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Best Practices for Reporting mtDNA Heteroplasmy when Using an MPS Approach: Considering Rates, DNA Damage & Drift





INTERNATIONAL SYMPOSIUM ON HUMAN IDENTIFICATION
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www.forensics.psu.edu

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Consultants (NIJ 2014-DN-BX-K022)





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Erica Pack (software solutions)
Emmy Demchak (molecular autopsy)
Irene Peng (Taiwan)

Research Group



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Thanks!!



illumina
Cydne Holt, Joe Valaro, etc



SoftGenetics – GeneMarker® HTS
John Fasnacht, Teresa Snyder-Leiby, etc



Promega
Doug Storts, Spencer Hermanson, Margaret Ewing

National Institute of Justice (NIJ 2014-DN-BX-K022)
National Institute of Justice (NIJ 2015-DN-BX-K025)


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AFDIL (Charla Marshall)
ATF (Todd Bille)
Mitotyping (Shelley Johnson & Gloria Dimick)
NIJ 2015-DN-BX-K025





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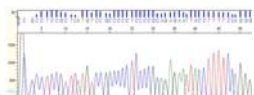
Forensic labs can now convert to a nextgen sequencing approach for mtDNA analysis, as the MPS approach produces reliable haplotypes



mtDNA + nextgen = 800+ articles




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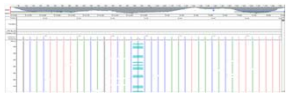



Forensic mtDNA Analysis Currently Requires:

- ▶ **Sensitive Amplification Approach** (HV1/HV2, mtgenome)
- ▶ **Sequencing Approach** (Sanger Dye Terminator)
- ▶ **Instrument** (CE, fragment separation)
- ▶ **Software for Data Analysis** (Mutation Surveyor, Sequencher)




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Forensic mtDNA MPS Analysis Requires:


- ▶ **Sensitive Amplification Approach** (HV1/HV2 up to mtgenome)
- ▶ **Sequencing Approach** (Library Preparation)
- ▶ **Instrument** (for example, Reverse Terminator MPS)
- ▶ **Software for Data Analysis** (GeneMarker® HTS)

We're there!


MISeq FGx 



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Sensitive Amplification Approach



PowerSeq™ Mito System

10-plex (multiplex) amplification across the entire control region

TruSeq® library preparation

"Nested" approach in development


Human mtDNA D-loop Hypervariable Region

4 separate amplification reactions across HV1/HV2

NexteraXT library preparation



Manuscript submitted

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PowerSeq™ Mito System

Sample Name	Size	Amplification Location
F1104024	164	15585-16122
F1104025	195	16158-16242
F1104026	227	16242-16428
F1104027	147	16242-16428
F1104028	172	16428-16712
F1104029	212	16428-16712
F1104030	165	16712-16923
F1104031	176	16923-17043
F1104032	164	17143-17243
F1104033	216	17243-17454





10 Overlapping Amplicons in a Multiplex Reaction

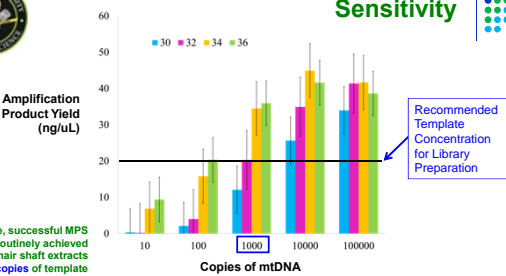
Using a Proprietary Non-Proofreading Polymerase

Gabriel et al. JFS 2001
Eichmann & Parson, JLM 2008

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Sensitivity



Amplification Product Yield (ng/uL)

Copies of mtDNA

1000 Copies = ~1 Cell's Worth of DNA

In practice, successful MPS was routinely achieved from hair shaft extracts with ~10 copies of template at 35 cycles of PCR

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Slide 10 features a composite image. At the top left is the logo of Pennsylvania State University. To its right is a DNA sequence alignment with colored bars above it. Below the alignment is a microarray chip with a grid of colored spots. An arrow points from the chip to the alignment. To the right of the chip is a white sequencing machine. Below the chip is a chemical structure of a molecule. At the bottom right is a sequencing machine with a screen.

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The "Pileup"

Slide 11 features a screenshot of a software interface. At the top left is the logo of Pennsylvania State University. To its right is the title "The 'Pileup'" in green. Below the title is a screenshot of a software interface showing a "Pileup" view of DNA sequence data. The interface includes a header with columns for "Position", "Reference", "Sample", and "Quality". Below the header is a grid of colored bars representing sequence variants. At the bottom of the screenshot is the text "What's up with software for forensic mito MPS analysis?"

What's up with software for forensic mito MPS analysis?

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Software Development

In collaboration with SoftGenetics, Inc.


Slide 12 features a screenshot of a software interface. At the top left is the logo of Pennsylvania State University. To its right is the title "Software Development" in green, followed by "In collaboration with SoftGenetics, Inc." in blue. Below the title is a screenshot of a software interface showing a "Zoom Global" view of DNA sequence data. The interface includes a header with columns for "Position", "Reference", "Sample", and "Quality". Below the header is a grid of colored bars representing sequence variants. A blue box with the text "Zoom Global" and an arrow points to the grid. At the bottom left of the screenshot is the logo for "Marker-ITS". At the bottom of the screenshot is the text "Uses a unique motif-based alignment strategy to identify the major haplotype and minor sequence variants".

Uses a unique motif-based alignment strategy to identify the major haplotype and minor sequence variants

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GeneMarker® HTS




Zoom Global

Global


Can view read depth in a Global or [Zoom Global](#) window

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Best Practice
Bottom Line

GeneMarker® HTS




Major Profile

Exportable Mutation Report


Minimal requirement for hands-on, manual interpretation

Manuscript in preparation



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Why bother converting to a nextgen approach?




Nextgen sequencing allows for vastly improved detection and **resolution** of mtDNA heteroplasmy


mtDNA + heteroplasmy + nextgen = 65+ articles

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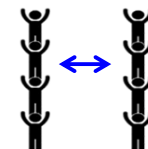


Goals for Leveraging Heteroplasmy

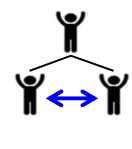
Better Discrimination Potential




Differentiate BETWEEN Maternal Lineages



Differentiate WITHIN Maternal Lineages



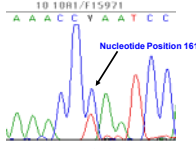
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Identification of Nicholas Romanov

10 1081/F15921

A A A C C Y A A T C C

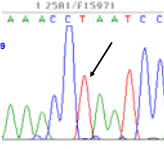


Nucleotide Position 16169



Tsar Nicholas II

1 2581/F15921

A A A C C T A A T C C




**Family Reference
5 Generations Removed**

Identification of the remains of the Romanov family by DNA analysis
Yakovlev, et al. 2008

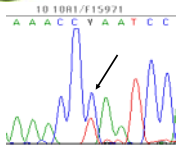
18



Identification of Nicholas Romanov

10 1081/F15921

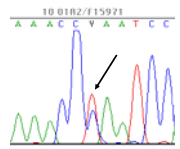
A A A C C Y A A T C C




Tsar Nicholas II

10 0182/F15921

A A A C C Y A A T C C



Georgij Romanov




Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of Tsar Nicholas II
Wright, et al. 2008

LR = 150


LR = 375,000
When heteroplasmy is considered

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With "read coverage" of 1000's of reads per nucleotide position, 2% minor components can be routinely resolved based on **instrument noise**


Err	Assumed error (%)	Error A (%)	Error C (%)	Error G (%)	Error T (%)	Error Insertions (%)	Error Deletions (%)
Average	2.19E-01	3.82E-02	7.06E-02	7.10E-02	3.99E-02	2.52E-02	2.12E-02
Std. Dev.	4.23E-02	1.24E-02	1.65E-02	7.55E-03	7.25E-03	9.18E-03	3.68E-03



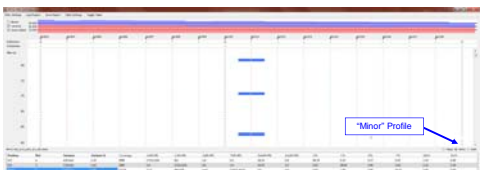
Requirements:
 200 total reads
 40 minor variant reads
 2% threshold if >2000 Reads
 Read depths average >10,000

Best Practice
Bottom Line

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GeneMarker® HTS

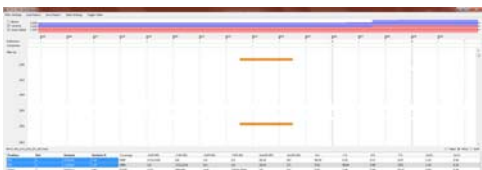


Provides a list of minor sequence variants that can be assessed as potential heteroplasmic positions

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GeneMarker® HTS



Dinucleotide repeat in the 515-524 Region ... including length-based variants

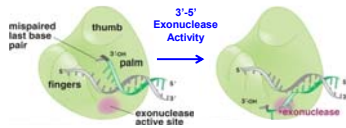
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Proofreading



Another potential source of “system noise“ is the use of a **non-proofreading polymerase** when performing the initial amplification



Can result in replication errors

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“Noise”



Nucleotide Position	Percentage of NP Samples >1% Noise	Percentage of P Samples >1% Noise	Percentage of NP Samples >2% Noise	Percentage of P Samples >2% Noise
53	93%	0%	0%	0%
181	100%	0%	0%	0%
316	53%	0%	40%	0%
461	83%	0%	0%	0%
16111	100%	0%	93%	0%
16153	100%	0%	100%	0%
16196	90%	0%	0%	0%
16362	100%	0%	27%	0%
16449	100%	0%	100%	0%

⊗ Primary haplotypes have been correct for all samples in every data set

⊗ “Noise” (replication errors/anomalies) seems to be reproducible

NP = non-proofreading P = proofreading (ExTaq from TaKaRa)

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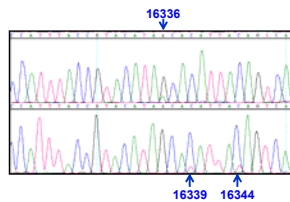


DNA Damage



1st Amplification

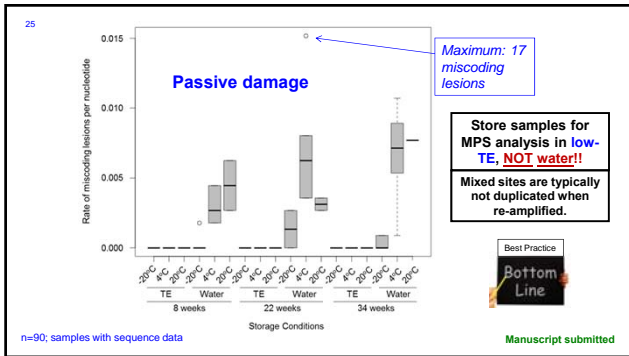
2nd Amplification

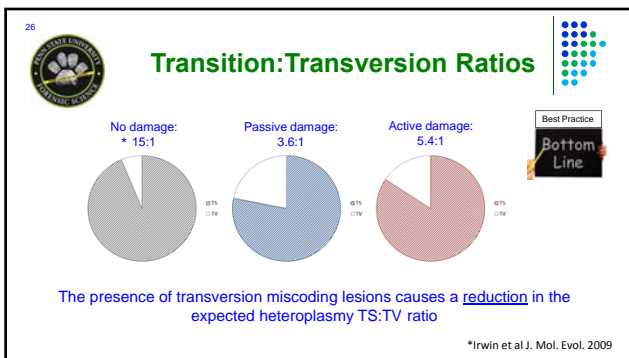


The mixed sites are not duplicated when re-amplified.

Sanger data showing DNA damage (Mitotyping Technologies). Bone sample from a 1985 missing persons case and tested in 2010.

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Rate Study

- mtDNA control region (1122 bps)
- Buccal swabs from ~550 unrelated individuals
- European decent
- Three age groups
 - 18-30, 31-50, >50 yoa
- Males & Females
- MiSeq/Nextera XT

Heteroplasmy rates per individual & per nucleotide position

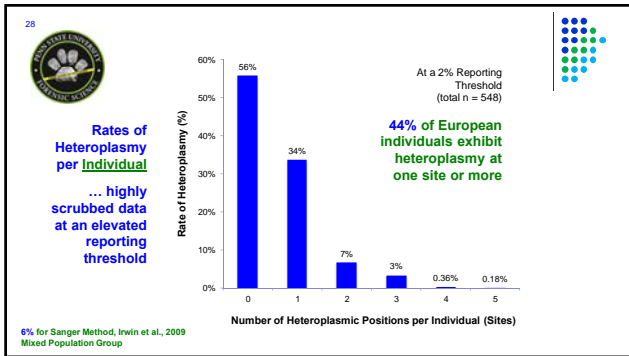
THE SWAB SQUAD

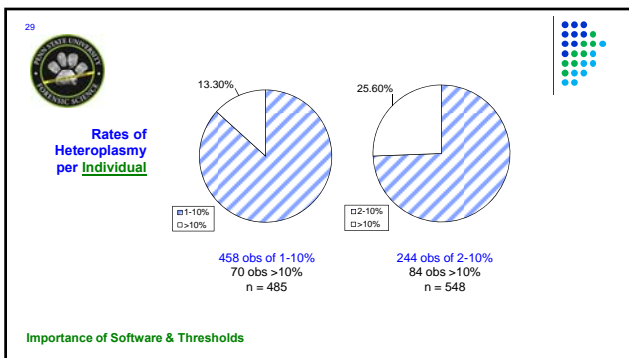
NIJ.GOV National Institute of Justice

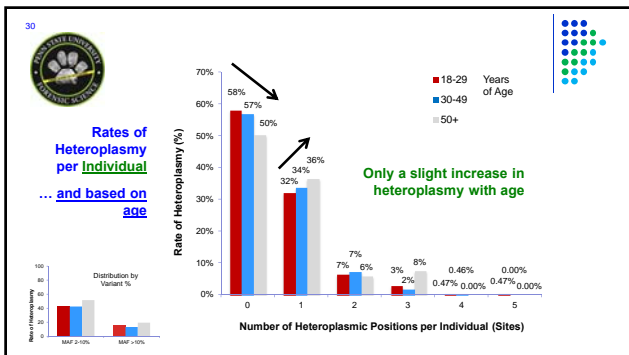
http://www.copelandtoday.com/blog/igglej2/03-06/00119600-awabing-check

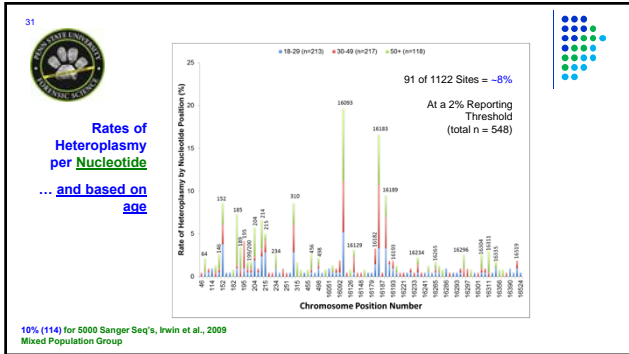
Bottom Line

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Frequency of Heteroplasmy

Number of Sites	Observation #	Frequency	Number of Sites	Observation #	Frequency
1031	0	0%	2	10	1.82%
43	1	0.18%	1	11	2.01%
18	2	0.36%	2	16	2.92%
5	3	0.55%	1	18	3.29%
12	4	0.73%	1	30	5.47%
1	5-7	0.91 - 1.28%	1	34	6.20%
2	9	1.64%			

Range of 0%-6.20%

Best Practice
Bottom Line

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- ▶ Software for Data Analysis (GeneMarker® HTS)

We're there! MiSeq FGx



Thanks!! 😊
mmh20@psu.edu
