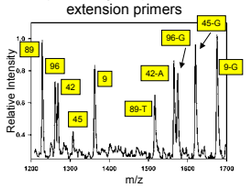
Shorter DNA fragments exhibit less Na+ adducts

Elimination of n-1 mer after UV cleavge

Mass range 1200-1650 Da (less than 500 Da!)

Number of laser shots required to obtain quality signal (less than 50) – rapid data collection

Pentaplex using UV photocleavable extension primers



Results of Typing 32 Samples

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

Typing results obtained from pentaplex extension reactions and genopure desalting

Sample	M9 (C/G)	M45 (G/A)	M96 (G/C)
17	G	A	G
18	G	A	G
19	G	A	G
20	G	A	G
21	G	A	G
22	G	A	G
23	G	A	G
24	C	G	C
25	C	G	C
26	C	G	C
27	C	G	C
28	C	G	C
29	C	G	C
30	C	G	G
31	G	G	G
32	G	G	G

Typing results obtained from singleplex extension reactions and biotin-strepavidin desalting (extension primers 5' labeled with biotin) data on M42 and M89 will be forthcoming

Conclusions and Future Plans

- Extension primers containing a UV photocleavable linker for five Y chromosome SNP markers worked successfully in single- and pentaplex reactions
- Biotin labeled extension primers provide a rapid and efficient means of salt removal
- Pentaplex extension reactions allow for rapid typing of the Y SNPs
- 6 haplogroups were observed in the 32 pilot samples

- Optimizing the multiplex PCR for the five markers
- Typing a panel of 96 male population samples for 5 (or more) Y SNPs

Acknowledgments

•Kristina Fahr (Bruker Saxonia Analytik GmbH)

•Funding from the National Institute of Justice through the NIST Office of Law Enforcement Standards

•Margaret Kline (NIST) for extraction and quantitation of DNA samples