# Next Generation Sequencing Analyses of Complex Dual Genome Mitochondrial Disorders: Technical Approach

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content:

1.Application of NGS to molecular diagnosis of mitochondrial disorders: nuclear genes and mitochondrial genome

2.Validation and Quality control of Clinical tests

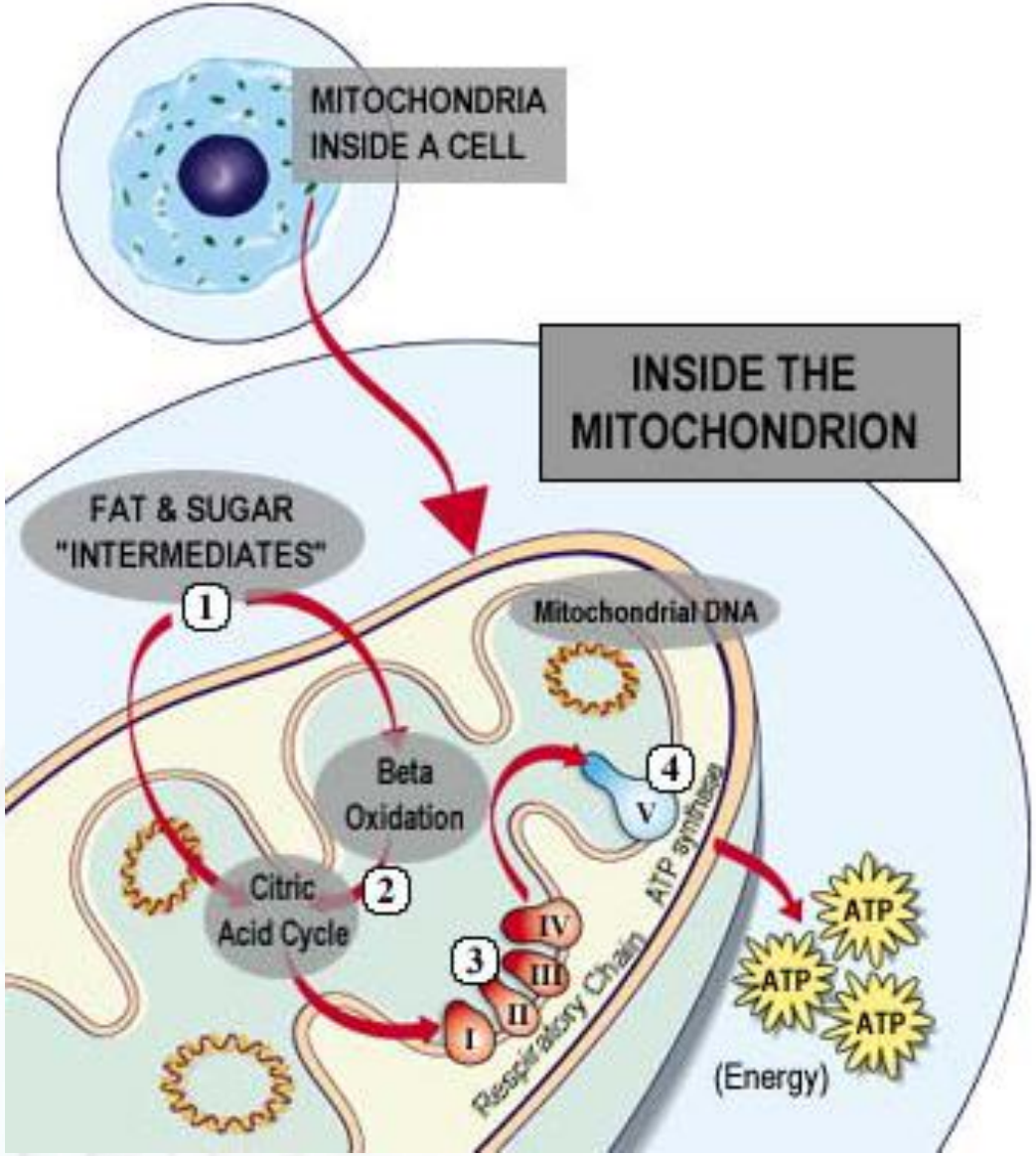
3.Types of mutations detected: point mutations, small indel, large deletions?

4.Target nuclear gene capture/sequencing

5.Mitochondrial genome: long range PCR of the whole mitochondrial genome

# Mitochondrial Function: produce energy

## Involves 2 genomes: mitochondrial and nuclear




**Fuels:**
**Fatty acids**
**carbohydrates**

↓

**Combustion:**
**Fatty acid oxidation**
**TCA cycle**

↓

**$NADH, FADH_2$**

↓

**electron transport chain**

↓

$O_2$ **Oxidative phosphorylation** $H_2O$

**ADP** **ATP**

↓

**NAD, FAD**

# Mitochondrial Disorders:
# Disease of Energy Deficiency
# Respiratory chain defect
# Defects in oxidative phosphorylation

**Preferentially affect tissues of high energy demand**

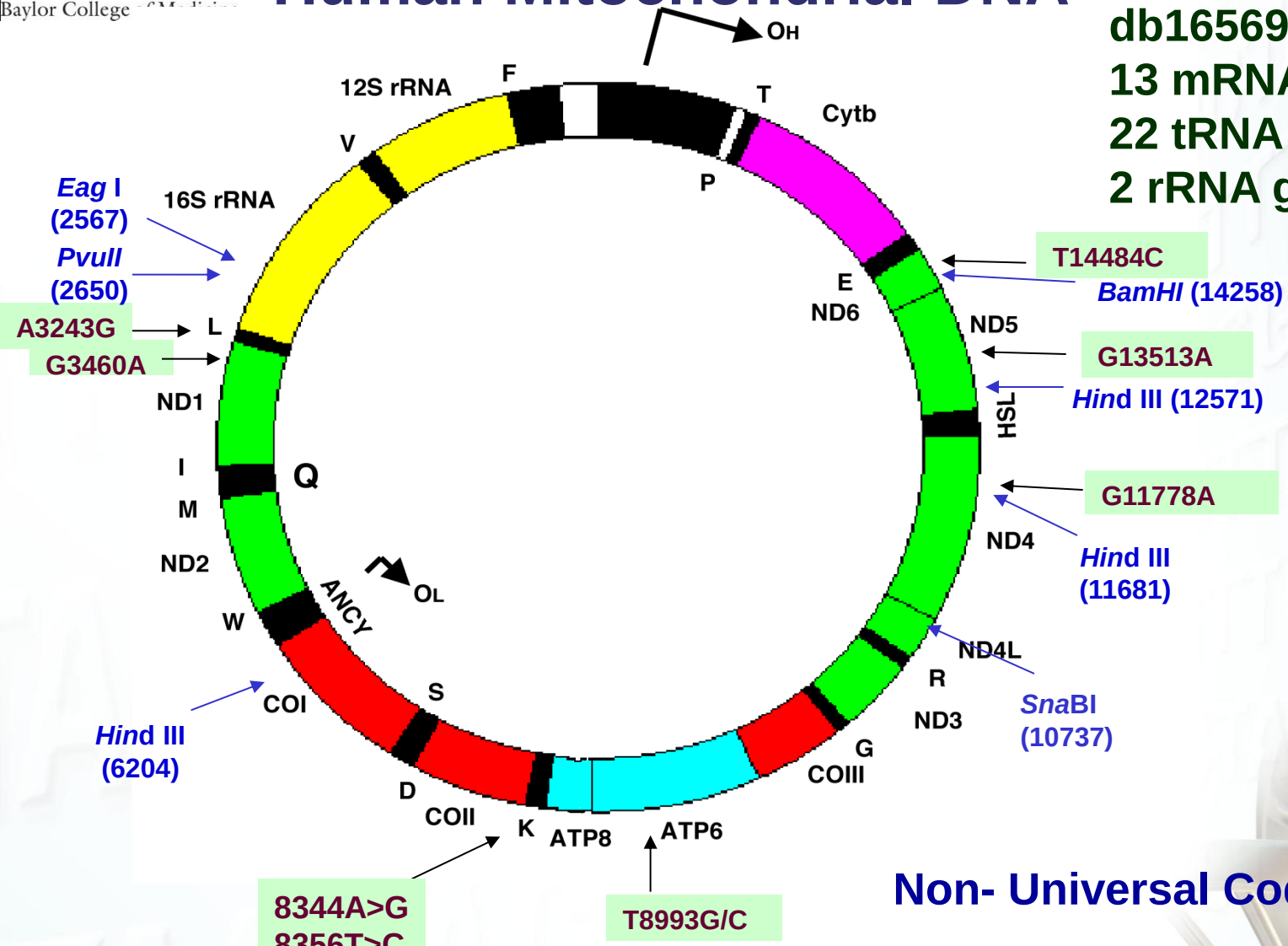
**Major clinical manifestation:**

**neuromuscular phenotype**

**CNS, Brain, skeletal muscle, heart, liver, etc.**

# Human Mitochondrial DNA

db16569 bp
13 mRNA genes
22 tRNA genes
2 rRNA genes



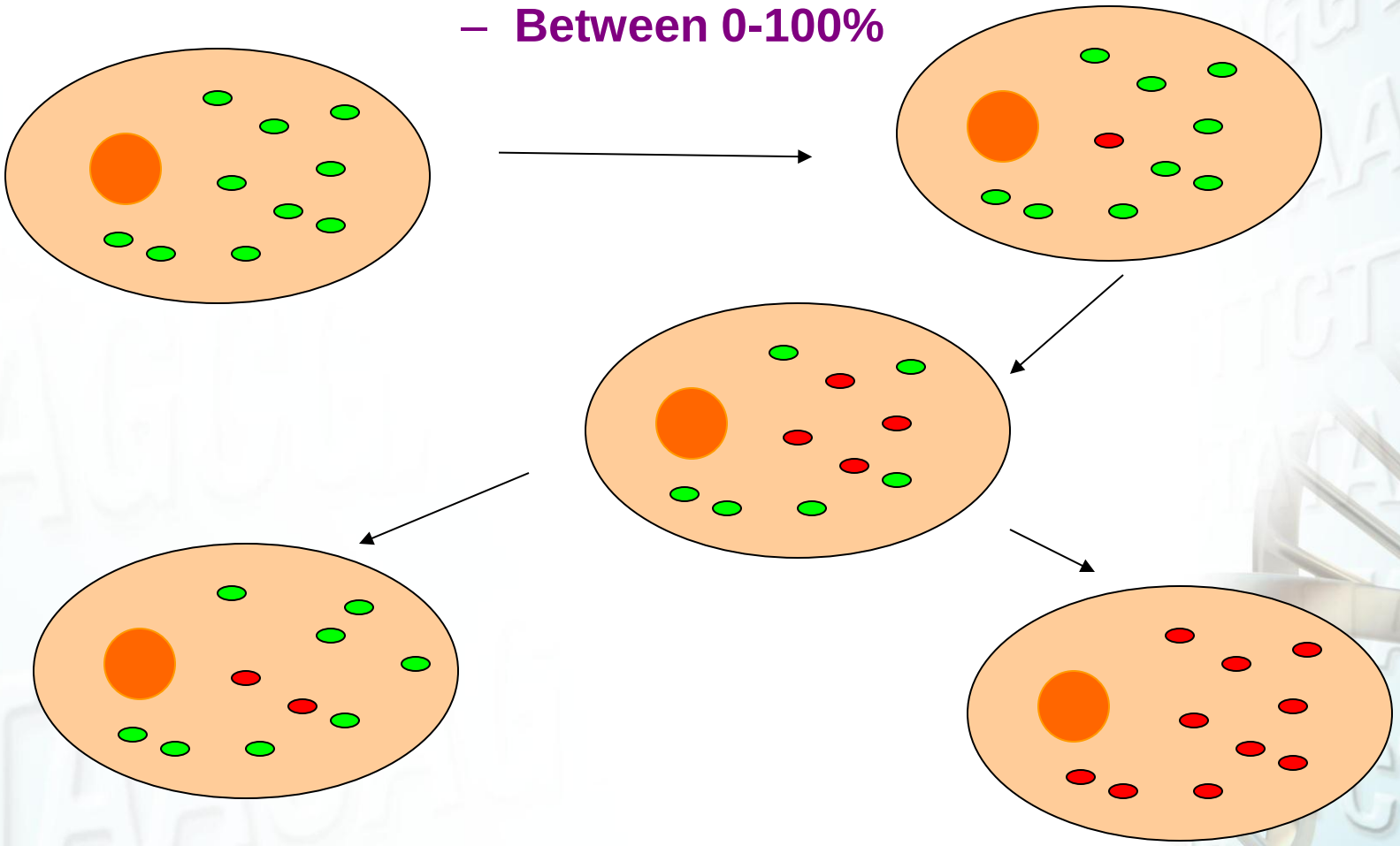

Non- Universal Codon

Light strand: 8 tRNA and 1 mRNA (ND6)
Heavy strand: 14 tRNA, 2 rRNA, and 12 mRNA
Polycistronic with posttranscriptional processing

Point mutations
Large deletions

# Homoplasmy and Heteroplasmy

- **Homoplasmy**
  - 0 or 100%
- **Heteroplasmy**
  - Between 0-100%



# Complex dual genome mitochondrial disorders:

- mtDNA biosynthesis+integrity maintenance
- Salvage synthesis of dNTP
- Complex assembly/Complex subunits
- Transcription/translation factors
- MRPLs/MRPSs (mito ribosomal proteins)
- Transcription and translation factors
- Mitochondrial aa-tRNA synthetases
- TIMMs and TOMMs, protein transporters
- dynamic fusion/fission proteins
- Apoptotic factors, protein kinases

**Majority of mitochondrial disorders are caused by Defects in nuclear genes**

**1500 nucear genes targeted to mitochondria**

**Currently about 200+ linked to known diseases**

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# Current Approaches: step-wise

1. Screen for mtDNA common point mutations: by PCR/ASO or other detection methods
2. mtDNA deletion: by Southern analysis
3. Quantification of heteroplasmic mtDNA point mutations: ARMS qPCR
4. Determination of mtDNA deletion and breakpoints: aCGH, PCR sequencing
5. Unknown mutations: sequence the whole mitochondrial genome by Sanger
6. mtDNA depletion: qPCR analysis for mtDNA copy number
7. Sequence relevant nuclear genes, one by one
8. aCGH to detect large deletions in nuclear genes

# Gold Standard Sanger Sequencing

***Pitfalls***

***1. Does not provide quantitative information***

***2. Sequence gene one by one***

***3. Does not detect deletions***

***4. Tedious and costly***

***5. Not comprehensive***

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# Mitochondrial Challenges

1. *The most clinically and genetically heterogeneous dual genome disorders*
2. *Primary defects in mitochondrial genome, common point mutations and large deletion*
3. *Quantification of mutation heteroplasmy*
4. *Majority (90%) of mitochondrial disorders are caused by one of ~1500 nuclear genes*
5. *Advances in technologies for diagnosis of complex disorders: array CGH and next generation sequencing approach*

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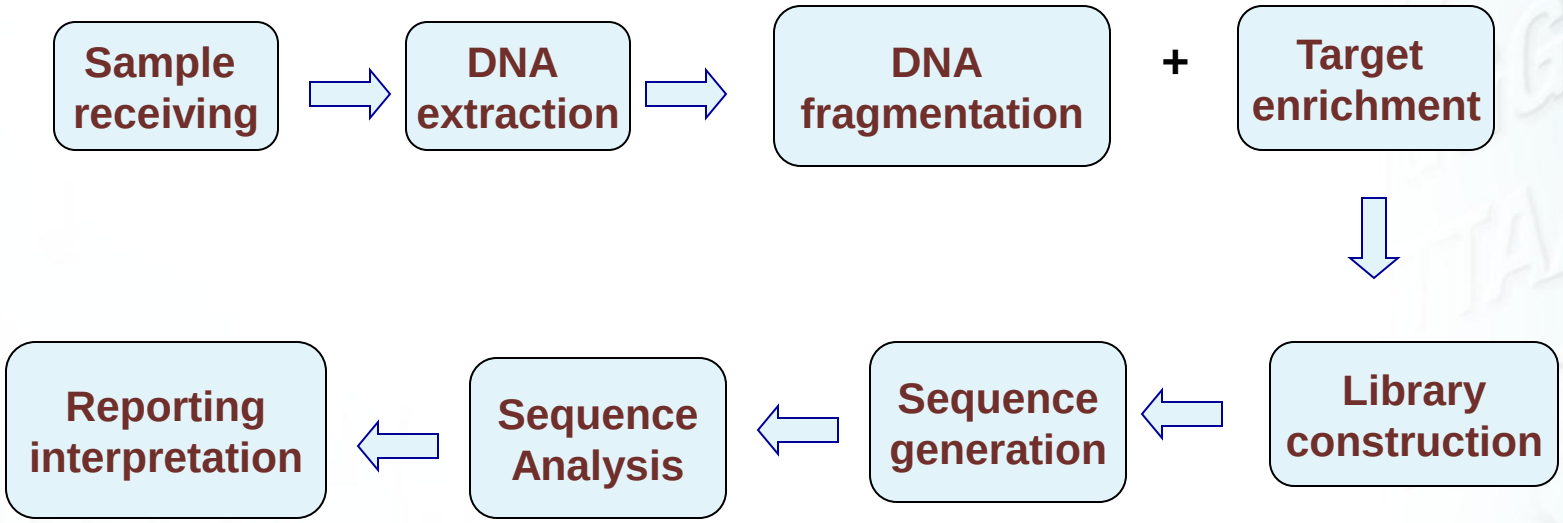
# Next Generation Massively Parallel Sequencing

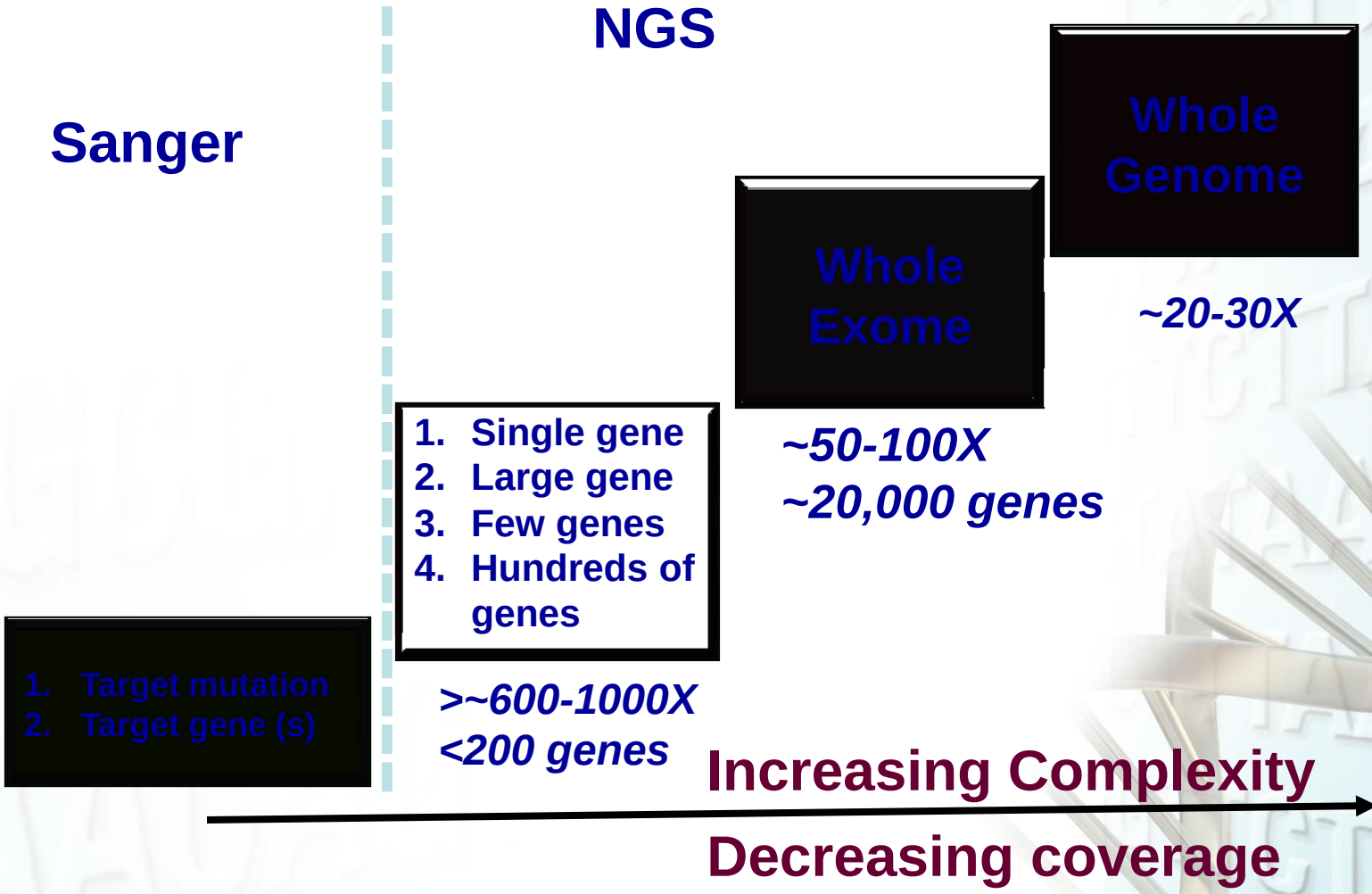
1. *Ability to sequence many genes in parallel*
2. *Identify new mutations in known genes*
3. *Discover new disease genes*
4. *Detect point mutations, small indels and large deletion/duplication (CNV)*
5. *Quantify mtDNA heteroplasmy, mosaicism*
6. *RNA sequencing, Gene expression*
7. *Quantitative DNA methylation analysis*

# NGS workflow

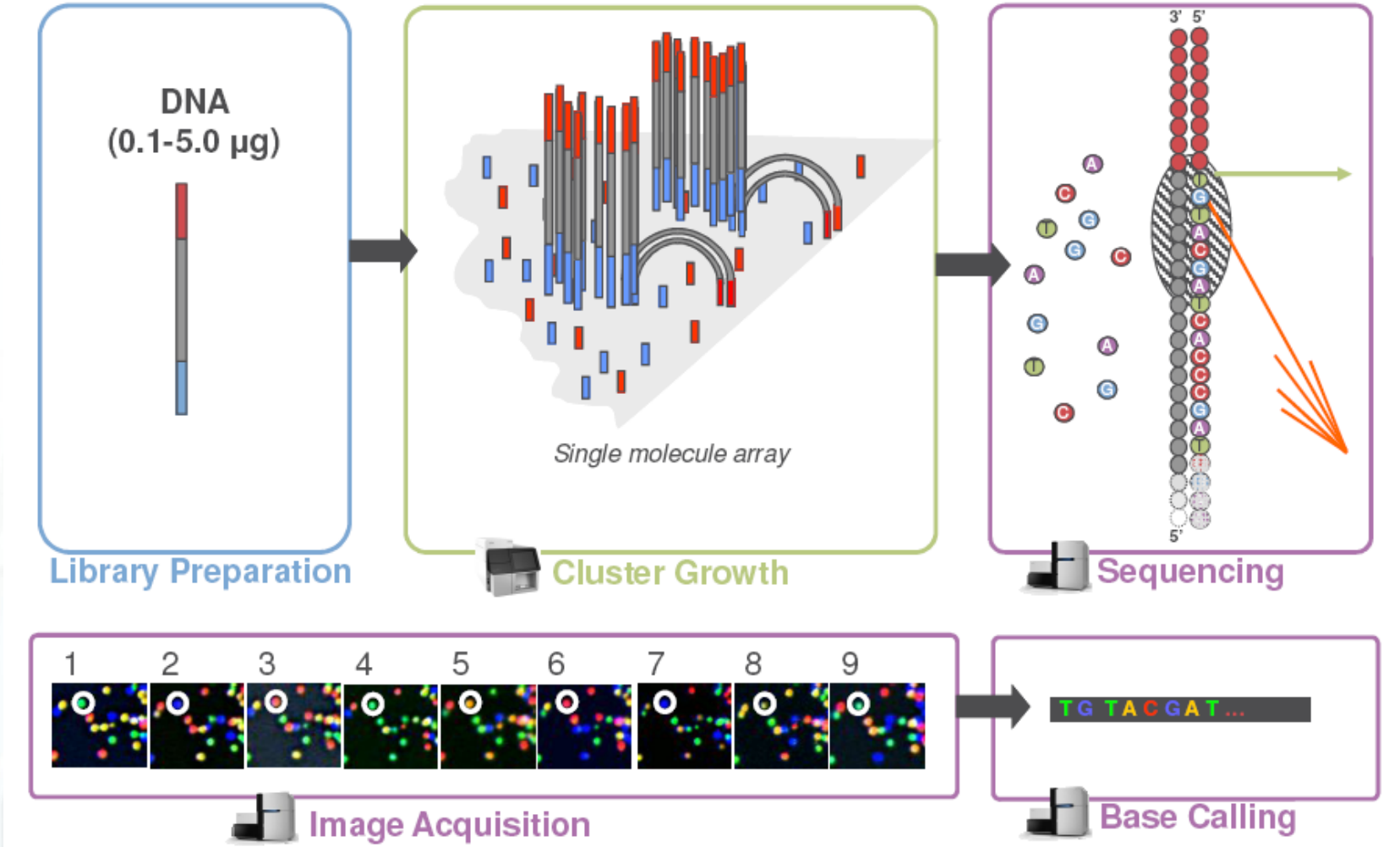



**Quality control procedures are required to assure that each step works properly and results are accurate for each patient's specimen analyzed**

# Bring NGS to Clinical Diagnosis



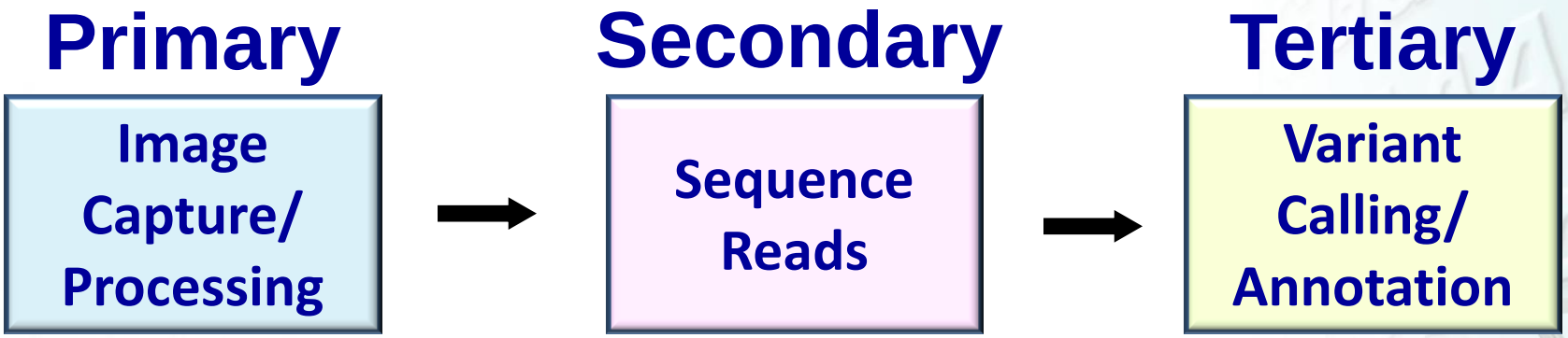
# Illumina Sequencing Technology Overview




Primary analysis

(Adapted from Illumina.inc.)

# sequence analysis: three steps
# With built-in QA/QC samples




Convert image to base calls
Base quality scores assigned

Filtering of reads
Based on quality
Alignment / Assembly

Results interpretation

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# *To bring NGS to clinical settings requires*

**1. Validation:**

**Cover all bases in all CDS + 20 bp in flanking introns all mutations/VUS confirmed by a second method**

**Phase I: specificity, sensitivity, reproducibility, accuracy, compared to Sanger**

**Phase II: detection of different mutation types using positive control samples**

**Phase III: Blinded Samples without molecular diagnosis to obtain diagnostic yield**

**2. Define experimental error, limit of detection, alignment and analytical steps**

**3. Variants interpretation and reporting**

# Nuclear Genes Capture Sequencing

1. Genes responsible for mtDNA Depletion and maintenance of integrity
2. GSD (liver and muscle forms)
3. Complex I-V panel, CoQ panel
4. Usher panel
5. PDH panels
6. Metabolic myopathy
7. RP (retinitis pigmentosa) panel 66 genes
8. Mitome200
9. Mitome500
10. Mitome1500
11. Exome
12. Whole Genome

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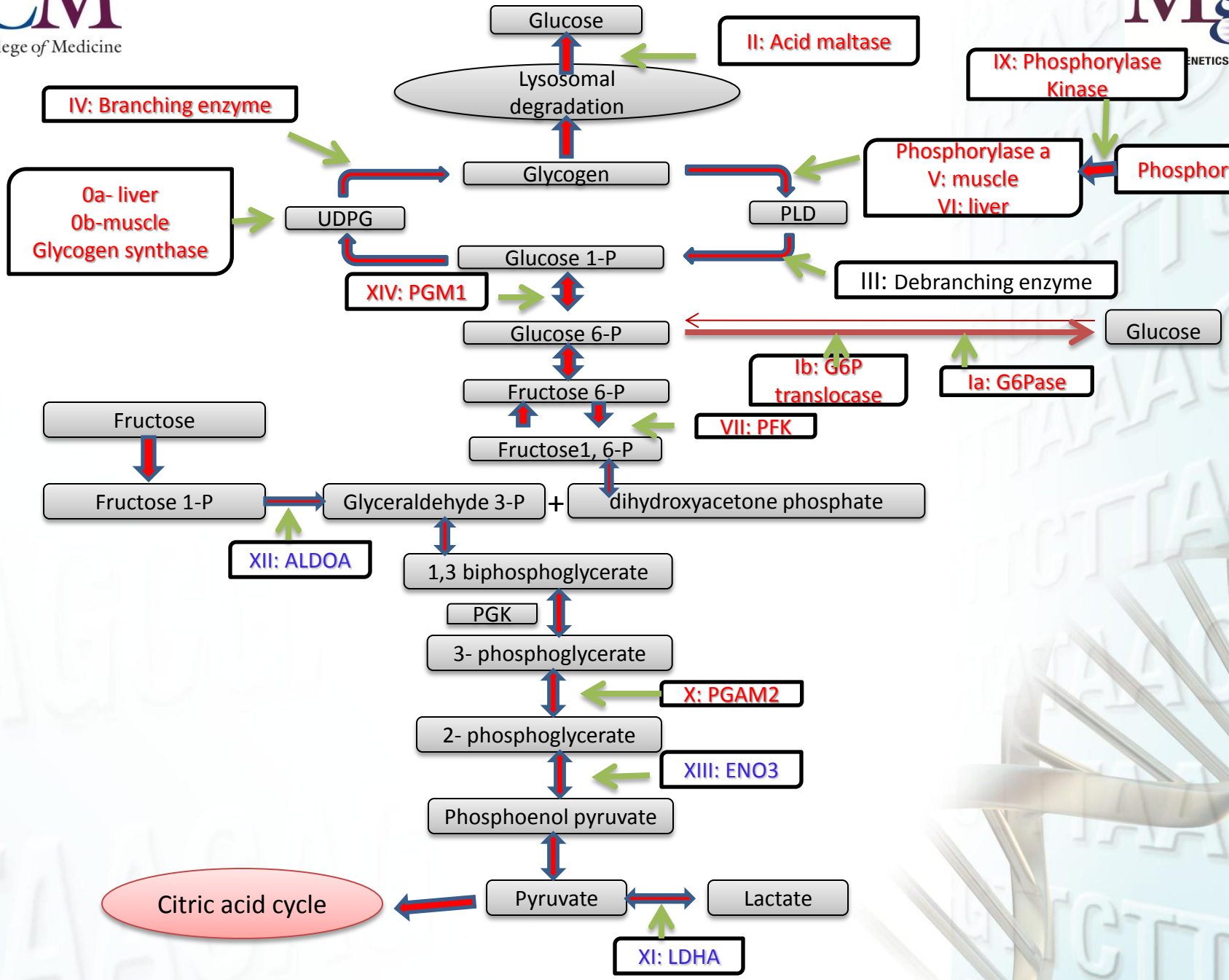
# Example of Nuclear Gene Capture Sequencing

1. Panel testing:
   NGS analysis of a group of genes involved in Glycogen Metabolism: synthesis and breakdown
   Glycogen Storage Disorders (GSD)

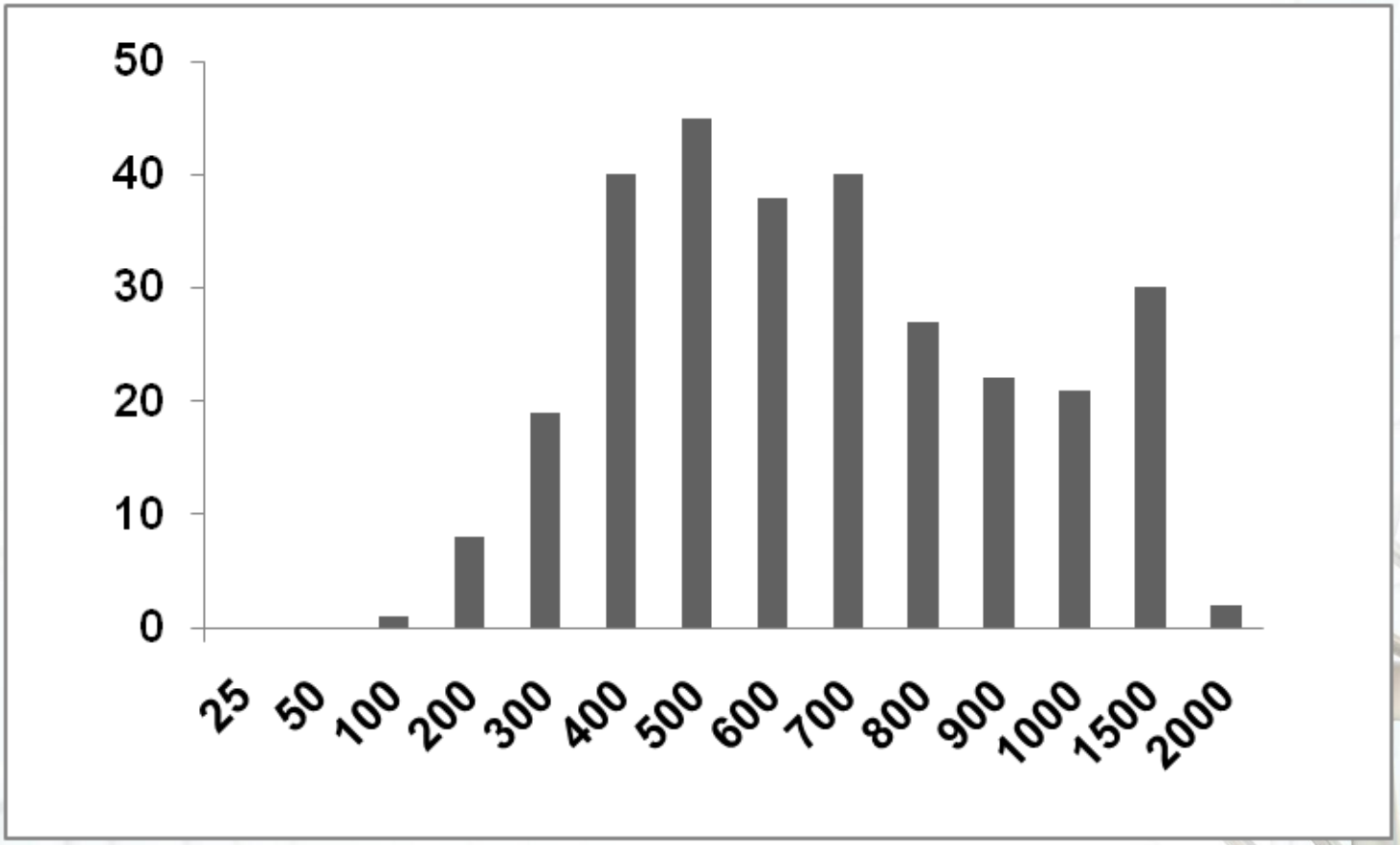

Glucose
II: Acid maltase
Lysosomal degradation
IX: Phosphorylase Kinase
IV: Branching enzyme
Glycogen
Phosphorylase a
V: muscle
VI: liver
Phosphorylase b
0a- liver
0b-muscle
Glycogen synthase
UDPG
PLD
Glucose 1-P
III: Debranching enzyme
XIV: PGM1
Glucose 6-P
Glucose
Ib: G6P translocase
Ia: G6Pase
Fructose 6-P
Fructose
VII: PFK
Fructose1, 6-P
Fructose 1-P
Glyceraldehyde 3-P
+
dihydroxyacetone phosphate
XII: ALDOA
1,3 biphosphoglycerate
PGK
3- phosphoglycerate
X: PGAM2
2- phosphoglycerate
XIII: ENO3
Phosphoenol pyruvate
Citric acid cycle
Pyruvate
Lactate
XI: LDHA

# Genes involved in Glycogen Metabolism

| GSD Types | Genes | Liver Panel | Muscle panel | NM# |
|---|---|---|---|---|
| GSD 0A | *GYS2* | √ | | NM_021957.3 |
| GSD 0B | *GYS1* | | √ | NM_002103.4 |
| GSD IA | *G6PC* | √ | | NM_000151.2 |
| GSD IB | *SLC37A4* | √ | | NM_001467.5 |
| GSD II | *GAA* | √ | √ | NM_000152.3 |
| GSD III | *AGL* | √ | √ | NM_000642.2 |
| GSD IV | *GBE1* | √ | | NM_000158.3 |
| GSD V | *PYGM* | | √ | NM_005609.2 |
| GSD VI | *PYGL* | √ | | NM_002863.4 |
| GSD VII | *PFKM* | | √ | NM_000289.5 |
| GSD IX A | *PHKA2* | √ | | NM_000292.2 |
| GSD IX B | *PHKB* | √ | √, mild | NM_000293.2 |
| GSD IX C | *PHKG2* | √ | | NM_000294.2 |
| GSD IX D | *PHKA1* | | √ | NM_002637.3 |
| GSD X | *PGAM2* | | √ | NM_000290.3 |
| GSDXIV | *PGM1* | | √ | NM_002633.2 |

| NGS Panel name | GSD-16gene-panel |
|---|---|
| Genes included | *AGL, G6PC, GAA, GBE1, GYS1, GYS2, PFKM, PGAM2, PGM1, PHKA1, PHKA2, PHKB, PHKG2, PYGL, PYGM, SLC37A4* (16 genes) |
| Number of CDS | 294 |
| Target size | 50,062 bp (CDS ± 20 bp) |
| Enrichment | In solution capture library |
| Sequencing info | Illumina HiSeq 2000, 75 cycle, single-end |

# Minimal coverage per base of Exons



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# Phase I Validation: known samples

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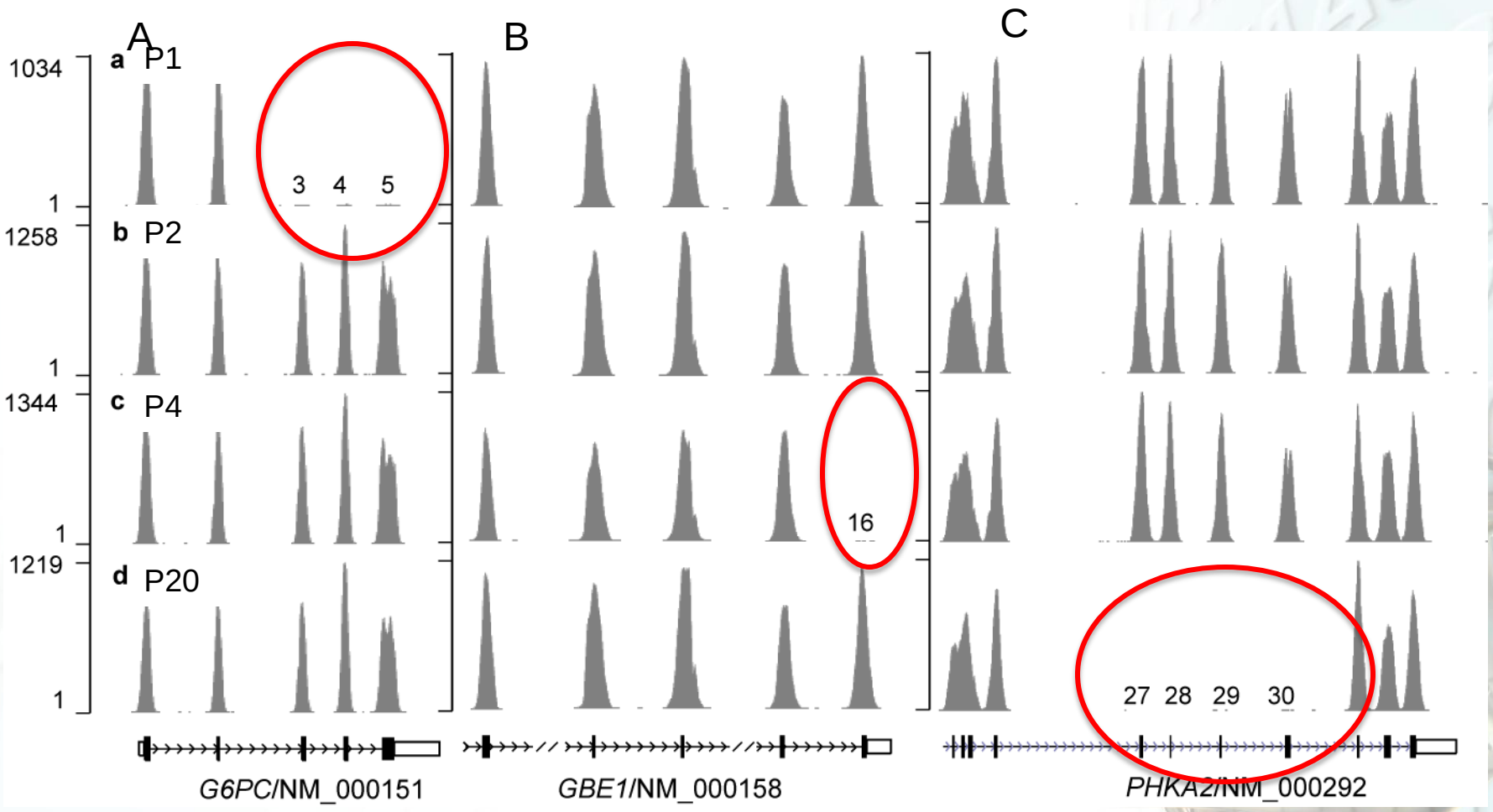
| Sample ID | Mean coverage | Total reads Per 100 bp | **Min coverage** | **# of CDS < 20X** | **Multiplexing factor** |
|---|---|---|---|---|---|
| 547 | 722±233 | 988±317 | 68 | 0 | 8 |
| 755 | 837±195 | 1146±266 | 77 | 0 | 8 |
| 700 | 783±187 | 1072±255 | 93 | 0 | 8 |
| 833 | 803±189 | 1099±258 | 74 | 0 | 8 |
| 264 | 686±225 | 939±306 | 47 | 0 | 8 |
| 041 | 841±220 | 1151±300 | 51 | 0 | 8 |
| 203 | 747±238 | 1021±324 | 92 | 0 | 8 |
| 941 | 674±220 | 921±299 | 1/92 | 3 | 8 |
| 545 | 731±230 | 1004±313 | 77 | 0 | 8 |
| 206 | 727±243 | 998±330 | 1/77 | 1 | 8 |
| 067 | 706±228 | 971±311 | 93 | 0 | 8 |
| 504 | 623±177 | 856±244 | 59 | 0 | 8 |
| 531 | 850±345 | 1169±418 | 69 | 0 | 8 |
| 255 | 878±267 | 1028±355 | 93 | 0 | 8 |


A
B
C
a P1
b P2
c P4
d P20
1034
1
1258
1
1344
1
1219
1
3 4 5
16
27 28 29 30
G6PC/NM_000151
GBE1/NM_000158
PHKA2/NM_000292

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# GSD1A negative

| Patients | Age | Gender | Clinical Indication |
|---|---|---|---|
| 24547 P8 | 4m | M | hypoglycemia, hepatomegaly |
| 28755 P9 | 13yr | F | fat,encephalopathy, abnormal liver function |
| 30700 P10 | 1.5yr | F | hyperlipidemia, hyperlactatemia,ftt, hepatomegaly |
| 31833 P11 | 10m | F | hypoglycemia, hepatomegaly |
| 34264 P12 | 3yr | M | hypoglycemia, hyperuricemia, reccurent infections, bone fractures |
| 36041 P13 | 3m | F | pulmonary hypertension, large liver, elevated lipids/uric acid/lactate |
| 37203 P14 | 2yr | M | Hepatomegaly |

# NGS results summary

| Patient | Gene | CDS | exons | mutations |
|---|---|---|---|---|
| 30700 P10 | SLC37A4 | 5 | 6 | c.817G>A (p.G273S) |
| | | 7 | 8 | c.1042_1043delCT (p.L348Vfs*53) |
| 31833 P11 | SLC37A4 | 5 | 6 | c.785-3_786del5 |
| | | 5 | 6 | c.785-3_786del5 |
| 37203 P14 | AGL | 2 | 4 | c.256C>T (p.Q86X) |
| | | 20 | 22 | c.2723T>G (p.L908R) |

c.817G>A (p.G273S): conserved from C. elegans to human, predicted to be deleterious.
c.2723T>G (p.L908R): conserved from yeast to human, predicted to be deleterious.

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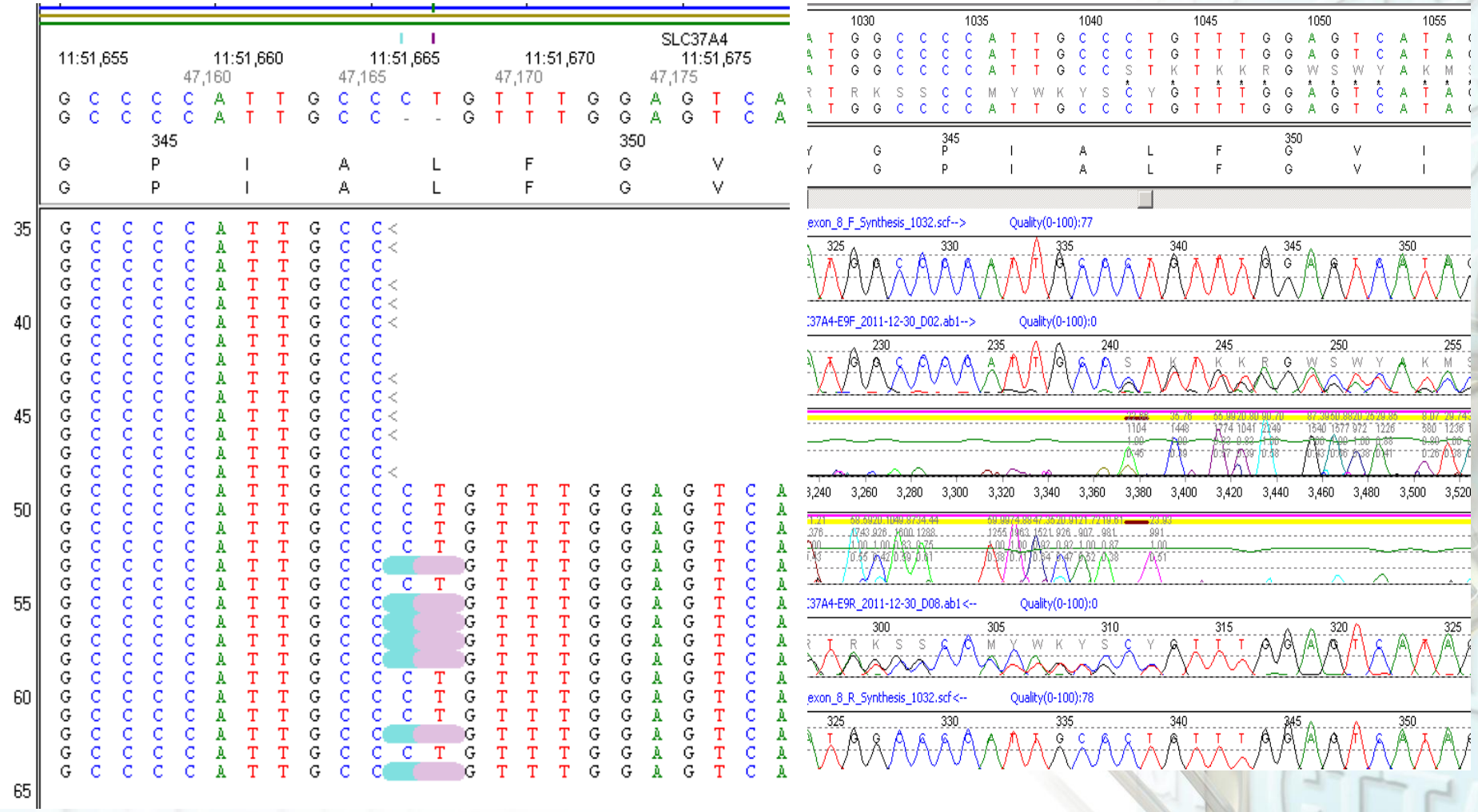
# P10 SLC37A4
# c.1042_1043delCT het



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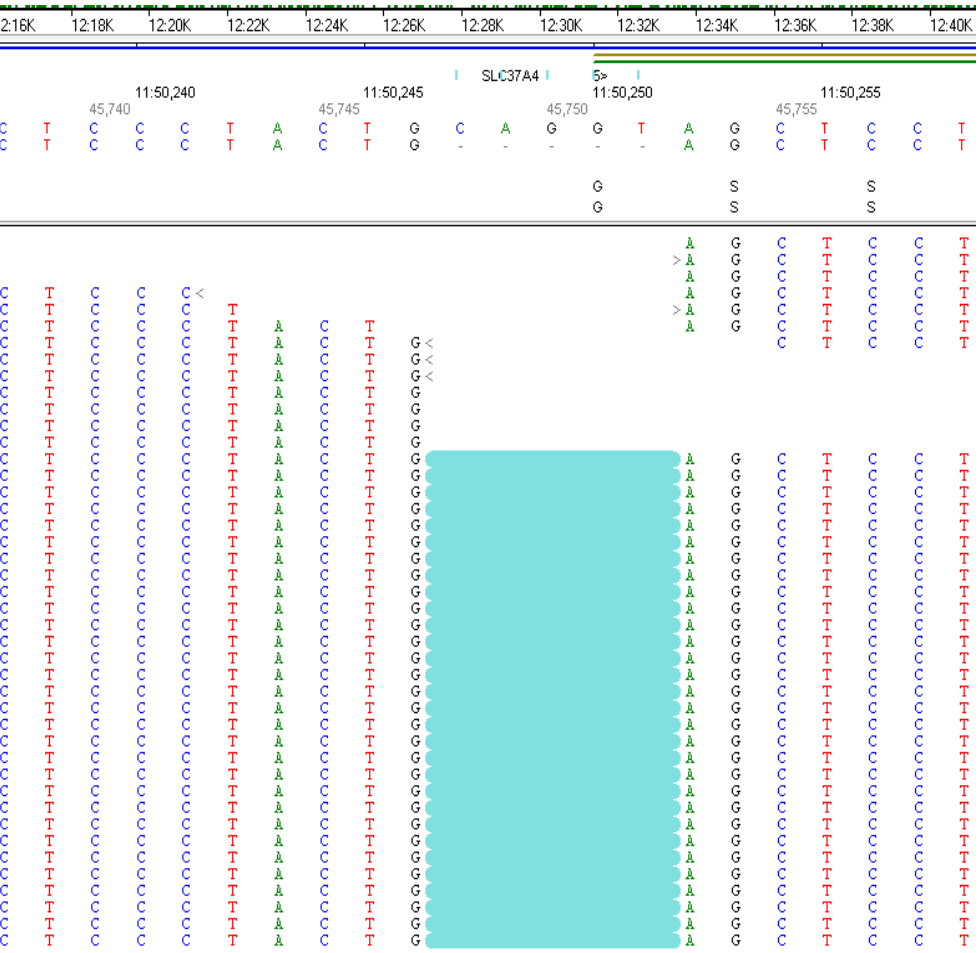
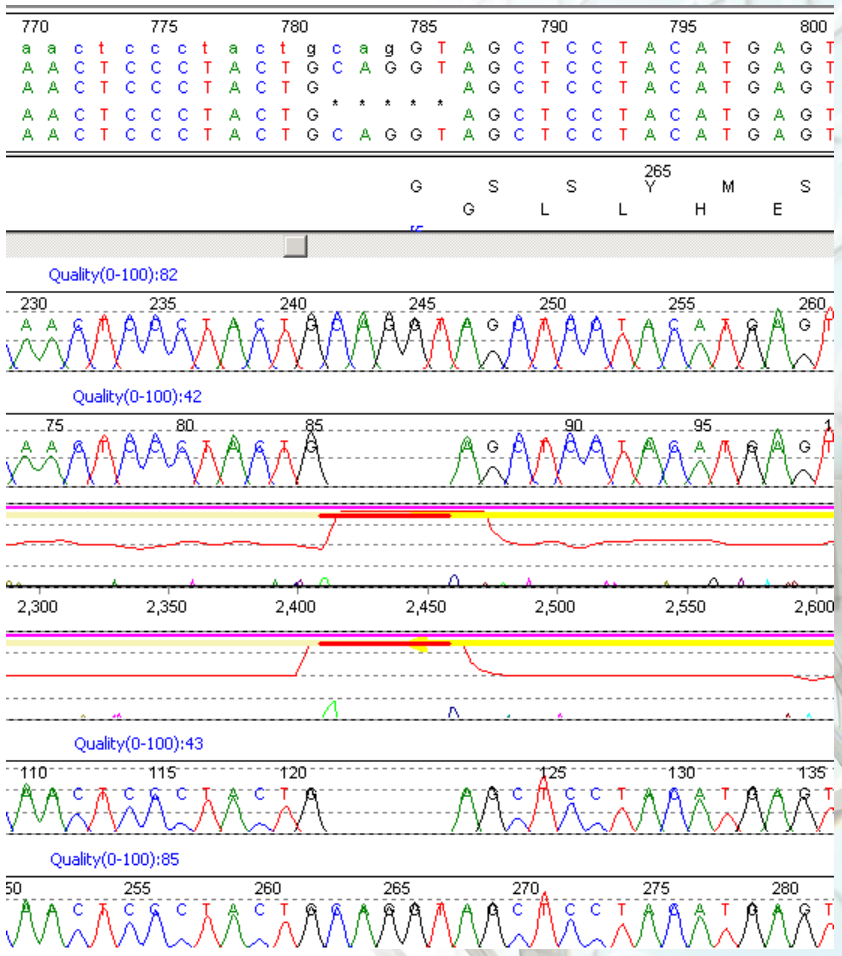
# P11: SLC37A4
# c.785-3_786del5 homozygous




GSD by panel NGS diagnostic yield: >65%

# Usher syndrome

**Hearing loss and retinitis pigmentosa**
**USH1, USH2, USH3**
**9 huge genes**
**Clinical overlap**

**By NGS: diagnostic yield is >83%**
**10/12 found 2 deleterious mutant alleles**
**2/12: one heterozygous allele**

# Usher syndrome

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **NGS Panel name** | ***Usher panel-2195*** | | | | | | | |
| **Genes included** | **9 nuclear genes: CDH23, CLRN1, DFNB31, GPR98, MYO7A, PCDH15, USH1C, USH1G, USH2A** | | | | | | | |
| **Number of CDS** | **362CDS** | | | | | | | |
| **Target size** | **81,170 bp (CDS ± 20 bp)** | | | | | | | |
| **Enrichment** | **In solution capture library** | | | | | | | |
| **Sequencing info** | **Illumina HiSeq 2000, 75 cycle, single-end** | | | | | | | |
| **Sample ID#** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| Mean coverage / base | 749± 235 | 1564± 499 | 902± 297 | 1636± 539 | 1623 ± 505 | 1433± 515 | 1345± 425 | 1627± 536 |
| Number of CDS < 20X | 4 | 3 | 4 | 4 | 3 | 4 | 3 | 3 |

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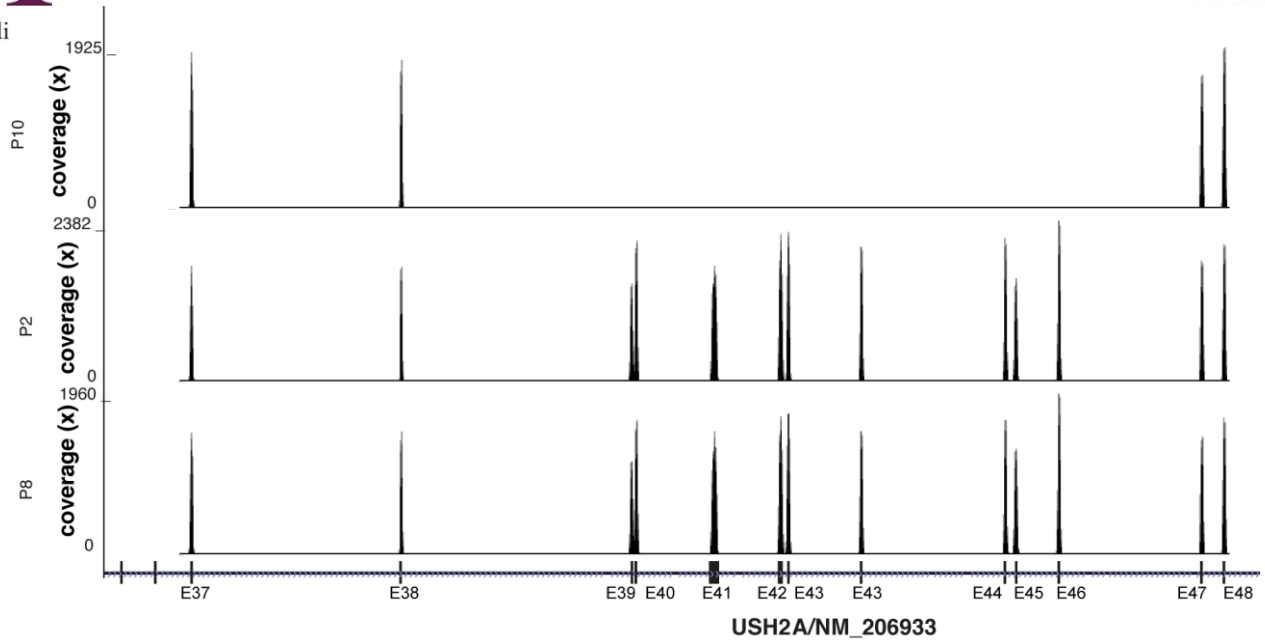
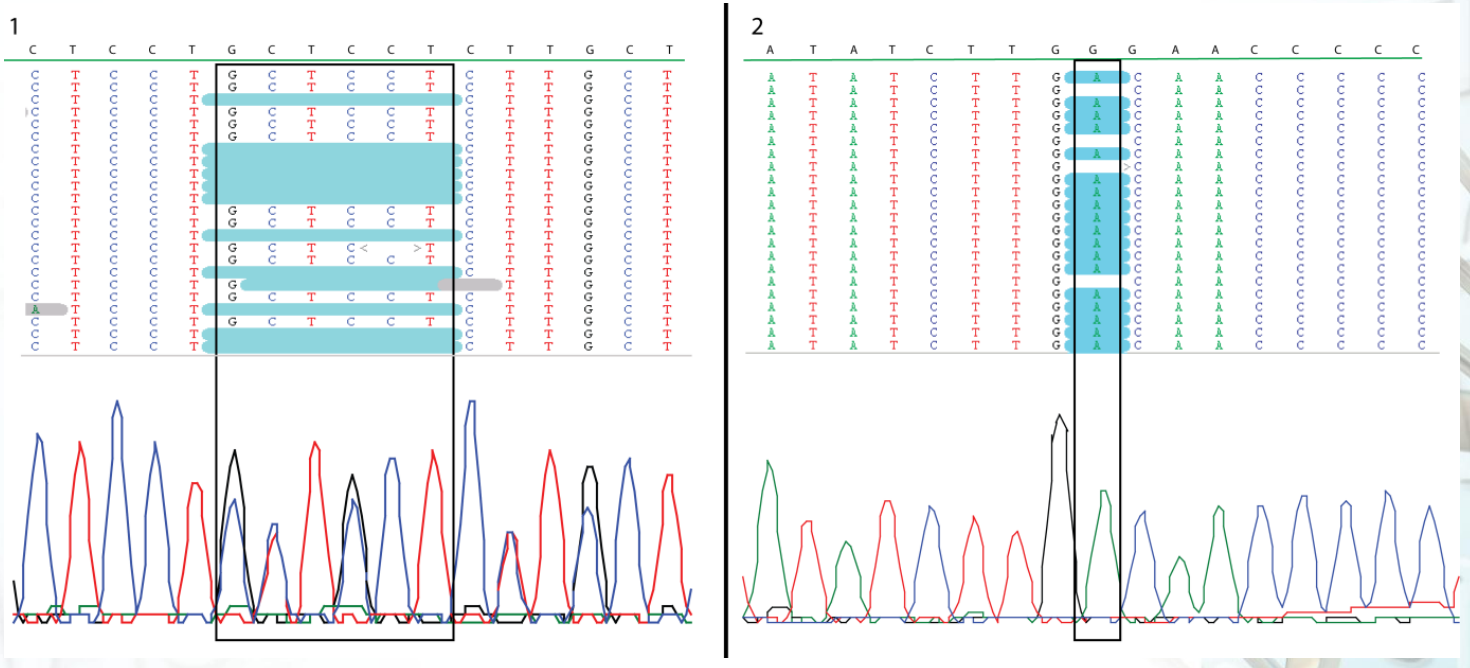
a




b



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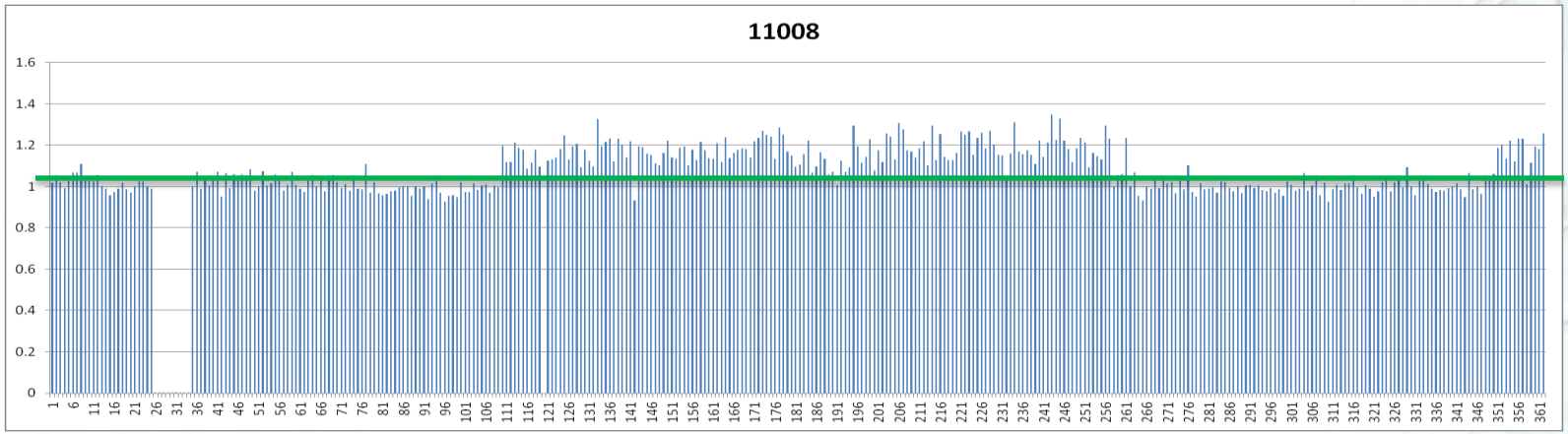
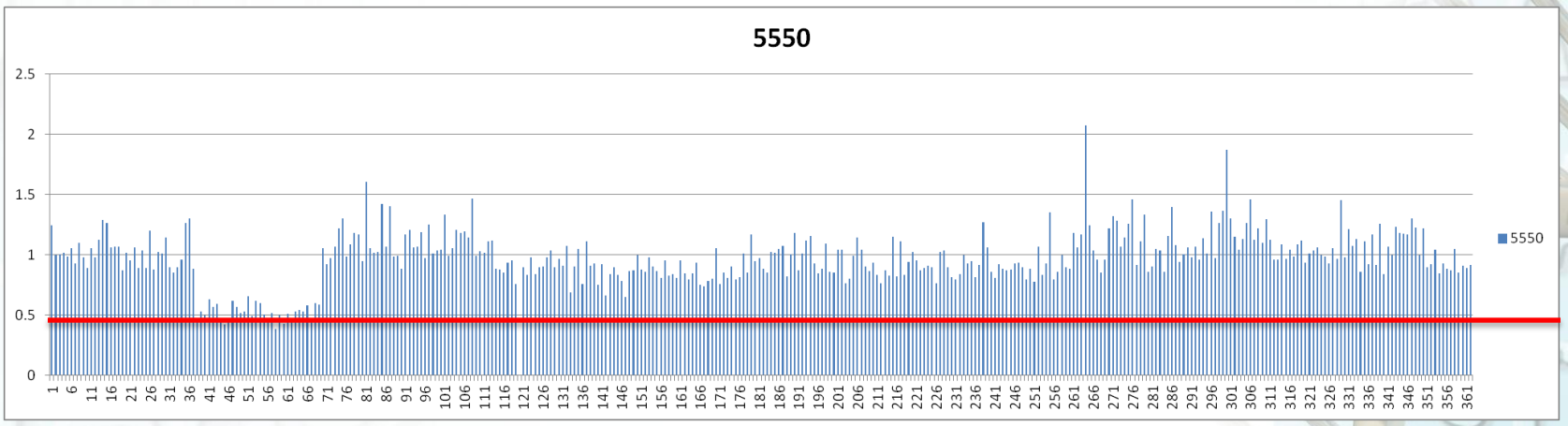
# NGS detects large deletions (CNV) Usher panel (previously not identified)

## USH2A: CDS38-46 homozygous del




## USH2A: CDS3-34 heterozygous deletion.



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# Abnormal Bone Mass related disease

## High Bone Mass Panel (14 genes)

*ANKH, CA2, CLCN7, CTSK, FAM123B, FAM20C, LEMD3, OSTM1, SOST, TCIRG1, TGFB1, TNFRSF11A, TNFSF11, TYROBP*

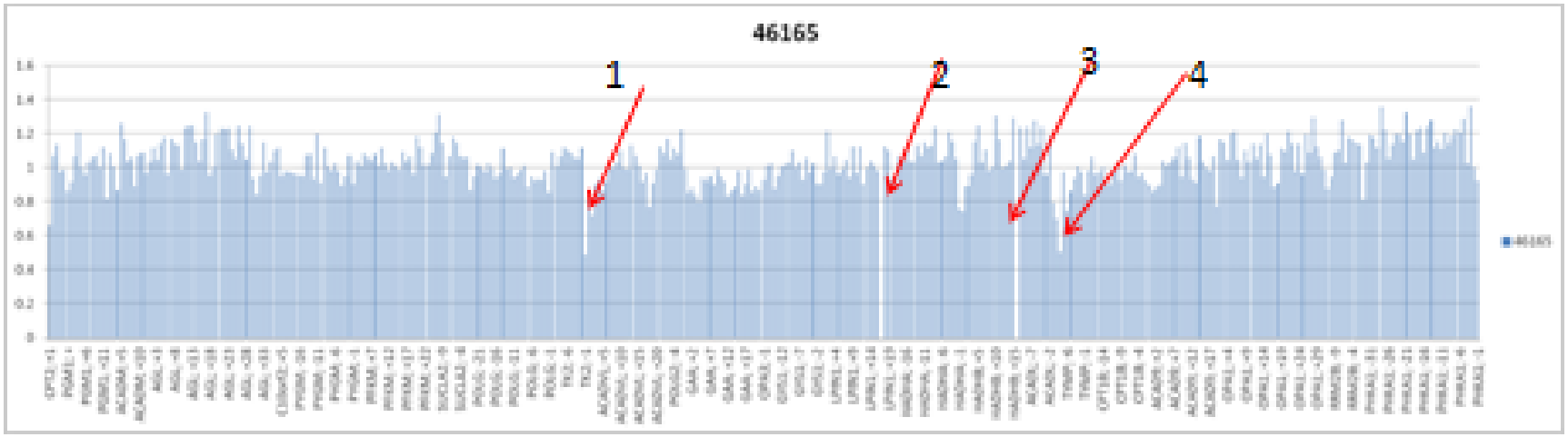
## Low Bone Mass Panel (21 genes)

*ALPL, B4GALT7, COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, CRTAP, FBN1, FKBP10, LEPRE1, PLOD2, PLOD3, PPIB, SERPINF1, SLC34A1, SLC39A13, SLC9A3R1, SP7, TNFRSF11A, TNFRSF11B*

# Abnormal Bone Mass related disease

| | |
|---|---|
| **NGS Panel name** | ***Skeletal panel*** |
| **Genes included** | **34nuclear genes including highbonemass-panel +lowbonemass-panel** |
| **Number of CDS** | **602 CDS** |
| **Target size** | **98962bp (CDS ± 20 bp)** |
| **Enrichment** | **In solution capture library** |
| **Sequencing info** | **Illumina HiSeq 2000, 75 cycle, single-end** |

| | Mean coverage per base | # of Exons with one base covered <20X (Exon) | |
|---|---|---|---|
| **Panel** | **663x** | **20** | **3.3%** |
| **Reduced coverage** | **165x** | **134** | **22.1%** |




1:TK2, E1: GC-rich. Capture not consistent between samples

2: LPIN1: E18 homozygous deletion

3: ACADL:E1: many probes but consistently among samples never been captured and sequenced to sufficient depth

4: TYMP, CDS7-9 (E8-E10): low coverage

# NGS Panel testing)

| Tests | number of genes | # cds | target size (bp) | cds needs PCR/Seq |
|---|---|---|---|---|
| GSD | 16 | 294 | 50,062 | 0 |
| Usher Synd | 9 | 363 | 81,171 | 4 |
| Bone-High Mass | 14 | 129 | 27,318 | 13 |
| Bone-Low Mass | 20 | 432 | 67,419 | 6 |
| Myopathy/ rhabdomyolysis | 26 | 401 | 70,178 | 4 |
| RP | 66 | 939 | 202,800 | 16 |
| mtDNA Depletion | 14 | 145 | 26,537 | 4 |
| Mitome200 | 162 | 1,788 | 307,144 | 31 |

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# Mitochondrial Disease: a Complex Dual Genome Disorders

## Genetically and Clinically Heterogeneous

## Mitochondrial Genome: 16.6 kb

- Point mutations: common, novel
- % mutant loads: heteroplasmy
- large mtDNA deletions
- copy number changes: mtDNA depletion

## Nuclear Genes: ~ 1,300 genes

- Most commonly autosomal recessive
- Severe, present at early age of life
- Point mutations and large deletions
- Autosomal dominant, X-linked

# Mitochondrial Disease: a Complex Dual Genome Disorders

## Genetically and Clinically Heterogeneous

## Nuclear Genes: ~ 1,500 genes

- specific panels:
  - depletion
  - complex subunits and assembly genes
  - aa tRNA synthetases
- Mitome200
- Mitome 500
- Mitome1500
- Exome
- Whole genome

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# Example

- 11 month old girl
- Presented with hepatomegaly and hypoglycemia
- Previous tests revealed:
  - mtDNA depletion in liver: 9% of control
  - Whole mitochondrial genome sequencing is unremarkable

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# *NGS detects homozygous deletions: MPV17* (Exons 3-7)

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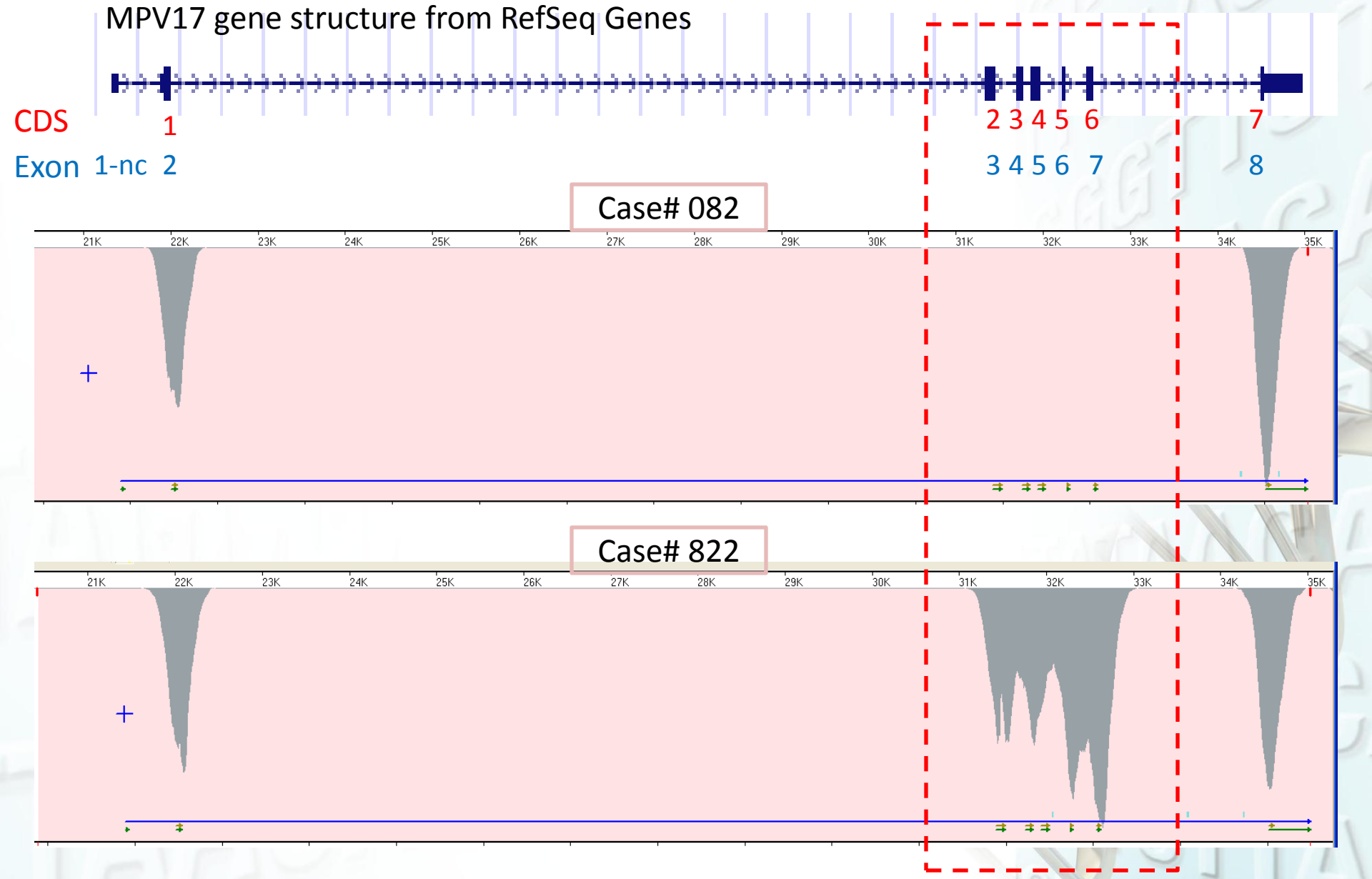

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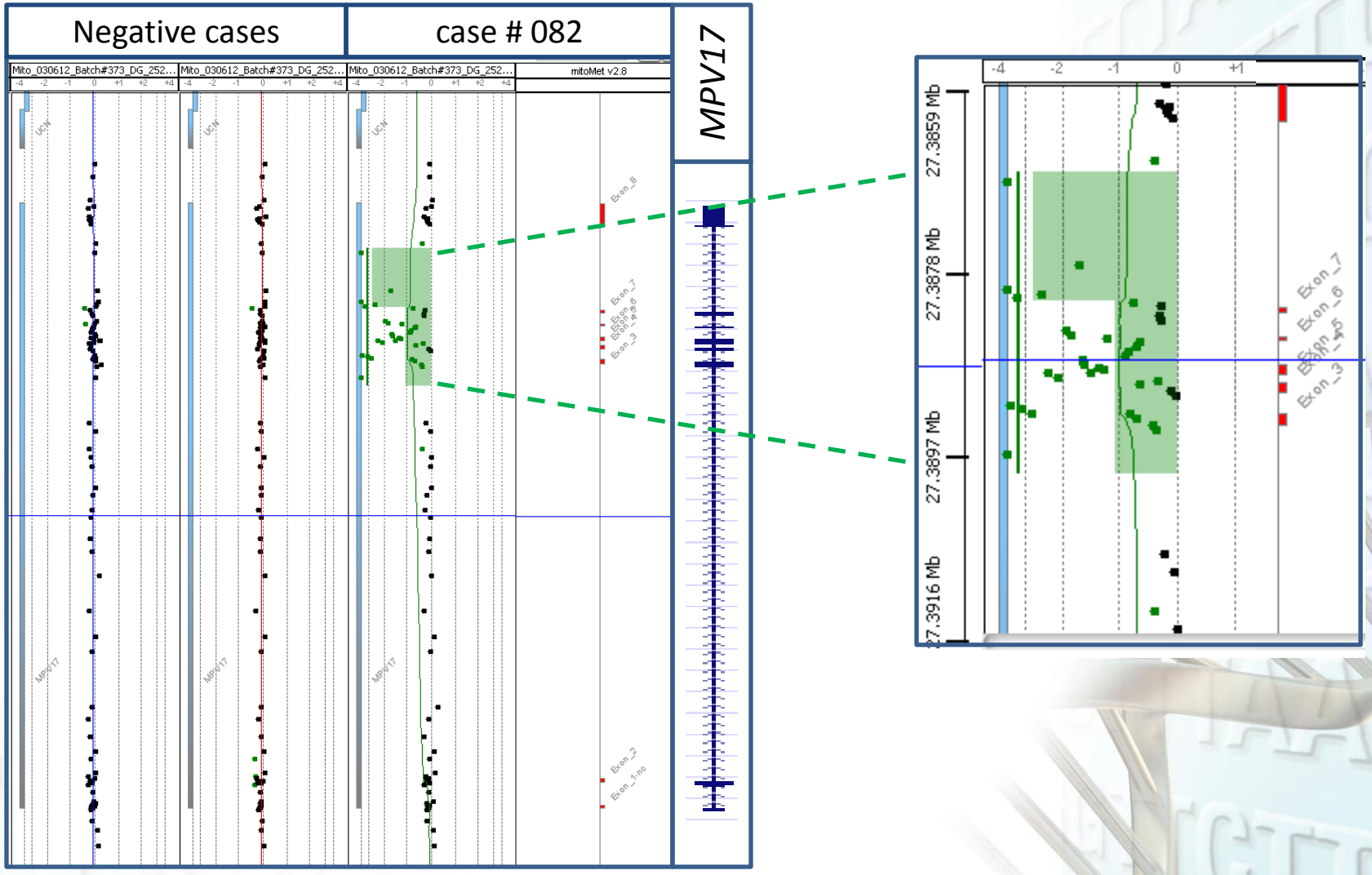
# Deletions in *MPV17* are confirmed by arrayCGH

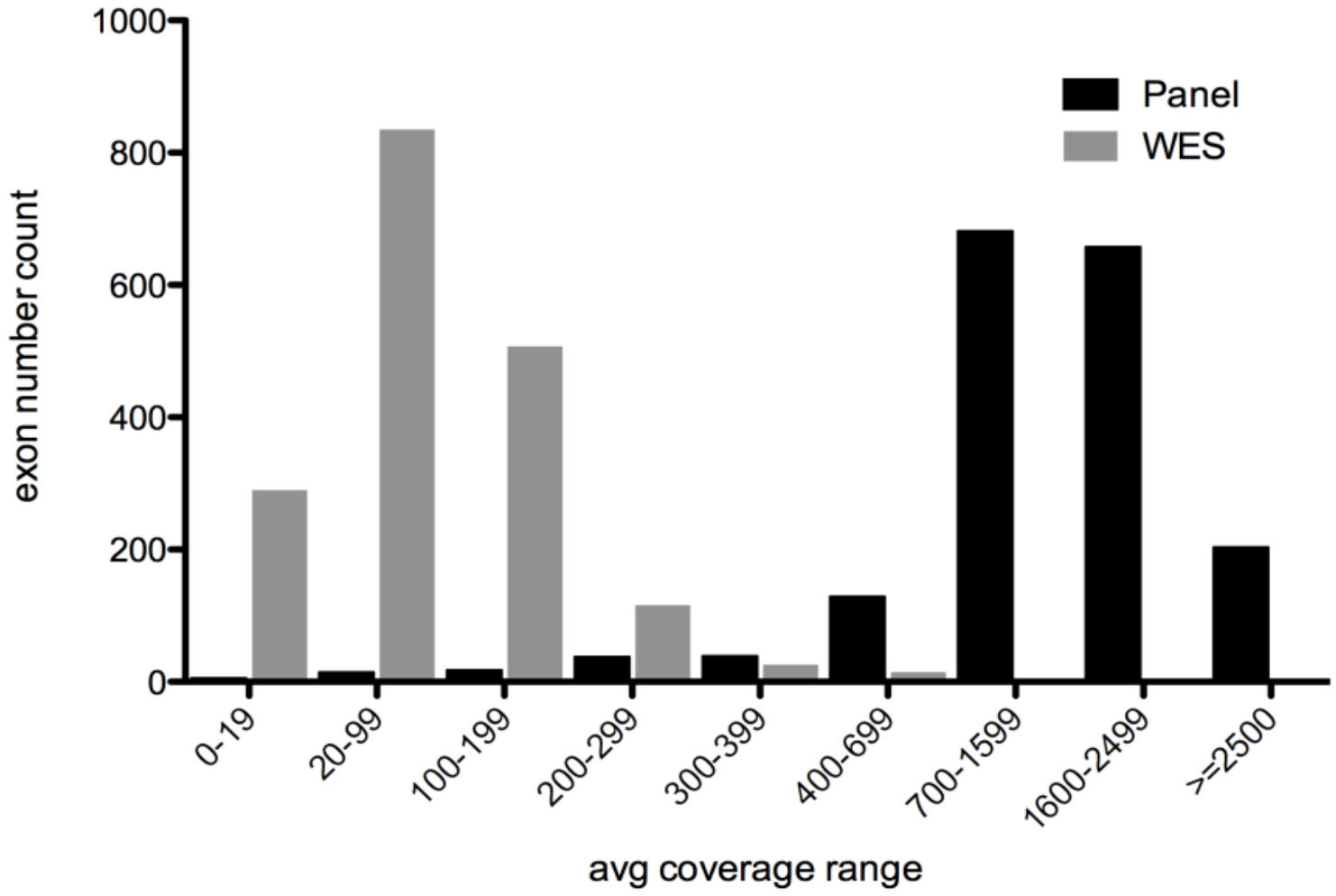


# Mitome200

| NGS Panel name | Mitome200 |
|---|---|
| Genes included | *162 nuclear genes related to mito diseases* |
| Number of CDS | 1,789 |
| Target size | 308,281 bp (CDS ± 20 bp) |
| Enrichment | In solution capture library |
| Sequencing info | Illumina HiSeq 2000, 75 cycle, single-end |

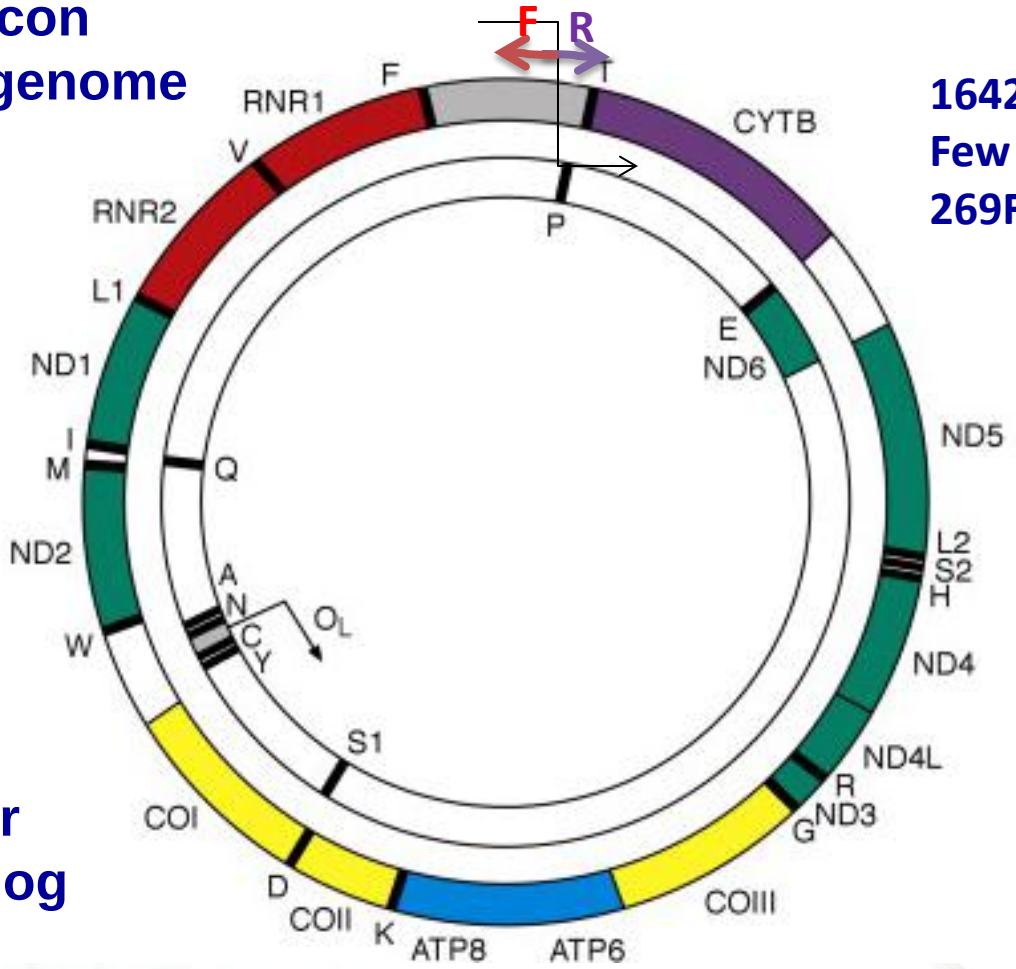
| | Mean coverage per base | # of Exons with one base covered <20X (Exon) | | # of bases covered <20X (Base) | |
|---|---|---|---|---|---|
| Panel | 1569x | 14 | 0.78% | 1,107 | 0.36% |
| Low coverage | 92x | 499 | 27.89% | 61,626 | 19.99% |

# Mitome200 vs low coverage exomes



# Next Generation deep seq
# 1 pair of primers to avoid NUMT

**Long Range PCR**
**Single amplicon**
**Whole mito genome**




**16426F/16425R**
**Few more backup sets**
**269F/268R**

**NUMT: nuclear mtDNA homolog**

# Sensitivity and specificity

| | NGS | | | | Sanger | NGS | |
|---|---|---|---|---|---|---|---|
| ID# | TP | FN | TN | FP | Positives | Sensitivity (%) | Specificity (%) |
| 309 | 15 (1het) | 0 | 16,554 | 0 | 14 | 100 | 100 |
| 286 | 15 | 0 | 16,554 | 0 | 15 | 100 | 100 |
| 964 | 41 | 0 | 16,528 | 0 | 41 | 100 | 100 |
| 614 | 45 | 0 | 16,524 | 0 | 45 | 100 | 100 |
| 798 | 37 | 0 | 16,531 | 0 | 37 | 100 | 100 |
| 914 | 16 (1het) | 0 | 16,553 | 0 | 16 | 100 | 100 |
| 085 | 38 | 0 | 16,531 | 0 | 38 | 100 | 100 |
| 799 | 32 | 0 | 16,537 | 0 | 32 | 100 | 100 |
| 926 | 37 | 0 | 16,532 | 0 | 37 | 100 | 100 |
| 563 | 46 | 0 | 16,523 | 0 | 46 | 100 | 100 |
| 889 | 23 (1het) | 0 | 16,546 | 0 | 22 | 100 | 100 |
| 820 | 40 | 0 | 16,529 | 0 | 40 | 100 | 100 |
| **Sum** | **385** | **0** | **198,442** | **1** | **383** | **100%** | **100%** |

>500 samples analyzed by MPS so far

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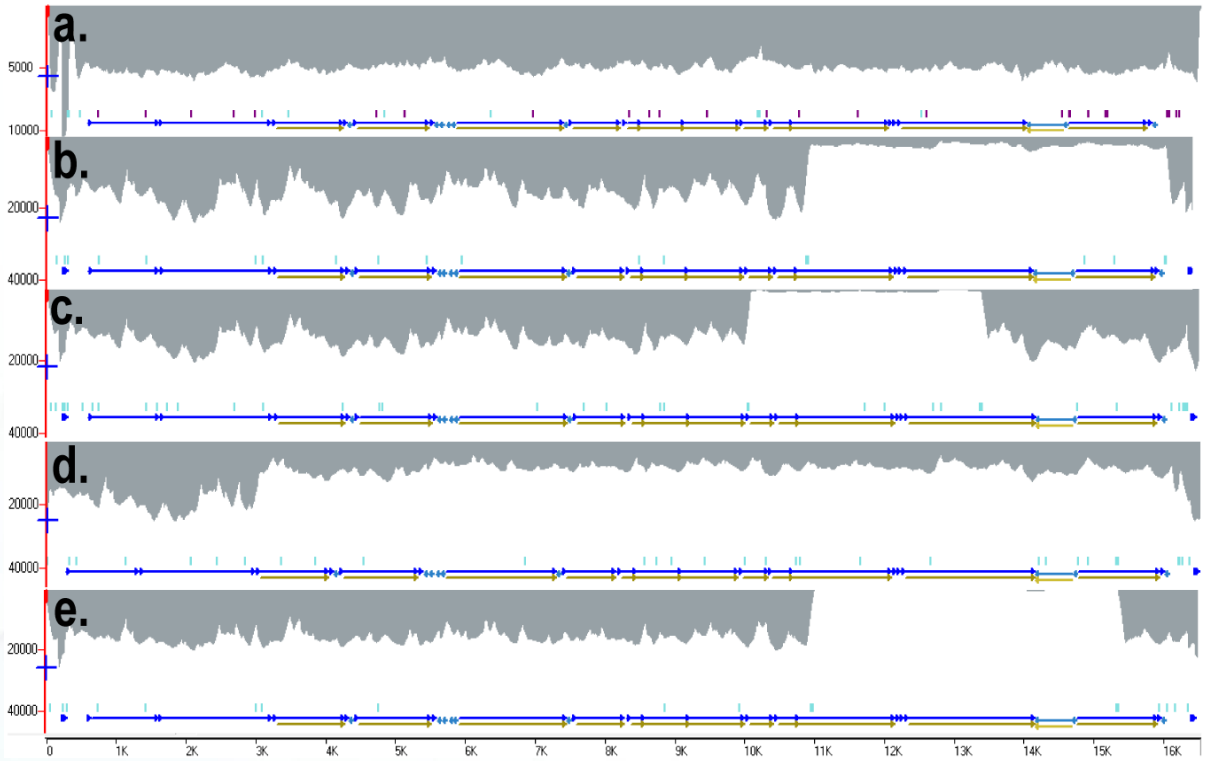
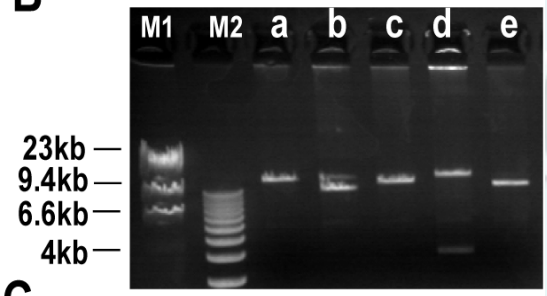
# Detection of mtDNA deletions by whole mtDNA amplification followed by NGS

A




B




C

| Sample ID | Size | Level |
|---|---|---|
| a | Normal | 0% |
| b | 5,182 bp | 82% |
| c | 3,361 bp | 90% |
| d | 13,031 bp | 58% |
| e | 4,420 bp | 96% |

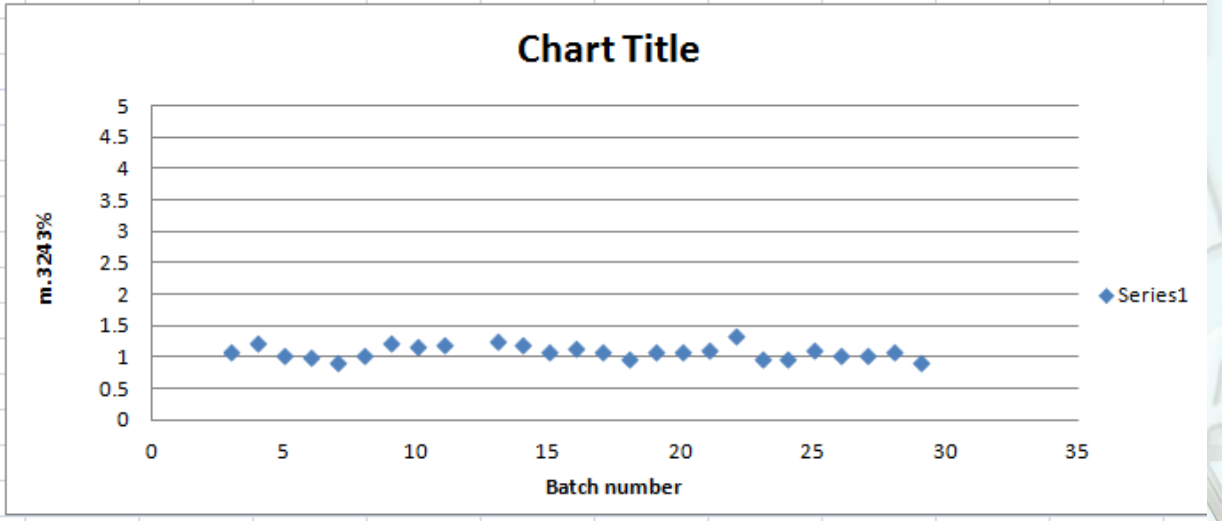
# Deletions are confirmed by MitoMet array CGH and PCR/sequencing

# Quantification of heteroplasmy

| ID# | Position | Base change | Heteroplasmy (%) | |
|---|---|---|---|---|
| | | | NGS | qPCR |
| 263 | normal | | NA | ND |
| 062 | m.3243 | A>G | 1.1 | 3 |
| 367 | m.3243 | A>G | 2.3 | 8 |
| 030 | m.3243 | A>G | 6.8 | 16 |
| 085 | m.3243 | A>G | 11 | 32 |
| 362 | m.3243 | A>G | 27 | 50 |
| 761 | m.3243 | A>G | 36 | 48 |
| 074 | m.3243 | A>G | 68 | 95 |
| 626 | m.8344 | A>G | 84 | 73 |
| 611 | m.8344 | A>G | 86 | 82 |
| 926 | m.8993 | T>C | 88 | 87 |
| 799 | m.10191 | T>C | 28 | ND |
| 994 | m.11778 | G>A | 90 | 83 |
| 027 | m.11778 | G>A | 91 | 91 |
| 285 | m.13513 | G>A | 37 | 84 |
| 487 | m.13513 | G>A | 54 | 95 |
| 563 | m.14484 | T>C | 45 | 20 |

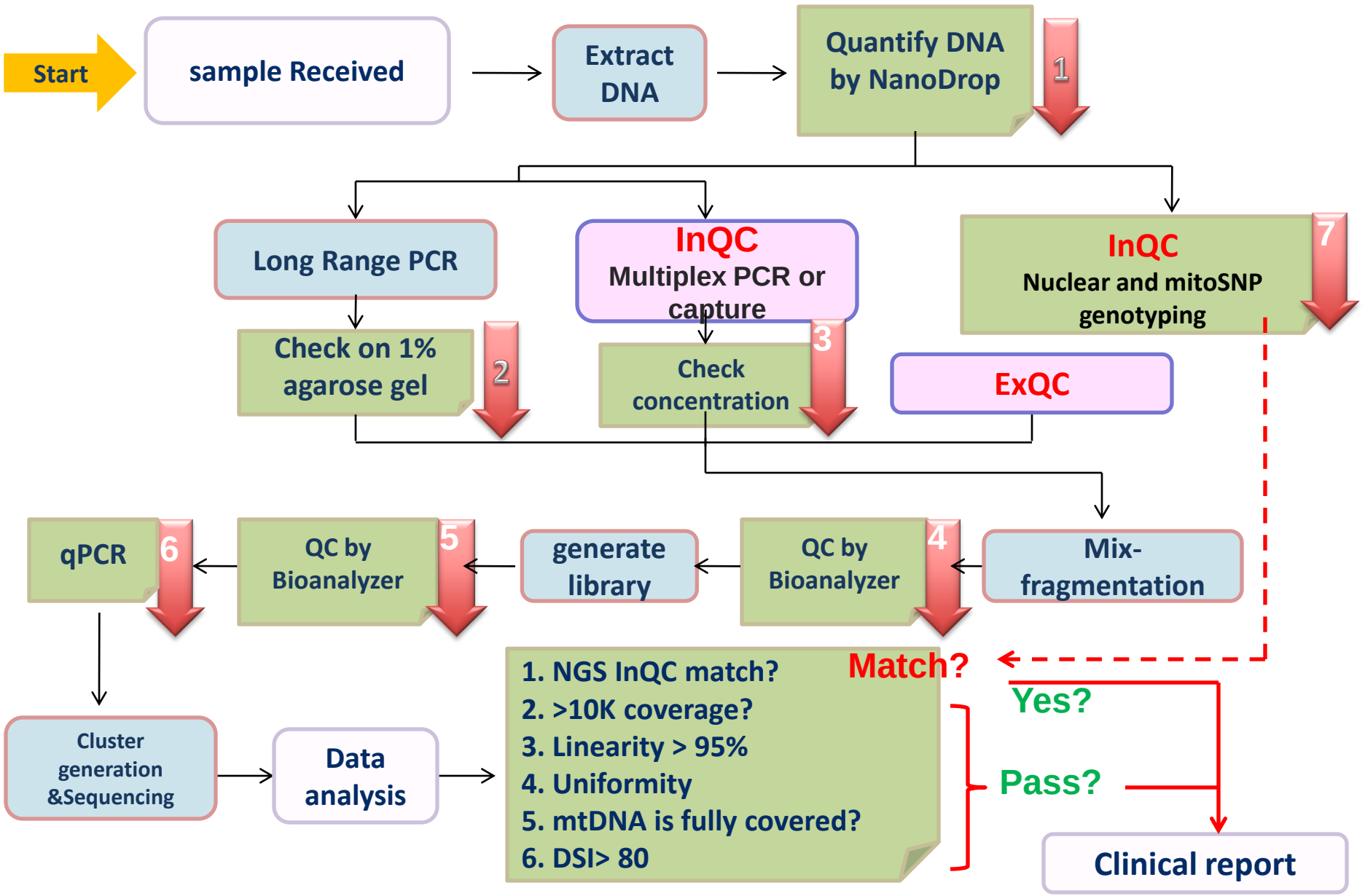
# Spike-in 1.1% positive control

| HiSeq run batch# | m.3243A>G% |
|---|---|
| 3 | 1.10 |
| 4 | 1.25 |
| 5 | 1.05 |
| 6 | 1.03 |
| 7 | 0.94 |
| 8 | 1.05 |
| 9 | 1.26 |
| 10 | 1.20 |
| 11 | 1.21 |
| 13 | 1.27 |
| 14 | 1.21 |
| 15 | 1.10 |
| 16 | 1.18 |
| 17 | 1.10 |
| 18 | 1.01 |
| 19 | 1.12 |
| 20 | 1.10 |
| 21 | 1.14 |
| 22 | 1.35 |
| 23 | 1.01 |
| 24 | 1.00 |
| 25 | 1.15 |
| 26 | 1.05 |
| 27 | 1.04 |
| 28 | 1.11 |
| 29 | 0.95 |
| average | 1.11 |
| STD | 0.10 |




**Average: 1.11+/- 0.1% heteroplasmy**

# Mito genome and Mitome NGS QA/QC workflow



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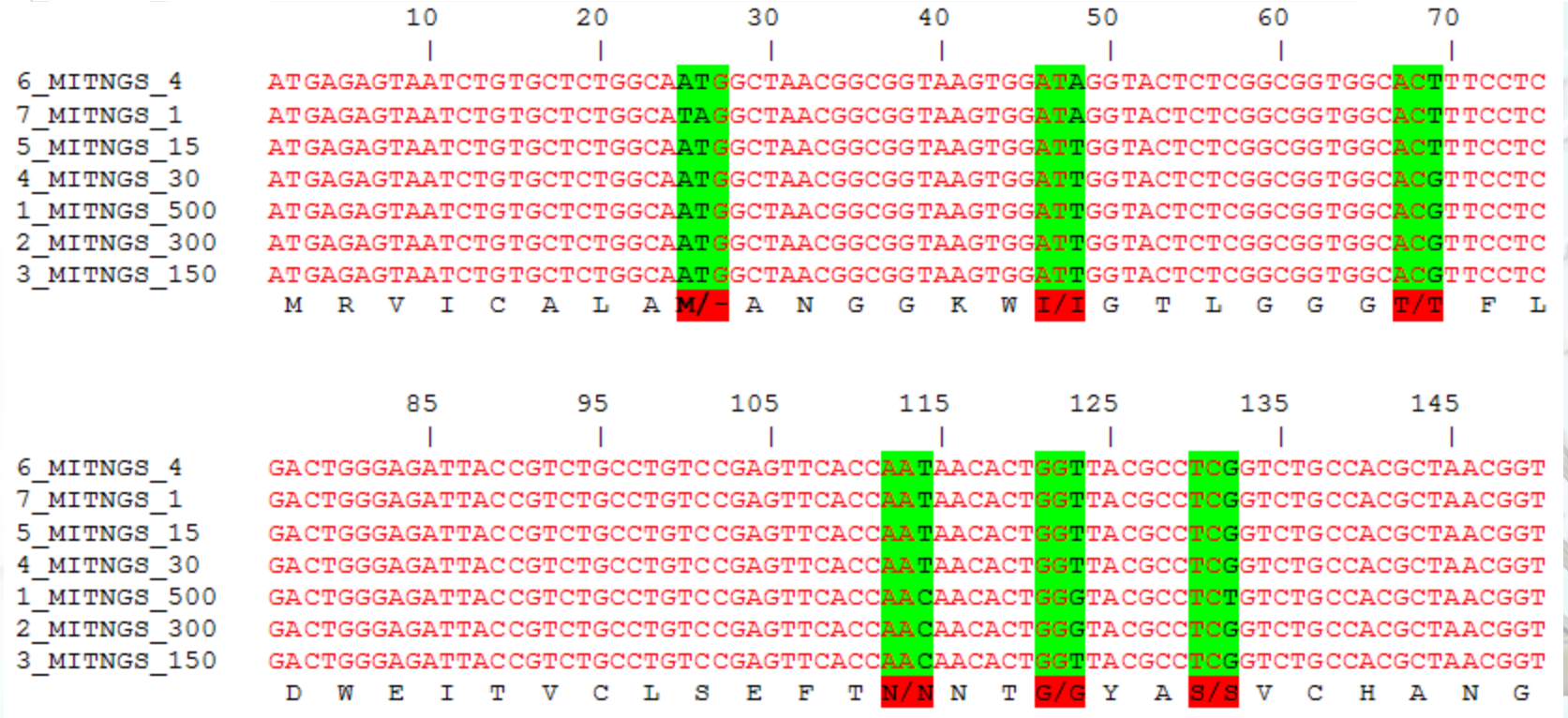
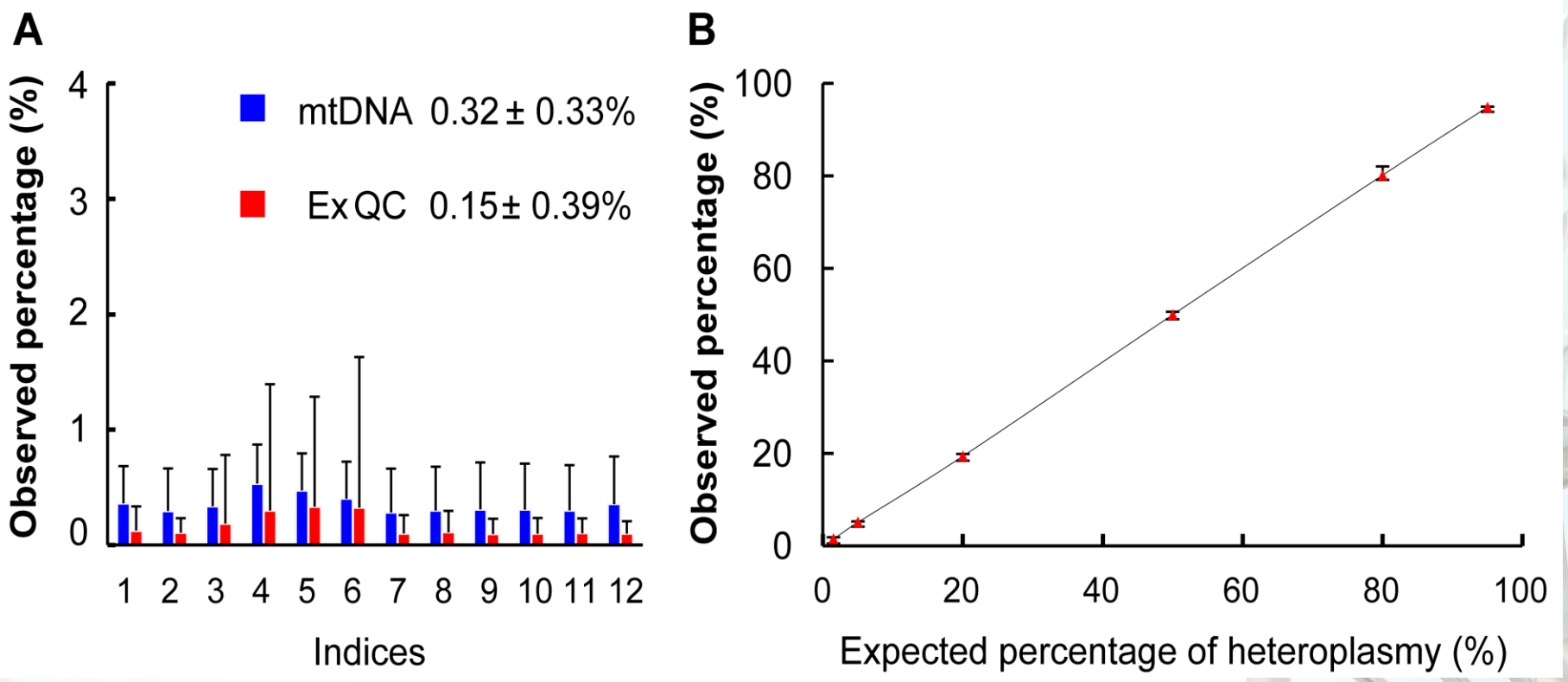
# "External quality control for each indexed sample"

**Spike in:** 7 fragments with different codons at 6 different sites on phage DNA sequence



# Spike in Quantitative control standards: for every sample

| | | | | | | 0.10% | 0.50% | 2.00% | 5.00% | 20.00% | 50.00% |
|---|---|---|---|---|---|---|---|---|---|---|---|
| X | I | T | W | G | S | 0.10% | 0.10% | 0.10% | 0.10% | 0.10% | 0.10% |
| M/X | I | T | W | G | S | 0.40% | 0.40% | 0.40% | 0.40% | 0.40% | 0.40% |
| M/X | I/I | T | W | G | S | 1.50% | 1.50% | 1.50% | 1.50% | 1.50% | 1.50% |
| M/X | I/I | T/T | W | G | S | 3% | 3% | 3% | 3% | 3% | 3% |
| M/X | I/I | T/T | W/W | G | S | 15% | 15% | 15% | 15% | 15% | 15% |
| M/X | I/I | T/T | W/W | G/G | S | 30% | 30% | 30% | 30% | 30% | 30% |
| M/X | I/I | T/T | W/W | G/G | S/S | 50% | 50% | 50% | 50% | 50% | 50% |
| | | | | | | 99.90% | 99.50% | 98.00% | 95.00% | 80.00% | 50.00% |
| | | | | | | M/X | I/I | T/T | W/W | G/G | S/S |




**Limit of detection~ 1.5%**

# Conclusion

1. Bringing NGS to clinical dx lab is practical. Proper QA/QC procedures should be instituted according to CLIA/CAP guidelines
2. Target gene capture/NGS: all procedures should be validated and positives confirmed
3. WES in research: discovery of new disease genes and/or new clinical phenotype
4. WES in clinical settings: currently report confirmed mutations in genes known to cause diseases (based on OMIM, HGMD db, and PubMed).
5. Novel gene/variants require functional confirmation.

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