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Former Director, Forensic Science Program  
Penn State University, University Park, PA




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



INTERNATIONAL SYMPOSIUM ON HUMAN IDENTIFICATION  
MINNEAPOLIS, MN • SEPT. 26-29, 2016



[www.forensics.psu.edu](http://www.forensics.psu.edu)

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**Walther Parson & Ann Gross**  
Consultants (NIJ 2014-DN-BX-K022)




**Jen McElhoe,**  
Research Associate (NIJ)

**Charity Holland,**  
Research Assistant (NIJ)

**Master's Students:**  
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Laura Wilson (D-loop val)  
Elena Zavala (bone extr, MPS SNPs)  
Jamie Gallimore (drift in hair, NIJ)

**UG Students:**  
Alyssa Duffy (Promega 10-Plex)  
Jillian Baker (Promega 10-Plex)  
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)**



**Consultants:**  
AFDIL (Charla Marshall)  
ATF (Todd Bille)

**Mitotyping** (Shelley Johnson & Gloria Dimick)  
NIJ 2015-DN-BX-K025



**Eberly College of Science, Department of Biochemistry & Molecular Biology, Forensic Science Program**

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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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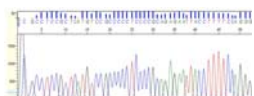
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**Forensic mtDNA Analysis Currently Requires:**

- ▶ **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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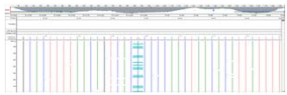
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**Forensic mtDNA MPS Analysis Requires:**

- ▶ **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 Protocol**  
4 separate amplification reactions across HV1/HV2  
NexteraXT library preparation  
Manuscript submitted

**Human mtDNA D-loop Hypervariable Region**  

 This document provides information for the application of the PowerSeq Mito System. It has been prepared as a general guide and is not intended to be used as a substitute for the manufacturer's instructions. The information contained herein is provided for informational purposes only and does not constitute an offer of any product or service. The information contained herein is subject to change without notice. The information contained herein is provided as a reference only and is not intended to be used as a substitute for the manufacturer's instructions. The information contained herein is provided as a reference only and is not intended to be used as a substitute for the manufacturer's instructions.

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## PowerSeq™ Mito System

Sample Name	Size	Amplification
F1100001	104	10000-10102
F1100002	110	10100-10202
F1100003	117	10100-10302
F1100004	124	10100-10402
F1100005	132	10100-10502
F1100006	141	10100-10602
F1100007	150	10100-10702
F1100008	159	10100-10802
F1100009	168	10100-10902
F1100010	178	10100-11002
F1100011	188	10100-11102
F1100012	198	10100-11202



**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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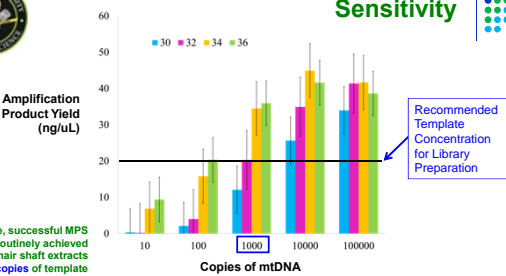
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## Sensitivity



**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

Recommended Template Concentration for Library Preparation

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### The "Pileup"

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

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### Software Development

In collaboration with SoftGenetics, Inc.

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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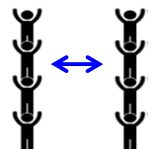


## Goals for Leveraging Heteroplasmy

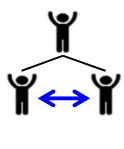
**Better Discrimination Potential**



**Differentiate BETWEEN Maternal Lineages**



**Differentiate WITHIN Maternal Lineages**



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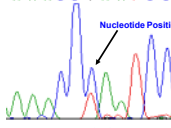
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## Identification of Nicholas Romanov

10 1081 / F15921

AAACCVAAATCC

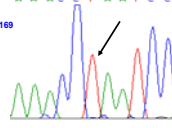


Nucleotide Position 16169



**Tsar Nicholas II**

1 2581 / F15921

AAACCTAAATCC



**Family Reference**  
5 Generations Removed

**Identification of the remains of the Romanov family by DNA analysis**

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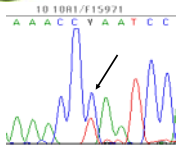
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## Identification of Nicholas Romanov

10 1081 / F15921

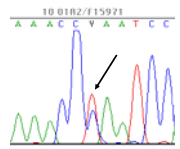
AAACCVAAATCC



**Tsar Nicholas II**

10 0182 / F15921

AAACCVAAATCC




**Georgij Romanov**

LR = 150

LR = 375,000

**When heteroplasmy is considered**



**Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of Tsar Nicholas II**

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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**

Run	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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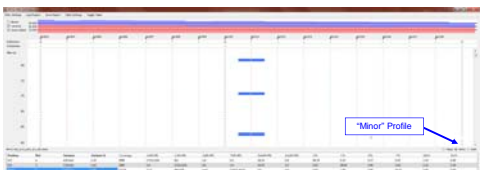
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**GeneMarker® HTS**



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

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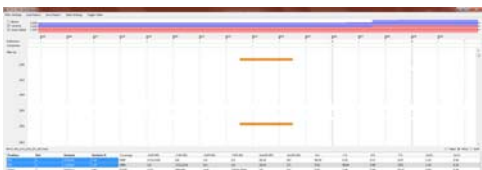
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**GeneMarker® HTS**



Dinucleotide repeat in the 515-524 Region

... including length-based variants

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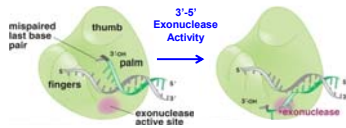
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### Proofreading



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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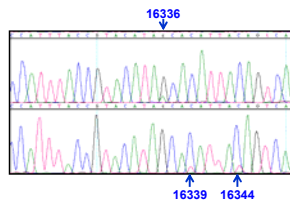


### DNA Damage



1<sup>st</sup> Amplification

2<sup>nd</sup> 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.

NJ 2015-DN-BX-K025

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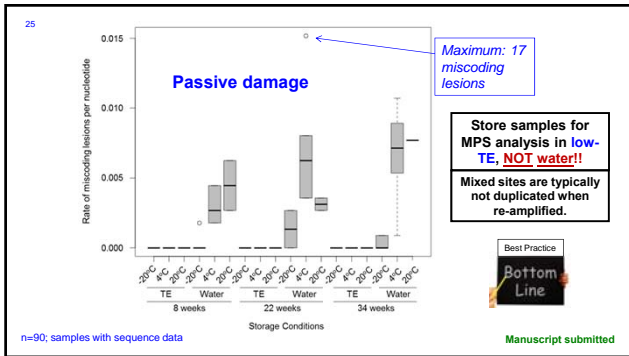
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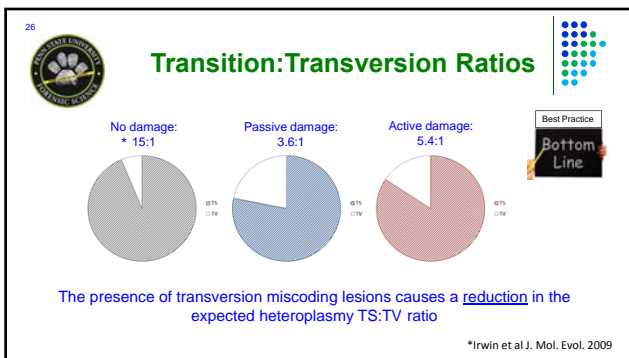
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Rate Study

- 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.copelandslab.com/blog/seqgly2/03.06/00119000-awabseq-check

NIJ 2014-DN-BX-K022

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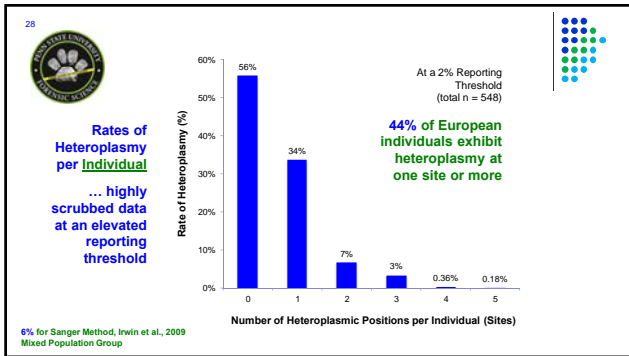
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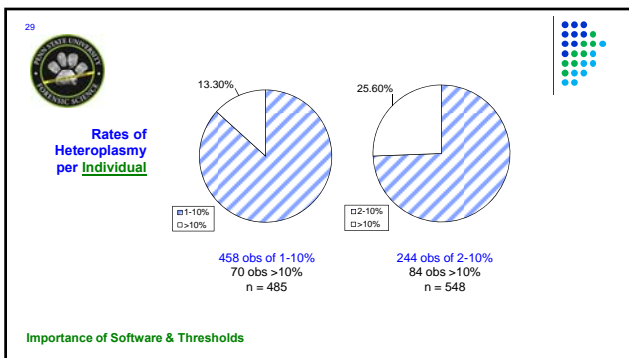
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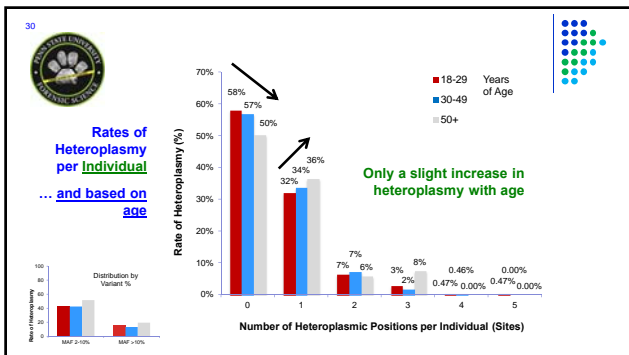
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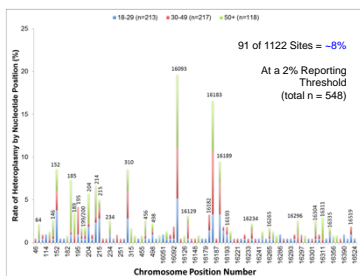
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**Rates of Heteroplasmy per Nucleotide ... and based on age**



10% (114) for 5000 Sanger Seq's, Irwin et al., 2009  
Mixed Population Group

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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%




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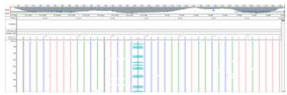
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**Forensic mtDNA MPS Analysis Requires:**

- ▶ **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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Thanks!! 😊  
mmh20@psu.edu

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