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Single nucleotide polymorphisms (SNPs) are the most frequent form of DNA sequence variation in the human genome and are becoming increasingly useful as genetic markers for genome mapping studies, medical diagnostics, and human identity testing. One technique for SNP detection currently employed relies on the mass resolution between a primer and its single base extension product(s) utilizing Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry or MALDI-TOF MS. The speed of data collection by this technique is on the order of 5 sec per sample and has the potential for high throughput when interfaced with a robotic system and automated data collection/analysis. At NIST we are developing assays for SNP detection on the Y chromosome and mitochondrial genome. Robust multiplexed SNP assays are designed to be compatible with existing SNP genotyping technologies such as mass spectrometry and capillary electrophoresis.

SNP Genotyping Goals at NIST

- **Explore the use of SNPs for human identification** purposes using Y chromosome and mitochondrial DNA markers (sites of interest obtained from collaborators and the literature)
- **Evaluate SNP typing technologies**
- **Improve multiplex assay development** (both PCR and SNP detection)
- **Y Chromosome**
  - Focusing on SNP sites shown to resolve major haplogroups (M9, M42, M45, M89, M96 published by Peter Underhill at Stanford)
  - Sites recommended by Mike Hammer (U. AZ.)
- **Mitochondrial DNA**
  - 18 sites from control region provided by Mark Wilson (FBI Laboratory)
  - 29 sites from coding region provided by Tom Parsons and Mike Coble (AFDIL)

General Guidelines for Developing Multiplex PCR Assays

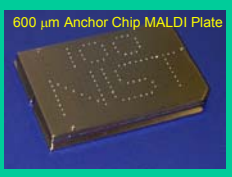
Multiplexed PCR and SNP detection assays are essential for rapidly genotyping multiple markers

We are developing new algorithms and strategies for rapid multiplex assay development

Obtain loci of interest (literature/collaborator/SNP discovery)
Retrieve DNA sequence(s) from GenBank®
Use publicly available Primer3 software for generating singleplex PCR primer pairs
Simultaneously check all PCR primer pairs for undesirable interactions (primer-dimers, hairpins, primer-template)
Redesign singleplex primer pairs that exhibit potential side interactions
Purchase primers, perform singleplex PCR (redesign failures)
Perform multiplex PCR, balance PCR product yields for even amplification across all loci (redesign failures)

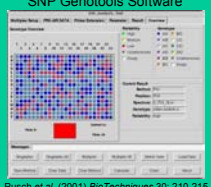
Under Investigation with SNP Typing by MALDI-TOF MS

Examination of various MS sample purification approaches and size reduction chemistries
Use of anchor chip technology (Bruker) to produce more uniform sample matrix spots
Optimization of automated data collection (AutoX) and automated SNP allele calling (Genotools)



384-well MALDI-TOF MS sample plate spotted with robot

SNP Genotools Software


Pusch *et al.* (2001) *BioTechniques* 30: 210-215

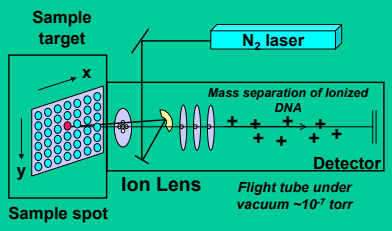
Steps in Primer Extension Assay

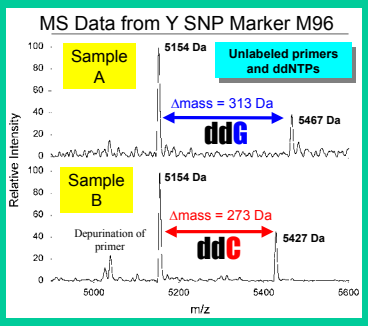
Select SNP site of interest and obtain DNA sequence
Design PCR primers and SNP extension primer
Perform PCR
Purification of PCR Template (SAP)
Add SNP reagents and perform single base extension
Purification with MS
Sample Preparation for Analysis (dilution)
Genotype based on measurement of mass difference or dye color

Mass Separation
ddCTP: 273 Da
ddTTP: 288 Da
ddATP: 297 Da
ddGTP: 313 Da

Color Separation
ddGTP-[dR110]
ddATP-[dR6G]
ddCTP-[dTAMRA]
ddTTP-[dROX]

Time-of-Flight Mass Spectrometry





MALDI-TOF MS

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry

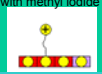
- Sub-picomolar amounts of DNA oligomers are ionized by a nitrogen laser and accelerated down a flight tube.
- The mass of the charged species is directly related to the time (in microseconds) it takes to reach the detector
- Primer extension assays products are ionized and genotyping is determined by mass difference between the primer and it's extension product(s)
- Rapid analysis technique (5-10 sec per sample)
- Multiplexing performed by using primers with distinguishable masses

GOOD Assay

Sauer *et al.*, (2000) *Nucleic Acids Res* 28:E13

Mixed-backbone primer w/ Charge Tag is extended with a-S-ddNTPs

Primer is digested with phosphodiesterase II and alkylated with methyl iodide



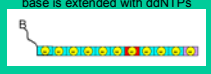
Shorter primer = better resolution
Charged primer = increased sensitivity

Phosphodiester
Phosphorothioate

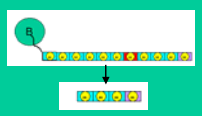
Cleavable Primer Assay

Monforte *et al.*, (1997) US Patent 5,700,642

Biotinylated primer w/ cleavable base is extended with ddNTPs



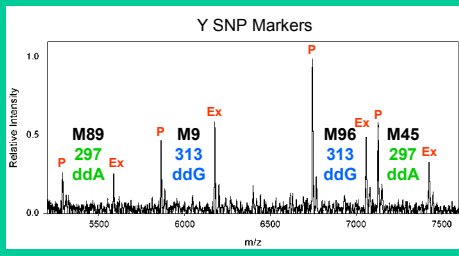
Bead capture and chemical cleavage



Shorter primer = better resolution

Cleavable base
Phosphodiester
B = Biotin

Multiplexing by Primer Mass Variation

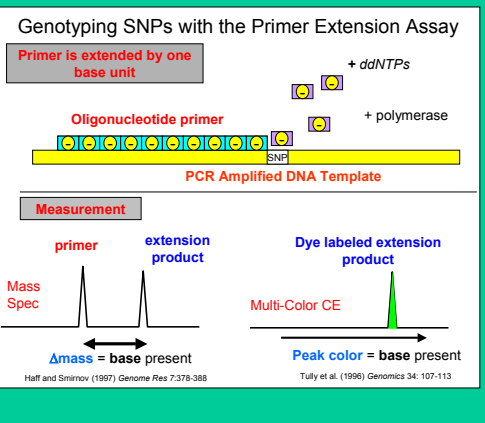


5 Y SNP markers for 16 samples

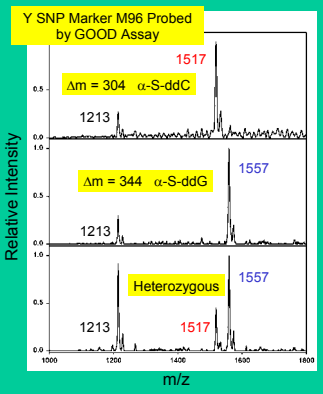
Sample ID	M9 (C/G)	M42 (A/T)	M45 (G/A)	M89 (C/T)	M96 (G/C)
Male 1	G	T	A	T	C
Male 2	G	T	A	T	C
Male 3	G	T	A	T	C
Male 4	G	T	A	T	C
Male 5	G	T	A	T	C
Male 6	G	T	A	T	C
Male 7	G	T	A	T	C
Male 8	C	T	G	C	G
Male 9	C	T	G	C	G
Male 10	C	T	G	C	G
Male 11	C	T	G	C	G
Male 12	C	T	G	C	G
Male 13	C	T	G	C	G
Male 14	G	T	G	T	C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

determined by MALDI-TOF MS

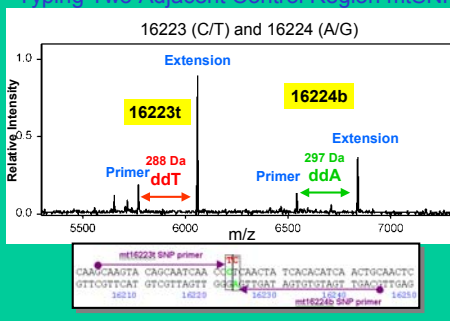
Genotyping SNPs with the Primer Extension Assay



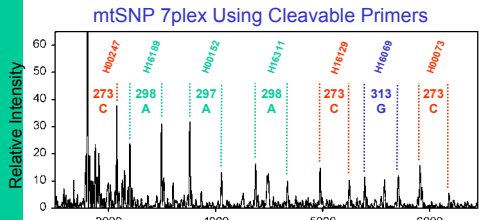
Haff and Smirnov (1997) *Genome Res* 7:378-388
Tully et al. (1996) *Genomics* 34: 107-113



Typing Two Adjacent Control Region mtSNPs



mtSNP 7plex Using Cleavable Primers



Future Plans

- Multiplex PCR and SNP detection with coding region mtSNPs in collaboration with AFDIL to increase power of discrimination for mtDNA typing

Acknowledgments
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Mark Wilson (FBI Laboratory) for mtDNA control region sites