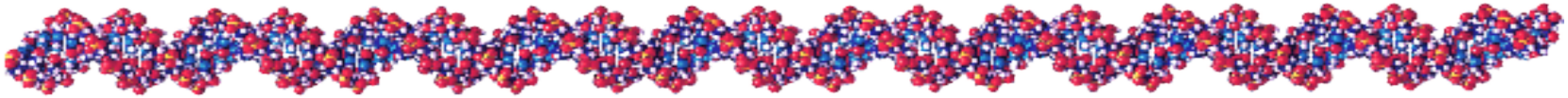
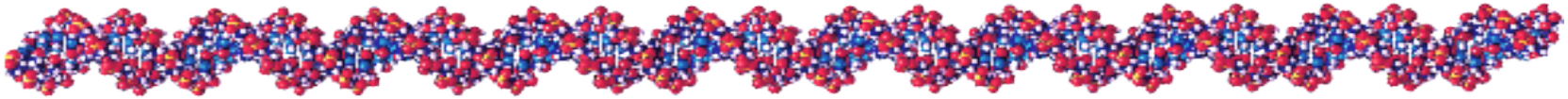


Capabilities of Next Generation Sequencing Instrumentation



Kevin Kiesler

Applied Genetics Group

U.S. National Institute of Standards and Technology

FBI Laboratory Seminars

May 23, 2013

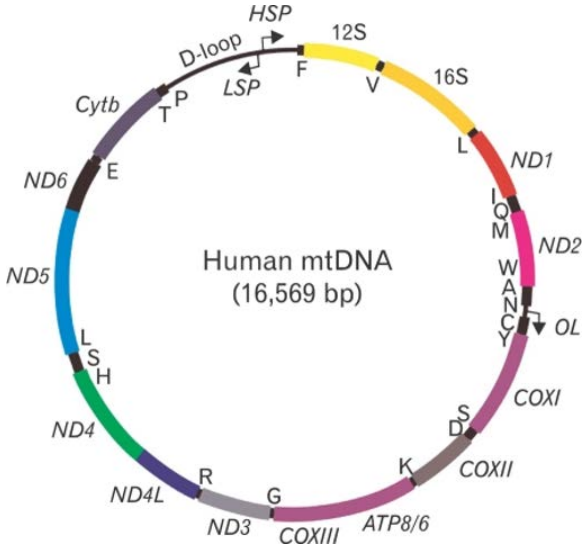
Quantico, VA

Outline

- Mitochondrial DNA background
- NIST SRM for mtDNA sequencing
- Next generation sequencing technology
- Experimental methods
- Experimental results
- Conclusions for mtDNA experiments
- Future directions – nuclear markers

mtDNA Sequencing

- Mitochondrial DNA (mtDNA) sequence information is sometimes used as an alternative to nuclear markers (STR) in forensic human identification
  - Automated Sanger sequencing
  - Non-coding “control region” ≈ 1122 bases
- Many copies of mtDNA (100 to 1000+) per cell
  - Useful when very small amount of tissue is available (i.e. hair, bone, tooth)
  - PCR amplification of mtDNA may be possible where nuclear markers (STR) fail to amplify
- Circular molecule is resistant to endonuclease activity
  - mtDNA may persist where nuclear DNA is degraded
- High levels of sequence diversity in non-coding region
  - Differentiate people based on sequence variations
- Maternally inherited
  - Cannot differentiate siblings
  - Sometimes used for missing persons I.D.



Yoon et. al. (2010) *Anat Cell Biol.; 43(2): 97 - 109*

NIST SRM 2392 & 2392-I

- Mitochondrial DNA sequencing Standard Reference Materials
  - Characterized for mtDNA whole genome sequence composition
  - Reference used to validate measurement techniques
  - Recommended by FBI as positive control for sequencing labs

- SRM 2392
  - Contains 3 components (extracted DNA)
    - 2392 A – From cell line CHR
    - 2392 B – From cell line 9947A
    - 2392 C – Cloned region of heteroplasmy

- SRM 2392–I
    - From cell line HL-60

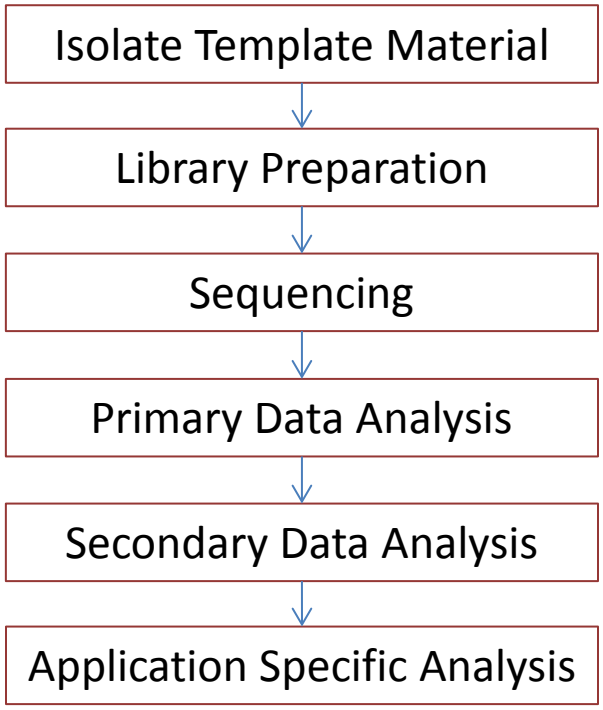
New Technology for Sequencing

- SRMs were initially characterized with Sanger sequencing
  - Levin et al. NIST Special Publication 260-155 (2003)
  - http://www.nist.gov/srm/upload/sp260-155.pdf

- Why use next generation sequencing (NGS)?
  - Whole mitochondrial genome analysis = more information
  - Potential for improved sensitivity
    - Detection of minor SNP variants - heteroplasmy
  - Confirm SRM sequence with an orthogonal technique

- Initial approach for NIST experiments
  - Sequence on multiple NGS platforms
  - Understand differences between platforms
  - Gain practical experience in library preparation, sequence data generation, and assembly/variant calling

Next Generation Sequencing Process



PCR, Hybridization Capture, gDNA, RNA/cDNA

Shear to size, ligate sequencing adaptors (barcode)

Multiple options

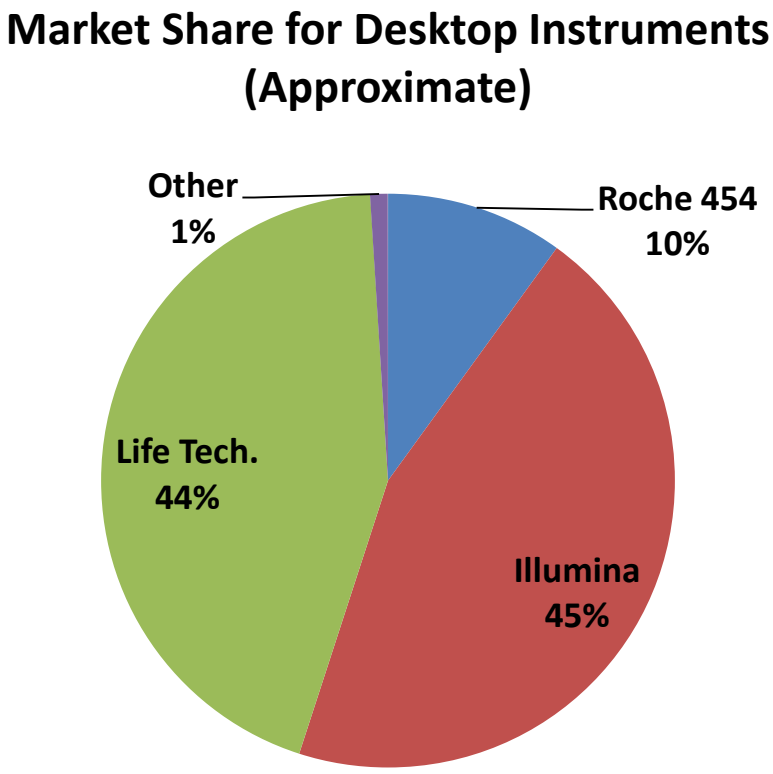
Image processing, signal processing

Map reads to reference sequence or de-novo assembly

SNP Calling, Structural Variation, RNA Expression

Four Common NGS Platforms

- Ion Torrent (Life Tech.)
  - Semiconductor sequencing
- SOLiD (Life Tech.)
  - Sequencing by ligation
- Illumina
  - Sequencing by synthesis
- Roche 454
  - Pyrosequencing



Source: DeciBio NGS Report (March 2013)
http://www.decibio.com/NGS_PR

Platform Output and Cost

Values estimated from manufacturer's specifications

	Output (Gb)	Run Time (Hours)	Cost Per Gb
Ion Torrent PGM ★	2	4.5	$
Ion Torrent Proton ★	10	4	$$
Illumina MiSeq ★	8	48	$
Illumina Genome Analyzer	95	336	$$
Illumina HiSeq 2000/2500	600	264	$$$
Life Technologies SOLiD	320	168	$$$
Roche 454 GS Junior ★	0.035	10	$$
Roche 454 GS FLX	0.7	23	$$$
Sanger sequencing (ABI 3730xl)	0.00005	2	$$$$

★ **"Benchtop sequencer" – lower instrument cost, smaller size, shorter run time**

Next Generation Sequencing Experiments

Approach

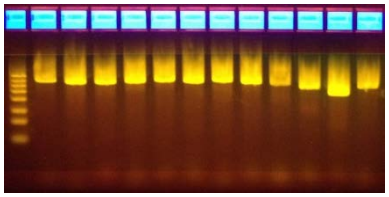
Pilot studies were performed on 4 platforms

- **Ion Torrent PGM**
  - Edge Biosystems (outsourced)
  - Instrument at NIST
- **Illumina HiSeq 2000**
  - Beckman-Coulter Genomics (outsourced)
- **Illumina MiSeq**
  - Edge Biosystems (outsourced)
- **SOLiD 5500**
  - Instrument at NIST

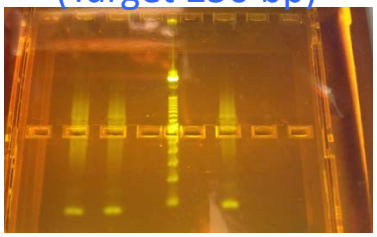
NIST is in the process of procuring both MiSeq and HiSeq instruments

Ion Torrent PGM Workflow

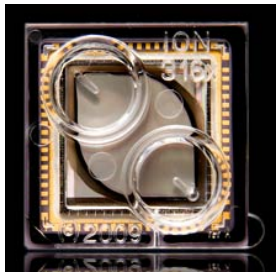
12 Amplicon PCR
0.8 kb to 1.5 kb



Size Selection Gel
(Target 250 bp)

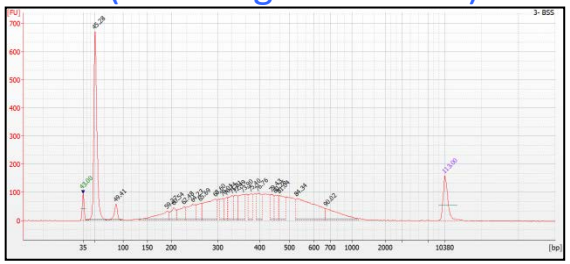


Ion 316 Chip
(6.1 Million Wells)

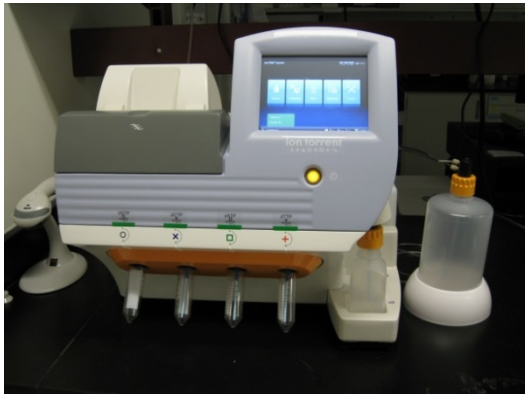


- Assay Design
- PCR Amplification
- Fragmentation
- Ligate Sequencing Adaptors
- Size Selection
- Quantitation of Library
- Emulsion PCR with Beads
- Enrichment
- Sequencing

Fragmentation
(Size range 100-1000)



Ion Torrent PGM Instrument



Signal Processing, Alignment, and Variant Calling

	Ion Torrent PGM	Illumina MiSeq	Illumina HiSeq	SOLiD 5500
Signal Processing Output: FASTQ	Torrent Server	MiSeq Reporter	HiSeq Control	LifeScope
Read Mapping Output: BAM	Torrent Server	Novoalign	BWA	LifeScope
Variant Calling Output: VCF	Torrent Server	GATK	GATK	GATK

Abbreviations:

FASTQ – Unaligned reads in text format with quality scores
BAM – Binary Alignment Map (Aligned reads)
VCF – Variant Call File
BWA – Burrows Wheeler Aligner
GATK – Genome Analysis Tool Kit

Sequence Coverage Summary

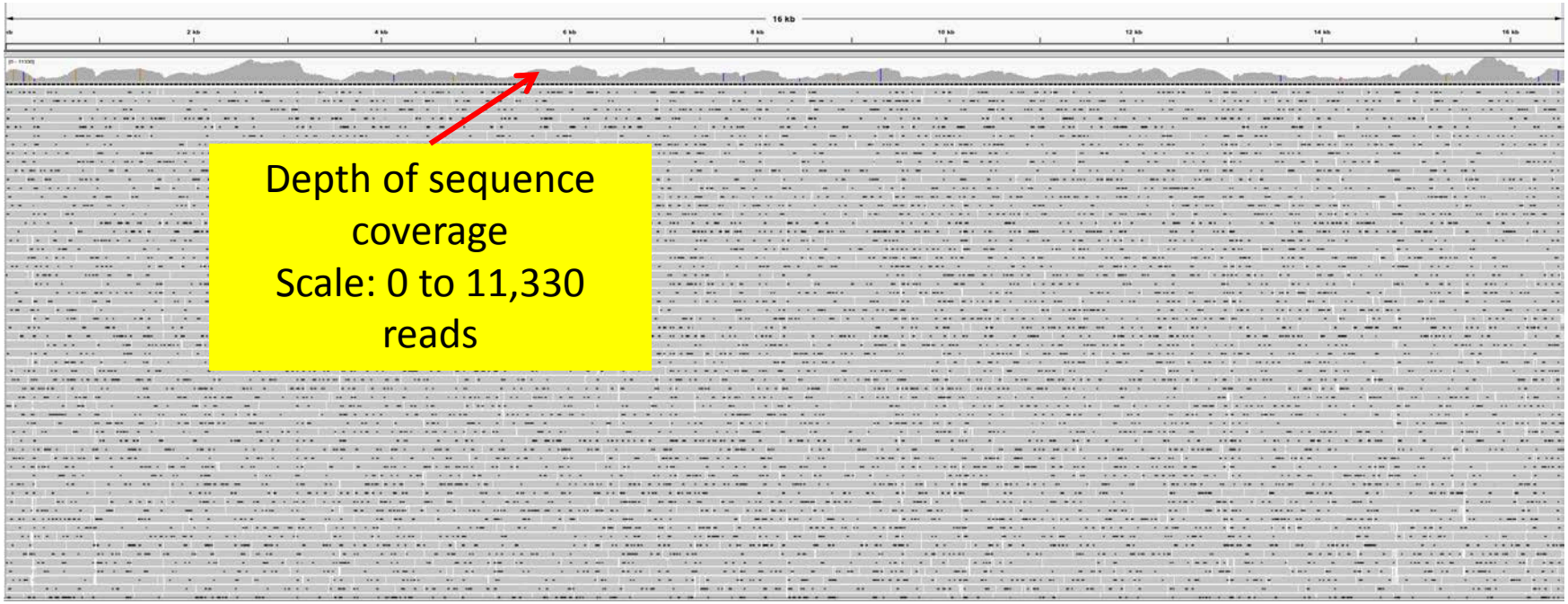
Experiment	Average Read Depth (AQ20*)	Experiment Design
EdgeBio PGM	280 x	Seven mtGenomes + spike-in controls**
NIST PGM Run 1	6,500 x	Three mtGenomes
NIST PGM Run 2	9,000 x	Three mtGenomes
Illumina MiSeq	49,000 x	Seven mtGenomes
Illumina HiSeq	41,000 x	Seven mtGenomes + spike-in controls**
NIST SOLiD	29,000 x	Seven mtGenomes + spike-in controls**

* AQ20 = reads with aligned quality score of 20 or above
= less than 1 error per 100 bases

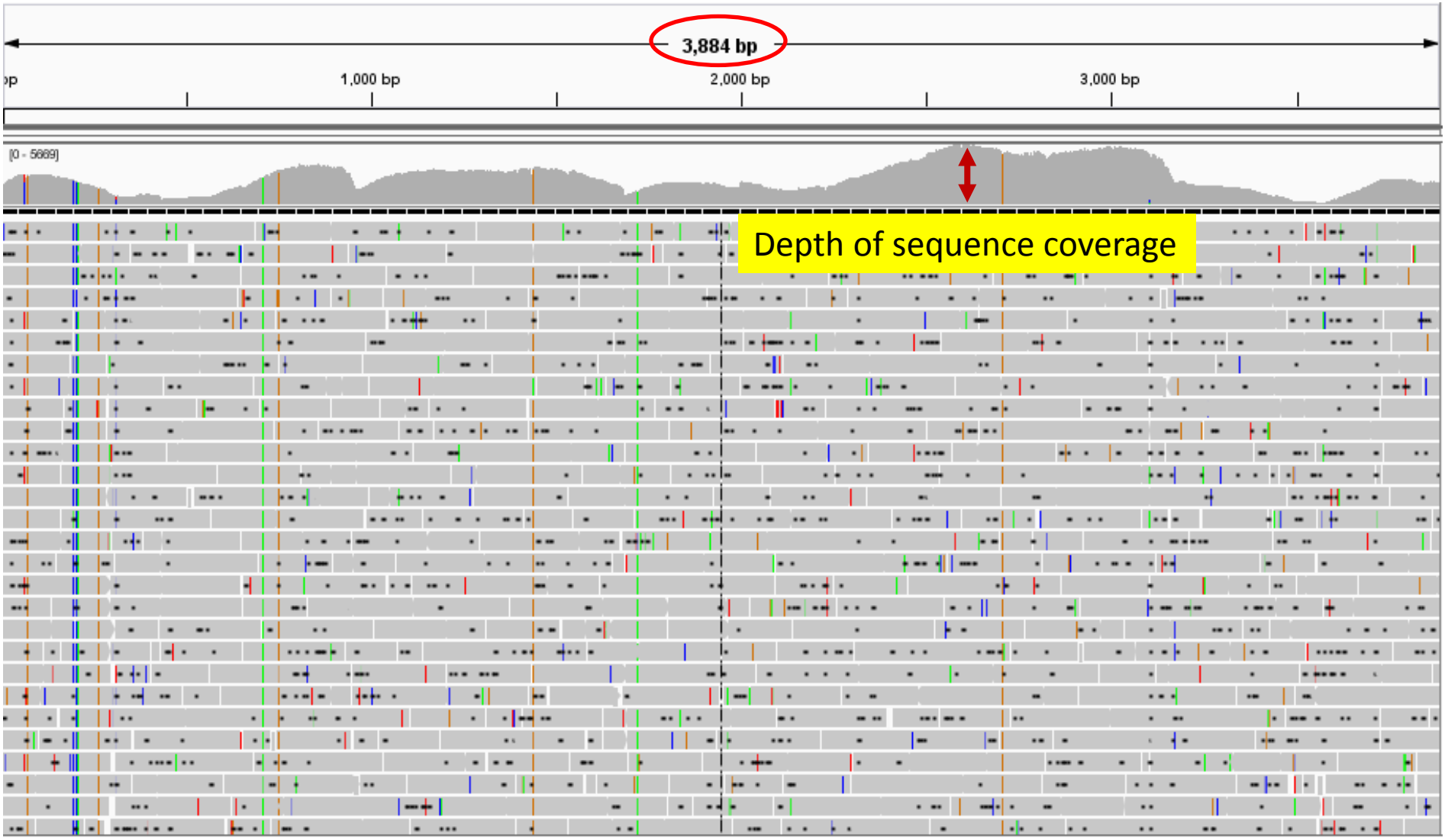
**Spike-in control was NIST SRM 2374: DNA Sequence Library for External RNA Controls

Broad Overview of Sequence Data

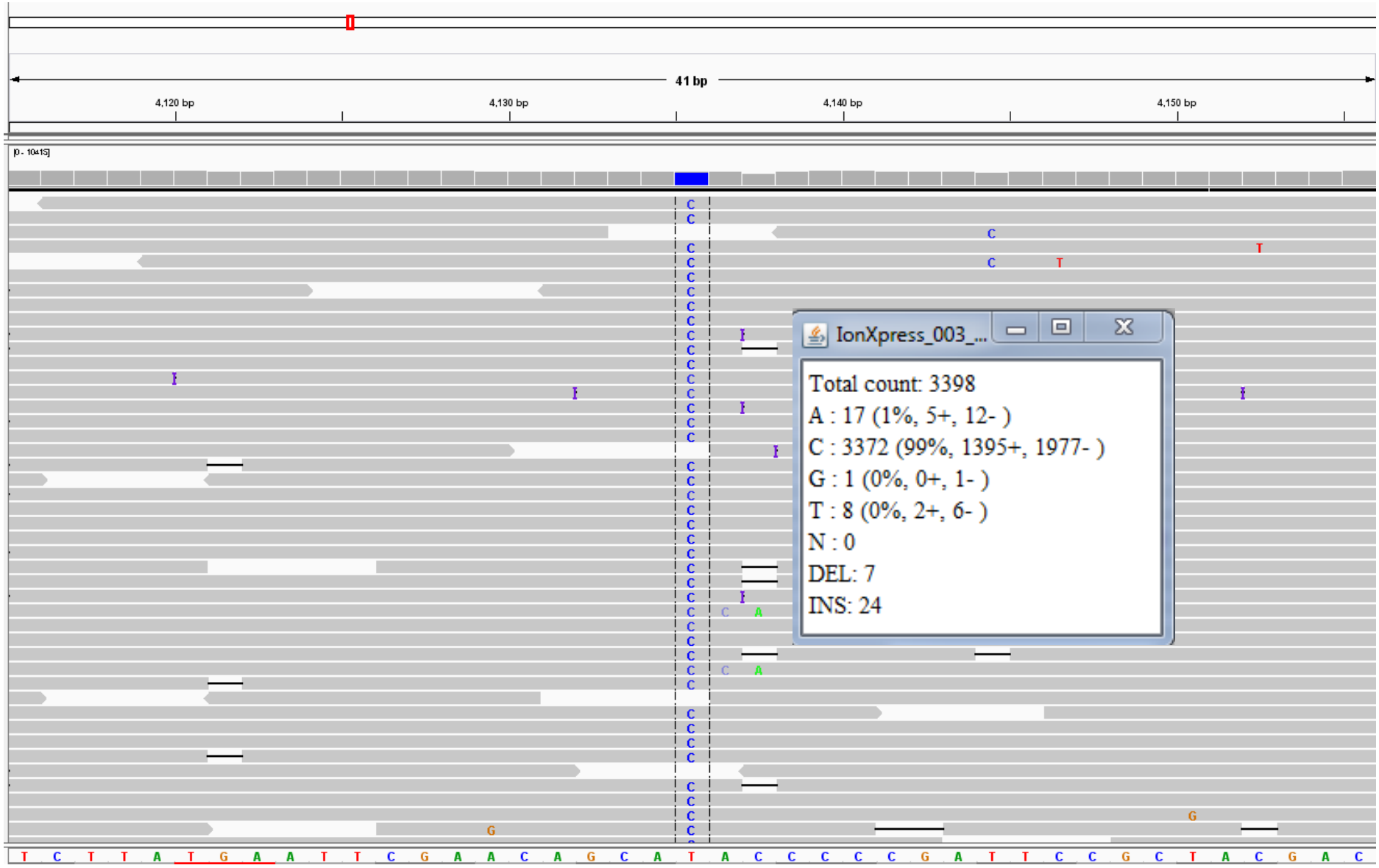
- Integrative Genomics Viewer (IGV)
  - Displays aligned sequences
    - Accepts BAM files
    - Sequence and coverage information displayed
  - Freely available from Broad Institute
    - Joint MIT/Harvard biomedical institute



Sequence Data - Zoomed In

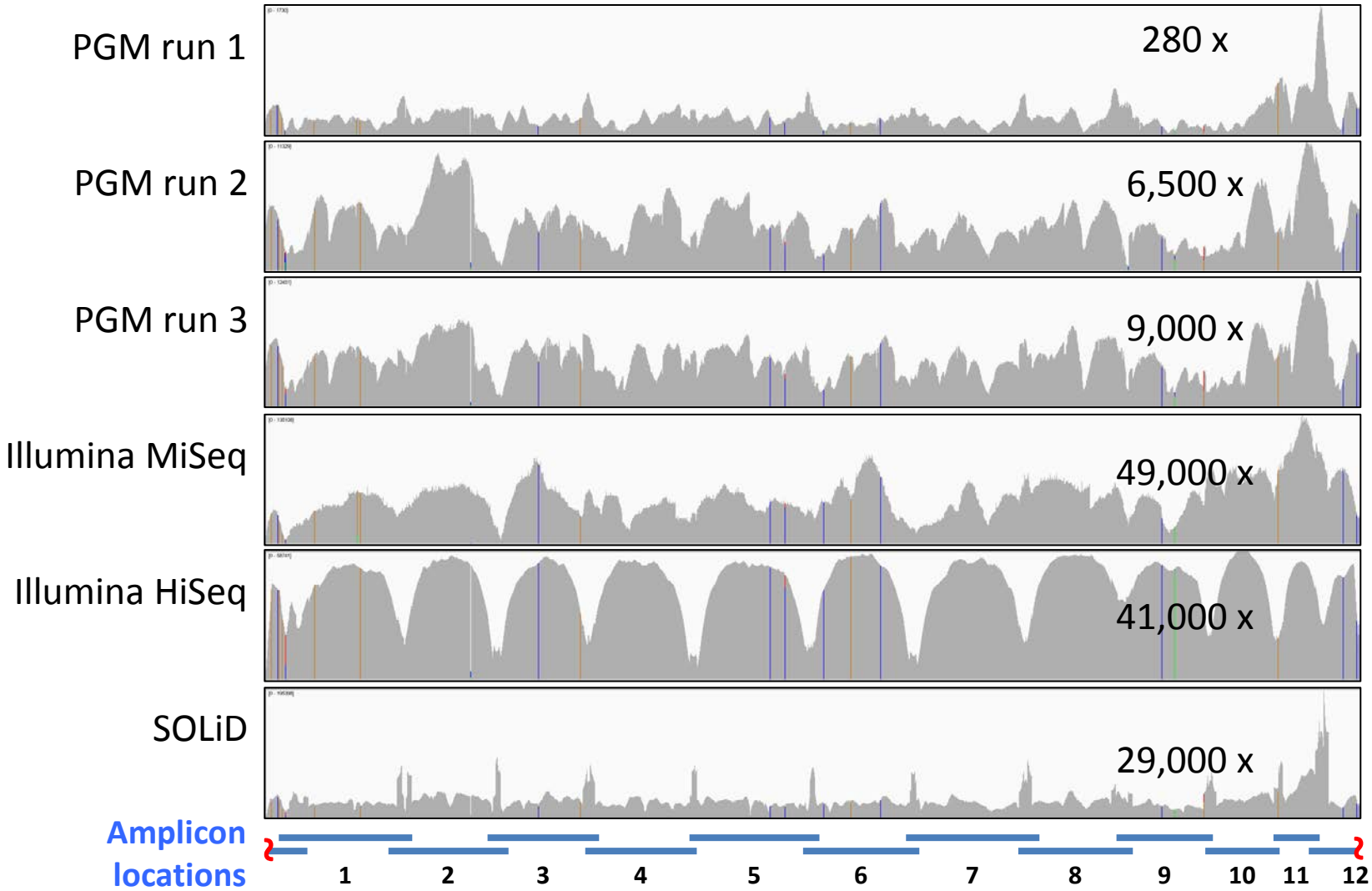


Sequence Data – Individual Bases



Coverage Across the mtDNA Genome

Data Shown for SRM 2392 Component B (9947a)



Variant Calls – Concordance

- Differences versus revised Cambridge Reference Sequence (rCRS) positions are reported
  - 16,569 bases
  - Only sites which differ are reported
  - All other sites are in agreement with reference
- Nucleotide position, reference & variant genotypes
- Other outputs: coverage statistics, quality metrics, p-values, etc.

Position	Type	Zygosity	Ref	Variant	Coverage	Ref Cov	Var Cov
93	SNP	Hom	A	G	4628	2	4626
195	SNP	Hom	T	C	3568	35	3533
214	SNP	Hom	A	G	3106	58	3046
263	SNP	Hom	A	G	2472	4	2460
750	SNP	Hom	A	G	3957	30	3919
1438	SNP	Hom	A	G	4826	57	4766
3106	SNP	Het	C	A	345	262	78
4135	SNP	Hom	T	C	2645	0	2642
4769	SNP	Hom	A	G	2620	10	2603
7645	SNP	Hom	T	C	3029	12	3016
7861	SNP	Het	T	C	1241	278	959
8448	SNP	Hom	T	C	1202	3	1187
8860	SNP	Hom	A	G	2991	5	2980
9315	SNP	Hom	T	C	4469	12	4456
13058	SNP	Het	C	A	198	131	58
13572	SNP	Hom	T	C	2528	8	2519
13759	SNP	Hom	G	A	814	2	758
14199	SNP	Het	T	G	1745	912	833
15326	SNP	Hom	A	G	3038	11	3023
16311	SNP	Hom	T	C	2097	10	2084
16519	SNP	Hom	T	C	3601	8	3582

Table output from Life Technologies "Torrent Server" software

Variant Calls – Concordance

SRM 2392 Component B (9947A)

Nucleotide Position	rCRS Reference Sequence	SRM 2392 Component B Sanger Call	EdgeBio PGM	NIST PGM run 1	NIST PGM run 2	EdgeBio Illumina MiSeq	Beckman Genomics Illumina HiSeq	NIST SOLiD
93	A	G	G	G	G	G	G	G
195	T	C	C	C	C	C	C	C
214	A	G	G	G	G	G	G	G
263	A	G	G	G	G	G	G	G
309.1	:	C						
309.2	:	C						
315.1	:	C						
750	A	G	G	G	G	G	G	G
1393	G	G	G/A	G/A	G/A	G/A	G/A	G/A
1438	A	G	G	G	G	G	G	G
4135	T	C	C	C	C	C	C	C
4769	A	G	G	G	G	G	G	G
7645	T	C	C	C	C	C	C	C
7861	T	C	C	C	C	C	C	C
8448	T	C	C	C	C	C	C	C
8860	A	G	G	G	G	G	G	G
9315	T	C	C	C	C	C	C	C
13572	T	C	C	C	C	C	C	C
13759	G	A	A		A	A	A	A
15326	A	G	G	G	G	G	G	G
16311	T	C	C	C	C	C	C	C
16519	T	C	C	C	C	C	C	C

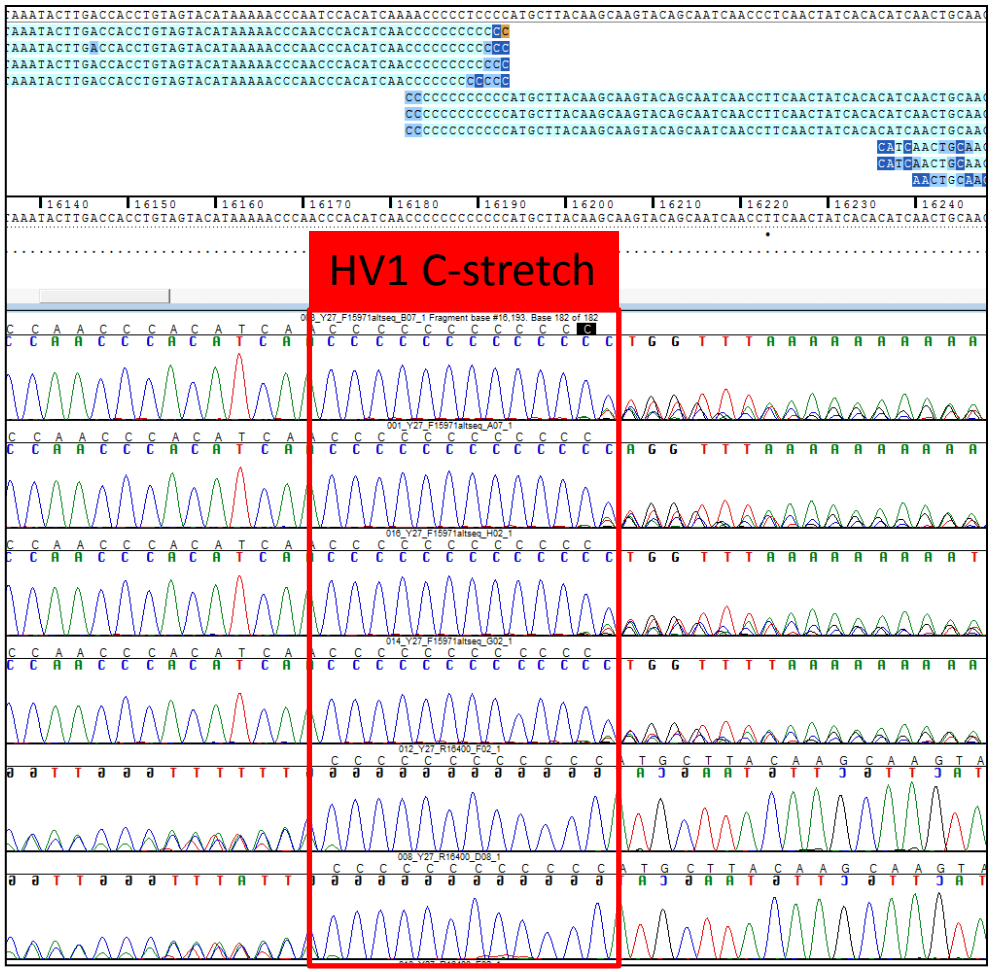
Insertions in HV2 C-stretch not correctly called according to forensic nomenclature rules

Nucleotide Position	rCRS Reference Sequence	SRM 2392 Component B Sanger Call	EdgeBio PGM	NIST PGM run 1	NIST PGM run 2	EdgeBio Illumina MiSeq	Beckman Genomics Illumina HiSeq	NIST SOLiD
93	A	G	G	G	G	G	G	G
195	T	C	C	C	C	C	C	C
214	A	G	G	G	G	G	G	G
263	A	G	G	G	G	G	G	G
309.1	:	C						
309.2	:	C						
315.1	:	C						
750	A	G	G	G	G	G	G	G
1393	G	G	G/A	G/A	G/A	G/A	G/A	G/A
1438	A	G	G	G	G	G	G	G
4135	T	C	C	C	C	C	C	C
4769	A	G	G	G	G	G	G	G
7645	T	C	C	C	C	C	C	C
7861	T	C	C	C	C	C	C	C
8448	T	C	C	C	C	C	C	C
8860	A	G	G	G	G	G	G	G
9315	T	C	C	C	C	C	C	C
13572	T	C	C	C	C	C	C	C
13759	G	A	A		A	A	A	A
15326	A	G	G	G	G	G	G	G
16311	T	C	C	C	C	C	C	C
16519	T	C	C	C	C	C	C	C

Control Region Homopolymers

- Two regions of poly **C** interrupted with one **T** in the middle, termed “C-stretch”
  - Difficult to sequence through with Sanger chemistry
- Challenge to align
  - There can be multiple length variants within one sample (heteroplasmy)
  - Strict nomenclature rules are used
- NGS variant calling software with forensic mtDNA nomenclature rules not yet available

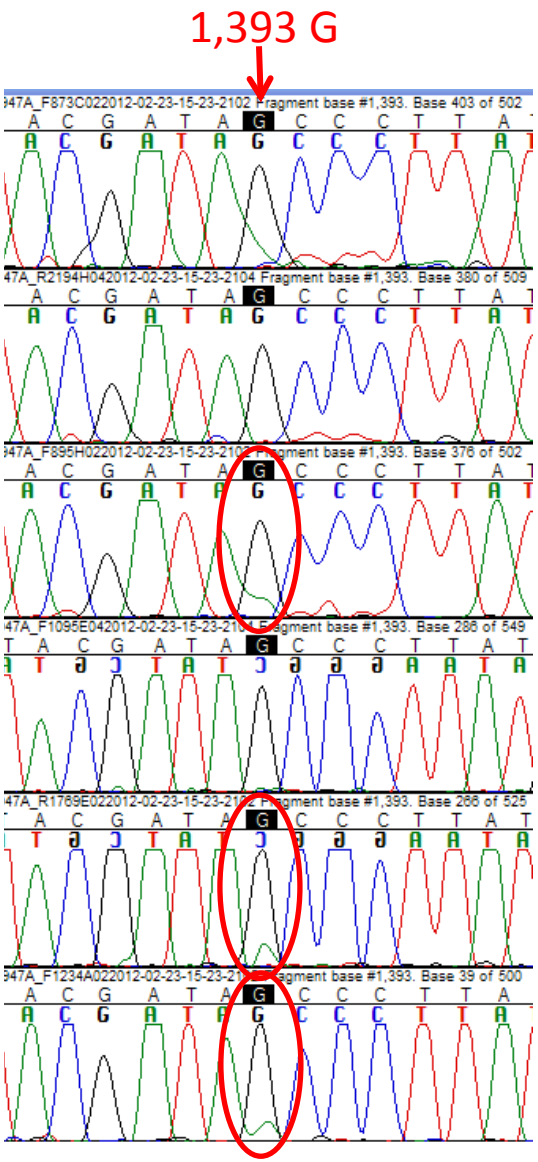
Reference sequence
HV1 = C C C C C **T** C C C C
HV2 = C C C C C C C **T** C C C C C



Heteroplasmy at Position 1,393

Nucleotide Position	rCRS Reference Sequence	SRM 2392 Component B Sanger Call	EdgeBio PGM	NIST PGM run 1	NIST PGM run 2	EdgeBio Illumina MiSeq	Beckman Genomics Illumina HiSeq	NIST SOLiD
93	A	G	G	G	G	G	G	G
195	T	C	C	C	C	C	C	C
214	A	G	G	G	G	G	G	G
263	A	G	G	G	G	G	G	G
309.1	:	C						
309.2	:	C						
315.1	:	C						
750	A	G	G	G	G	G	G	G
1393	G	G	G/A	G/A	G/A	G/A	G/A	G/A
1438	A	G	G	G	G	G	G	G
4135	T	C	C	C	C	C	C	C
4769	A	G	G	G	G	G	G	G
7645	T	C	C	C	C	C	C	C
7861	T	C	C	C	C	C	C	C
8448	T	C	C	C	C	C	C	C
8860	A	G	G	G	G	G	G	G
9315	T	C	C	C	C	C	C	C
13572	T	C	C	C	C	C	C	C
13759	G	A	A		A	A	A	A
15326	A	G	G	G	G	G	G	G
16311	T	C	C	C	C	C	C	C
16519	T	C	C	C	C	C	C	C

Heteroplasmy at 1,393?



- 3/6 of Sanger reads show possible low-level heteroplasmy
  - Red circles
- Not reproducible in all reads
  - Not always detected by Sanger sequencing

Heteroplasmy detected by NGS at Site 1,393

- Agreement across platforms
  - ≈ 17.6% (± 2.6%) minor component “A”

Experiment	Reference “G”	Variant “A”	Coverage at 1,393
EdgeBio PGM	77.3%	22.7%	97 x
NIST PGM Run 1	82.5%	17.5%	2940 x
NIST PGM Run 2	83.4%	16.6%	3275 x
Illumina MiSeq	83.7%	16.3%	26,234 x
Illumina HiSeq	84.4%	15.6%	62,186 x
NIST SOLiD	82.5%	16.9%	24,226 x

‘Missed Call’ at Position 13,759

Nucleotide Position	rCRS Reference Sequence	SRM 2392 Component B Sanger Call	EdgeBio PGM	NIST PGM run 1	NIST PGM run 2	EdgeBio Illumina MiSeq	Beckman Genomics Illumina HiSeq	NIST SOLiD
93	A	G	G	G	G	G	G	G
195	T	C	C	C	C	C	C	C
214	A	G	G	G	G	G	G	G
263	A	G	G	G	G	G	G	G
309.1	:							
309.2	:							
315.1	:							
750	A							G
1393	G							G/A
1438	A							G
4135	T							C
4769	A							G
7645	T							C
7861	T	C	C	C	C	C	C	C
8448	T	C	C	C	C	C	C	C
8860	A	G	G	G	G	G	G	G
9315	T	C	C	C	C	C	C	C
13572	T	C	C	C	C	C	C	C
13759	G	A	A		A	A	A	A
15326	A	G	G	G	G	G	G	G
16311	T	C	C	C	C	C	C	C
16519	T	C	C	C	C	C	C	C

On closer inspection of NIST PGM run 1:
1007 reads at this position were “A” (correct)
Only 3 forward strand reads were generated
Strand bias resulted in ‘missed call’

Variant Calls – False Positives

- Variants denoted by nucleotide position and base call
- Percentage of reads associated with variant calls shown
- Ion Torrent PGM variants called with Torrent Suite software
- Illumina and SOLiD variants called with GATK Unified Genotyper

PGM	%	MiSeq	%	HiSeq	%	SOLiD	%
3106 A	38.8%	302 INS C		302 INS C		301 INS C	
5744 C	3.9%	308 INS C		309 INS C		302 INS C	
7861 DEL	21.3%	310 INS C		310 INS C		309 INS C	
11512 A	6.6%	514 DEL		310 INS C		310 INS C	
13045 INS C	25.8%	515 DEL		360 T	0.4%	347 A	1.4%
13058 A	19.3%	515 G	0.9%	1992 T	1.1%	4722 G	0.4%
		3106 A	0.9%	3103 T	0.6%	6482 A	0.3%
		12418 C	0.4%	3104 C	1.2%	15284 C	0.1%
				3105 T	2.1%		
				4796 T	1.0%		
				6419 C	26.1%		
				8163 T	2.1%		
				9753 T	2.4%		
				14188 T	0.4%		
				15259 T	1.9%		
				15877 T	0.5%		
F.P. = 6		F.P. = 8		F.P. = 16		F.P. = 8	

Variant Calls – False Positives

- Many false positives are low-level
  - 0.1% to 2.4% of reads
  - Only in GATK variant calls
- These can be filtered out computationally
  - Evaluation of thresholds is recommended

PGM	%	MiSeq	%	HiSeq	%	SOLiD	%
3106 A	38.8%	302 INS C		302 INS C		301 INS C	
5744 C	3.9%	308 INS C		309 INS C		302 INS C	
7861 DEL	21.3%	310 INS C		310 INS C		309 INS C	
11512 A	6.6%	514 DEL		310 INS C		310 INS C	
13045 INS C	25.8%	515 DEL		360 T	0.4%	347 A	1.4%
13058 A	19.3%	515 G	0.9%	1992 T	1.1%	4722 G	0.4%
		3106 A	0.9%	3103 T	0.6%	6482 A	0.3%
		12418 C	0.4%	3104 C	1.2%	15284 C	0.1%
				3105 T	2.1%		
				4796 T	1.0%		
				6419 C	26.1%		
				8163 T	2.1%		
				9753 T	2.4%		
				14188 T	0.4%		
				15259 T	1.9%		
				15877 T	0.5%		
F.P. = 6		F.P. = 8		F.P. = 16		F.P. = 8	

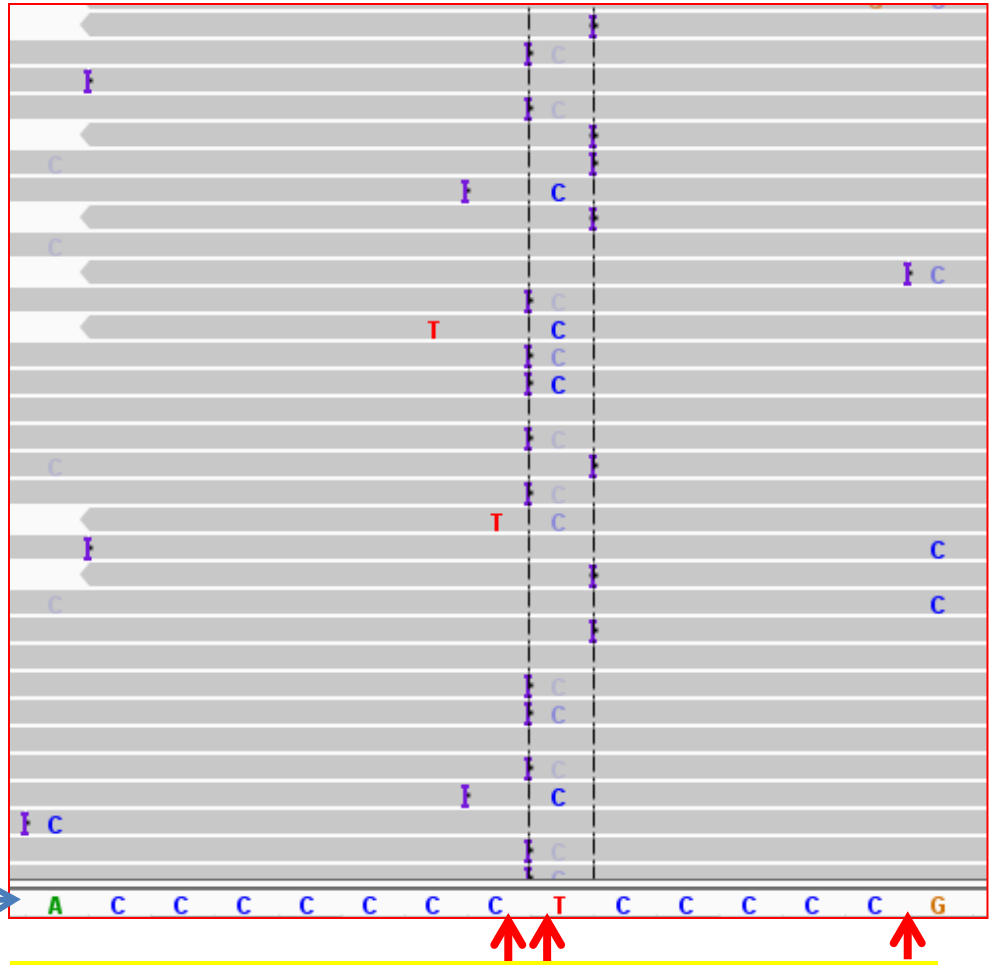
Variant Calls – False Positives

- Most of the remaining errors are related to HV2 C-stretch alignment
- This sample has three insertions in the HV2 C-stretch

PGM	%	MiSeq	%	HiSeq	%	SOLiD	%
3106 A	38.8%	302 INS C		302 INS C		301 INS C	
5744 C	3.9%	308 INS C		309 INS C		302 INS C	
7861 DEL	21.3%	310 INS C		310 INS C		309 INS C	
11512 A	6.6%	514 DEL		310 INS C		310 INS C	
13045 INS C	25.8%	515 DEL					
13058 A	19.3%						
				6419 C	26.1%		
F.P. = 6		F.P. = 5		F.P. = 5		F.P. = 4	

Variant Calls – False Positives

- HV2 Homopolymer associated alignment issue
- Software has difficulty aligning around insertions and deletions



Variant Calls – False Positives

- Homopolymers in other regions in the mtDNA genome
- GC rich regions
  - Low coverage
- Alignment
  - Deletion at 3,107
  - HV3 CA repeat

PGM	%	MiSeq	%	HiSeq	%	SOLiD	%
3106 A	38.8%	302 INS C		302 INS C		301 INS C	
5744 C	3.9%	308 INS C		309 INS C		302 INS C	
7861 DEL	21.3%	310 INS C		310 INS C		309 INS C	
11512 A	6.6%	514 DEL		310 INS C		310 INS C	
13045 INS C	25.8%	515 DEL					
13058 A	19.3%						
				6419 C	26.1%		
F.P. = 6		F.P. = 5		F.P. = 5		F.P. = 4	

Variant Calls – False Positives

- Homopolymers in other areas of the mtDNA genome
- GC rich regions
  - Low coverage
- Alignment
  - Deletion at 3,107
  - HV3 CA repeat

PGM	%	MiSeq	%	HiSeq	%	SOLiD	%
3106 A	38.8%	302 INS C		302 INS C		301 INS C	
5744 C	3.9%	308 INS C		309 INS C		302 INS C	
7861 DEL	21.3%	310 INS C		310 INS C		309 INS C	
11512 A	6.6%	514 DEL		310 INS C		310 INS C	
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Summary

- SRMs 2392 and 2392-I were successfully sequenced on four platforms
  - Sequence variants certified by Sanger were confirmed for 2392 Component B
    - Novel G/A heteroplasmy was detected at position 1393 (minor component ≈ 17%)
  - Other SRM components are still under analysis

- ≈ 16,569 bases measured correctly – average of 6 false positives per run
  - False positive rate = 0.04%
  - Without in-depth bioinformatics analysis

- Alignment is challenged by insertions/deletions/homopolymers
  - HV1 & HV2 C-stretch homopolymers not aligned according to forensic rules
  - Forensic mitochondrial DNA specific pipeline/solution is needed

- Software settings affect the sensitivity to minor component variants
  - Low sensitivity variant calling threshold increases false positives

Future plans - STR Sequencing

- Characterize sequence variation within repeat structure and flanking regions
  - Could aid in resolving mixtures
  - Increase information content (i.e. partial profiles)

- Our plan:
  - Characterize NIST SRM 2391c components
  - Start with CODIS loci
  - Amplify in single-plex using NIST primer designs
  - Pool and sequence

- Key considerations:
  - Only reads that fully cover the repeat structure are informative
  - Sequencing chemistry must be “long read” – now available

First Step – Reference "Genome"

- Create reference fasta file for read mapping
- Each allele is a separate line with:
  - Number of repeats for that allele
  - Flanking sequence on both sides of repeat
- Example: D16S539

>D16S539 (5)
ATACAGACAGACAGACAGGTG GATA GATA GATA GATA GATA TCATTGAAAGACAAAACAGAG

Flanking sequence | Repeats (5 units) | Flanking sequence

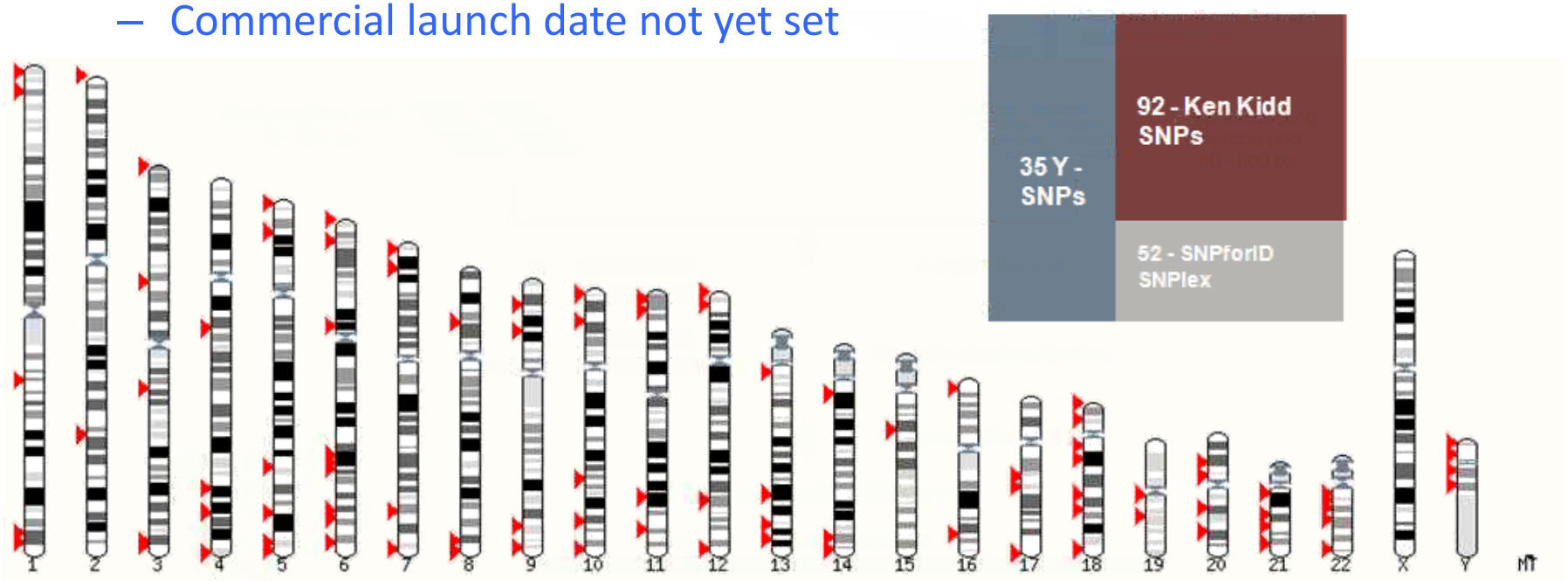
>D16S539 (6)
ATACAGACAGACAGACAGGTG GATA GATA GATA GATA GATA GATA TCATTGAAAGACAAAACAGAG

Repeats (6 units)

Life Technologies

Coming Soon for PGM

- HID SNP Panel v2.2
  - 179 loci amplified in a single multiplex PCR
  - Autosomal loci chosen for high heterozygosity and low Fst
  - Genotype match probability 10^{-31} to 10^{-35}
  - Uses “Ampliseq” design strategy from Ion Torrent
  - Short amplicons ≈ 150 bp
  - Commercial launch date not yet set



Life Technologies

Future Plans

- Ancestry informative and phenotypic SNP panel
- For generating investigative leads, subject exclusion
- 245 SNPs
  - 202 Ancestral SNPs
  - 45 Hair and eye color SNPs




Thanks for your attention!

Questions?

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