UMASS MEDICAL SCHOOL Commonwealth Medicine

Discordant MPS1 Sequencing Results from Newborn Screening and Diagnostic Labs: Investigation and Resolution

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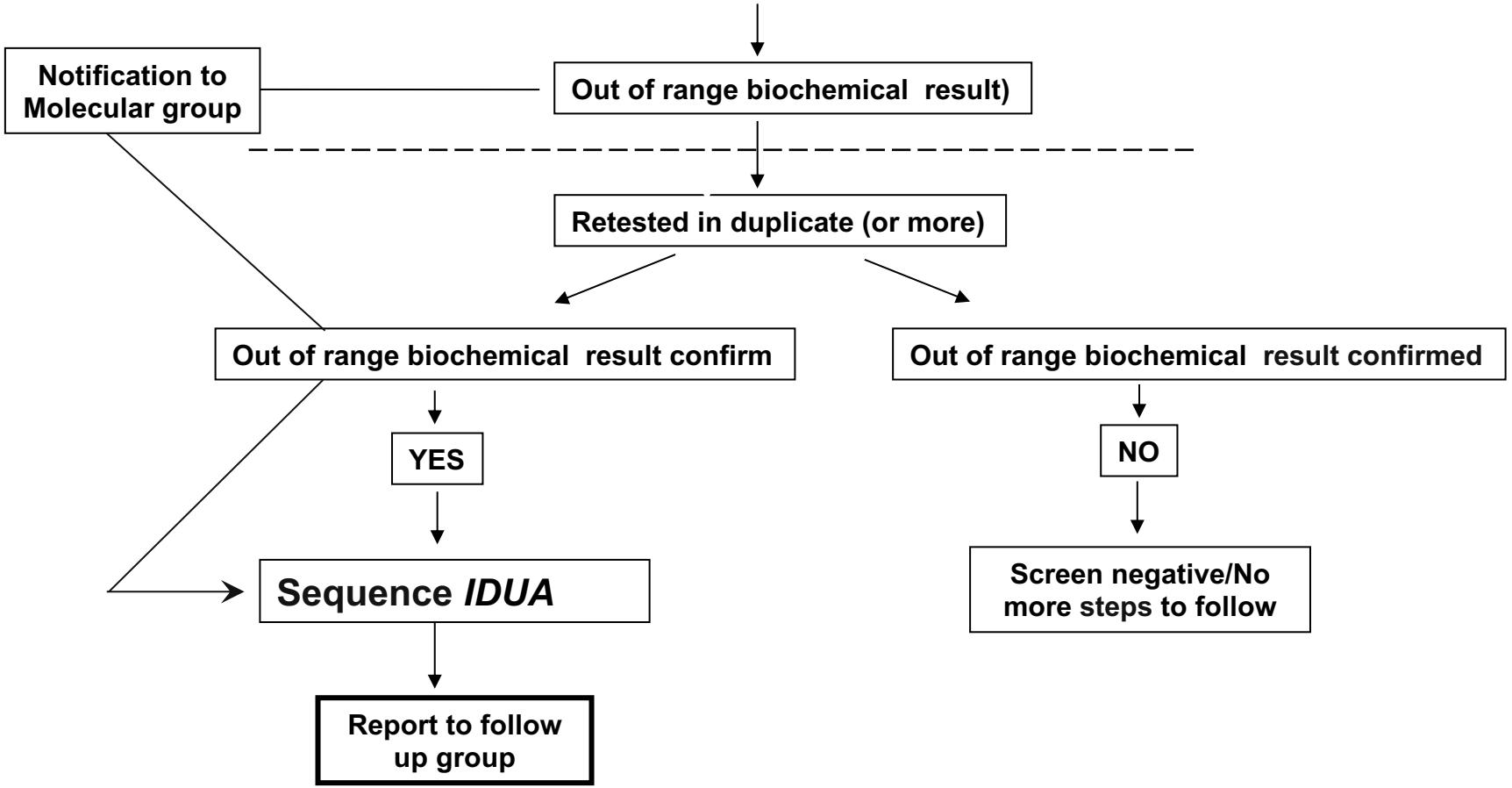
Topics to be covered:

- Allele dropout event during routine MPS1 sequencing
- Lessons learned and Technical Strategy
- Algorithm developed to address when sequencing should include a retest with alternate primers set for genotype confirmation

Algorithm for the prompting of IDUA sequencing

Program-wide Specimens tested for IDUA enzyme activity



Observations and Challenges

- Routine sequencing yielded one pathogenic variant that was reported as homozygous by our program.
- Diagnostic lab reported discordant zygosity for the same variant. They suggested a possible case of allele dropout for this allele.
- We confirmed alleleic dropout and issued a corrected report.
- We re-reviewed all homozygous variants that we had reported for MPS1.
- We developed an algorithm to determine when such retests with alternate primer pairs might be indicated to ensure the correct genotype.

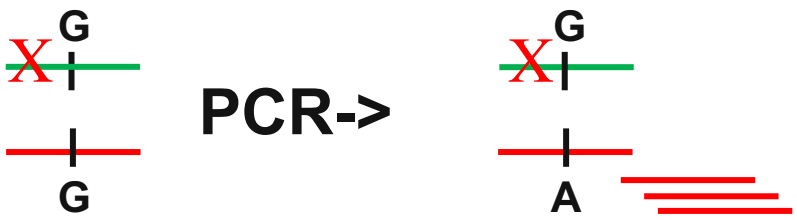
What is Allele Drop out?

Allele dropout results from a failure of amplification of one of the two alleles at a given locus. Possible causes for such failures in amplification include:

- One or more polymorphisms at the primer binding site
- Target Sequence secondary structure
- Non-primer-site SNV affecting PCR amplification
- G-Quadruplex Structures
- DNA Methylation at Imprinted Human Loci

Allele dropout led to erroneous genotype at position 1148G

a)

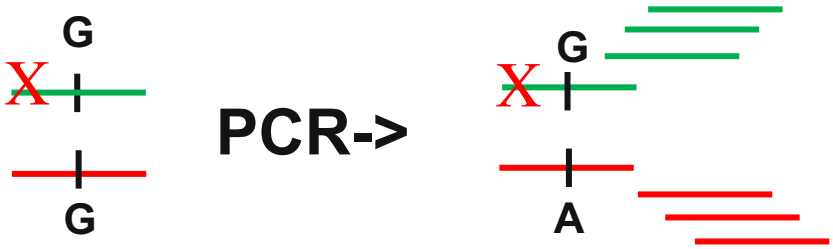


Regular-MPS primers set

No amplification of allele with G

Only "A" variant observed

b)

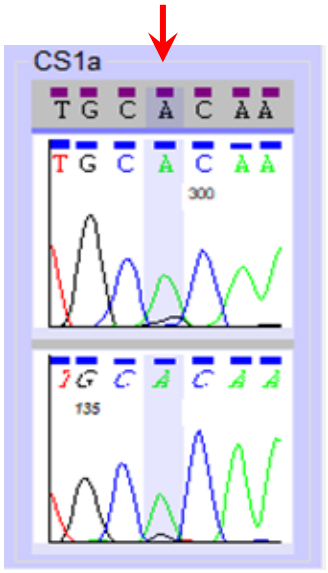


Genotype reported by other source using alternate primers set

Alternate primers allow amplification of both alleles

Both G and A observed

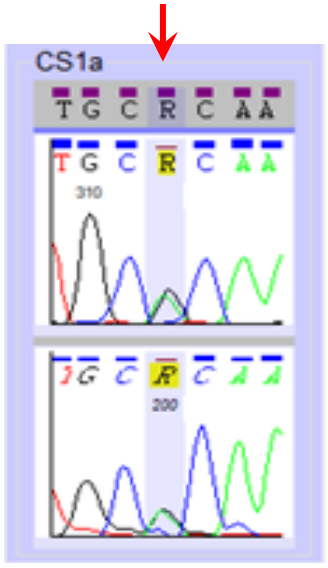
Alternate primer set detected the correct genotype



Regular-MPS primers set

c.1148g>A

Homozygous
G>A

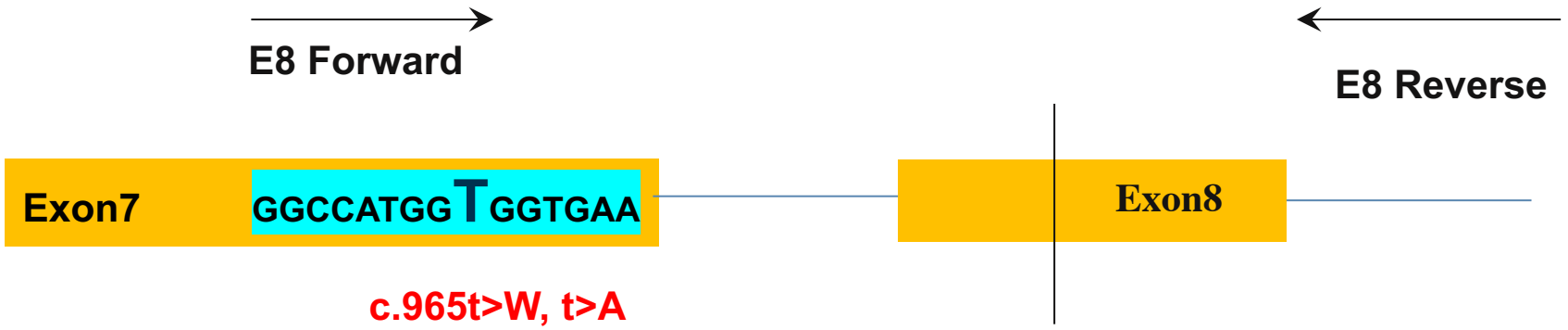


Alternate primers set

c.1148g>R

Heterozygous
G>A

Primer-site SNP may have led to Allelic dropout and erroneous genotyping



(SNP at primer binding site)

***IDUA* gene**

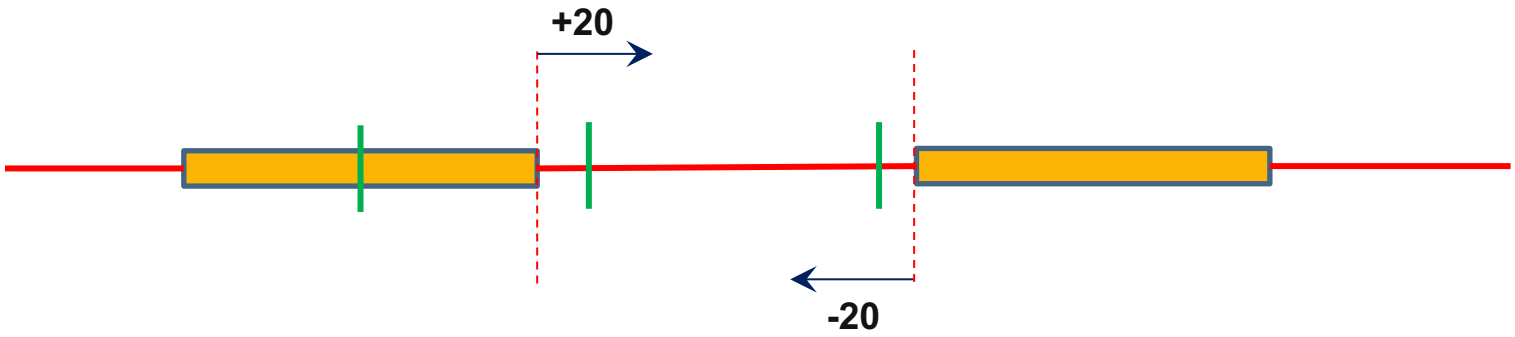
Which sequences should be subjected to retesting with alternate primers?

One Diagnostic Laboratory:

- Any pathogenic variant that appears to be homozygous

Is that too much? ($)
Is that enough? What do we risk missing?
(dominant pathogenic variant on dropout allele)

NENSP current algorithm for use of an alternate primer set



Whenever a sequenced variant :

- Appears to be homozygous AND
- is present within an Exon or within an intron +/- 20 from the exon/intron border AND
- is observed on an amplicon in which no heterozygous alleles are observed AND
- has been classified by our program as pathogenic or VOUS with low allele frequency

Whenever a sequence

- Shows no variant(s) consistent with phenotype (possible dominant variant on dropped allele)

Algorithm for use of alternate primer set

If variant is homozygous, present on exon or intron within the splice junction (+/- 20 from the exon intron border)

Yes

No heterozygous allele observed along with homozygous allele on same amplicon

Yes

Variant/s is classified as pathogenic or VOUS with low allele frequency by our program

Yes

Retest with alternate primer pairs for allele dropout

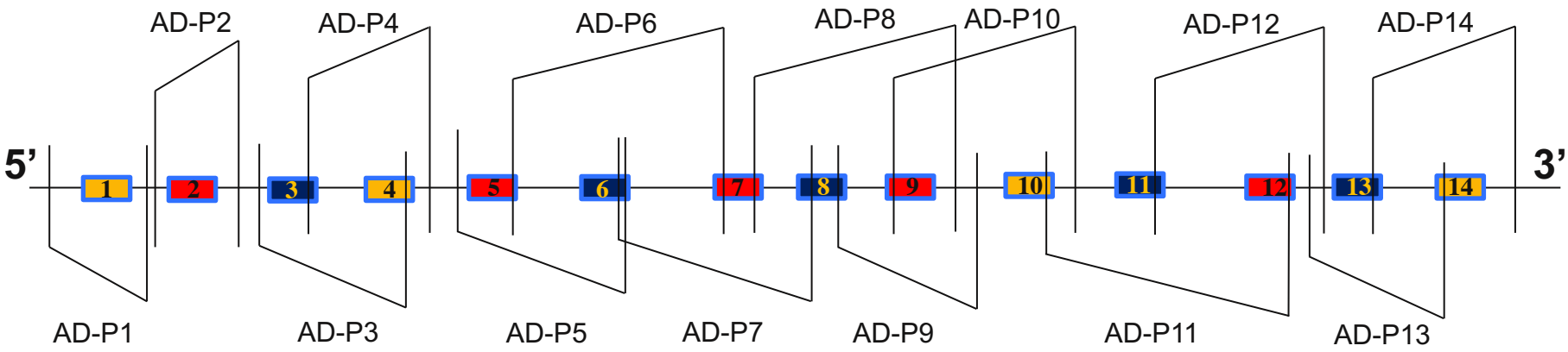
Experience to date

58	**Massachusetts specimens prompted IDUA sequencing**
31	**specimens appeared to have a homozygous variant**
9	**specimens appeared to have a pathogenic homozygous variant**
2	**of the 9 were on amplicons with an independent heterozygous variant**
7	**specimens were subjected to allele dropout evaluation**

List of 7 variants appearing to be homozygotes

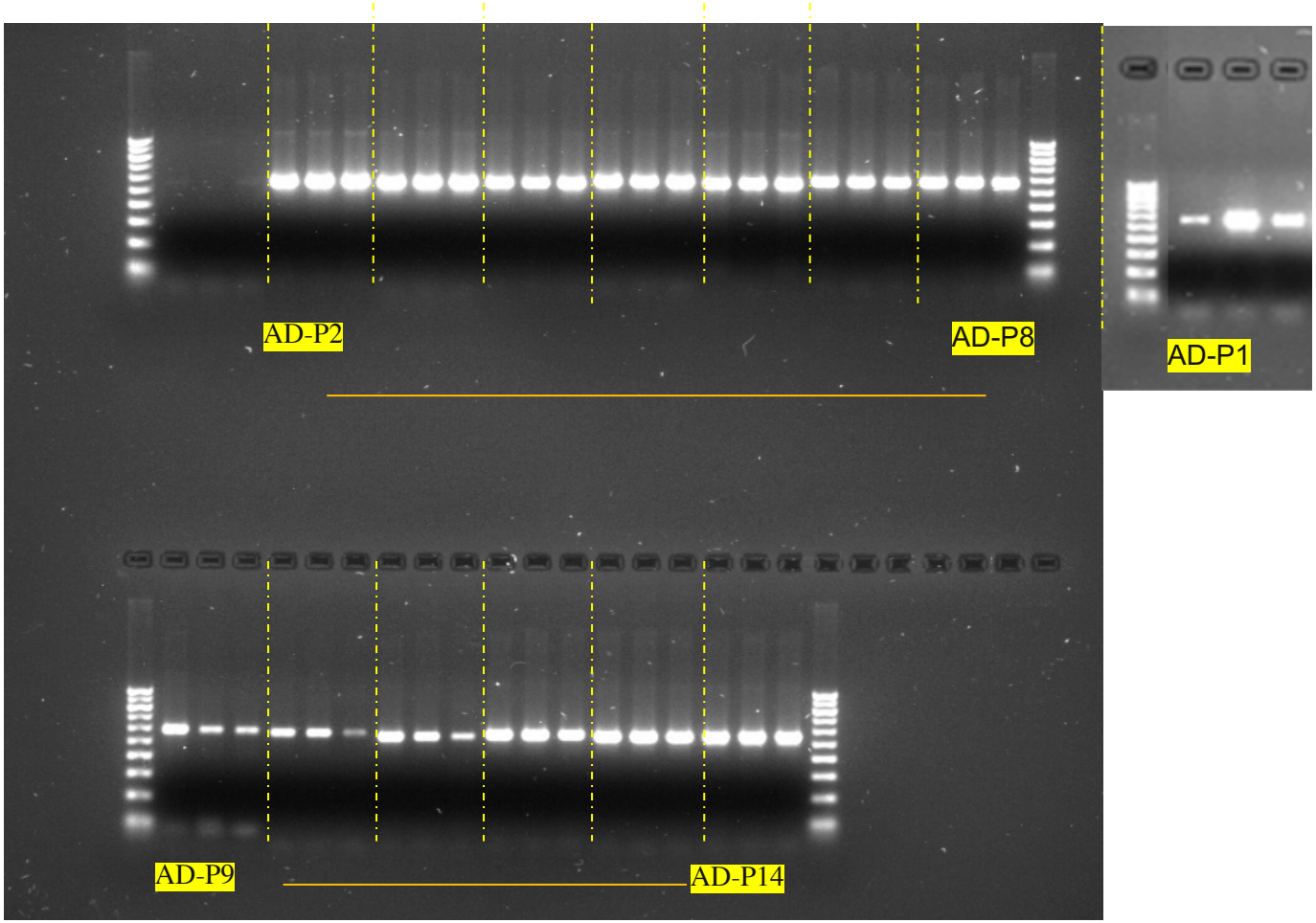
Variants	No. of Specimens in which the variant was observed	Allele Frequency	Classification
c.235 g>A	5	0.0030	VOUS
c.246 c>G	2	0.0029	VOUS
c.965 t>A	1	0.0009	VOUS
c.1148 g>A	1	0.00003	Pathogenic
c.1205 g>A	1	0.0006	Pathogenic
c. 1225 g>C	1	0.0072	VOUS

Alternate primers to find any MPS1 allele dropout



IDUA Gene

Optimization of PCR condition for Alternate primers

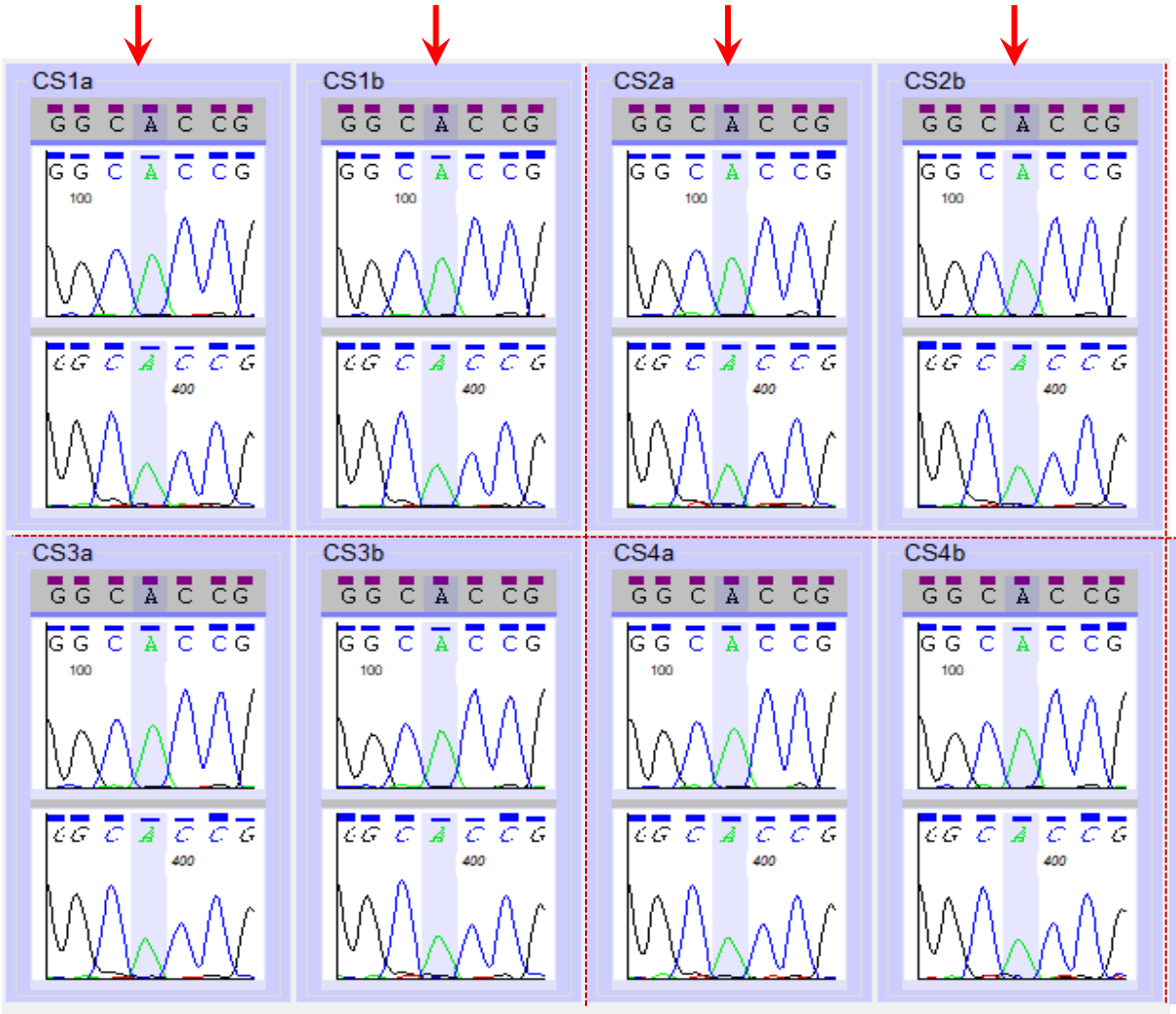


Investigating allele dropout for the homozygous variants observed for MPS1 disease at NENSP

- DNAs already extracted from the specimens or DNA eluates were used whenever it was available.
- Sequencing was performed using the alternate primers set(1) or sets(2).

Genotype retest using alternate primers for variant c.235g>A

Variants	No. of Specimens variant observed	Allele Frequency	Classification
c.235 g>A	5	0.0030	VOUS

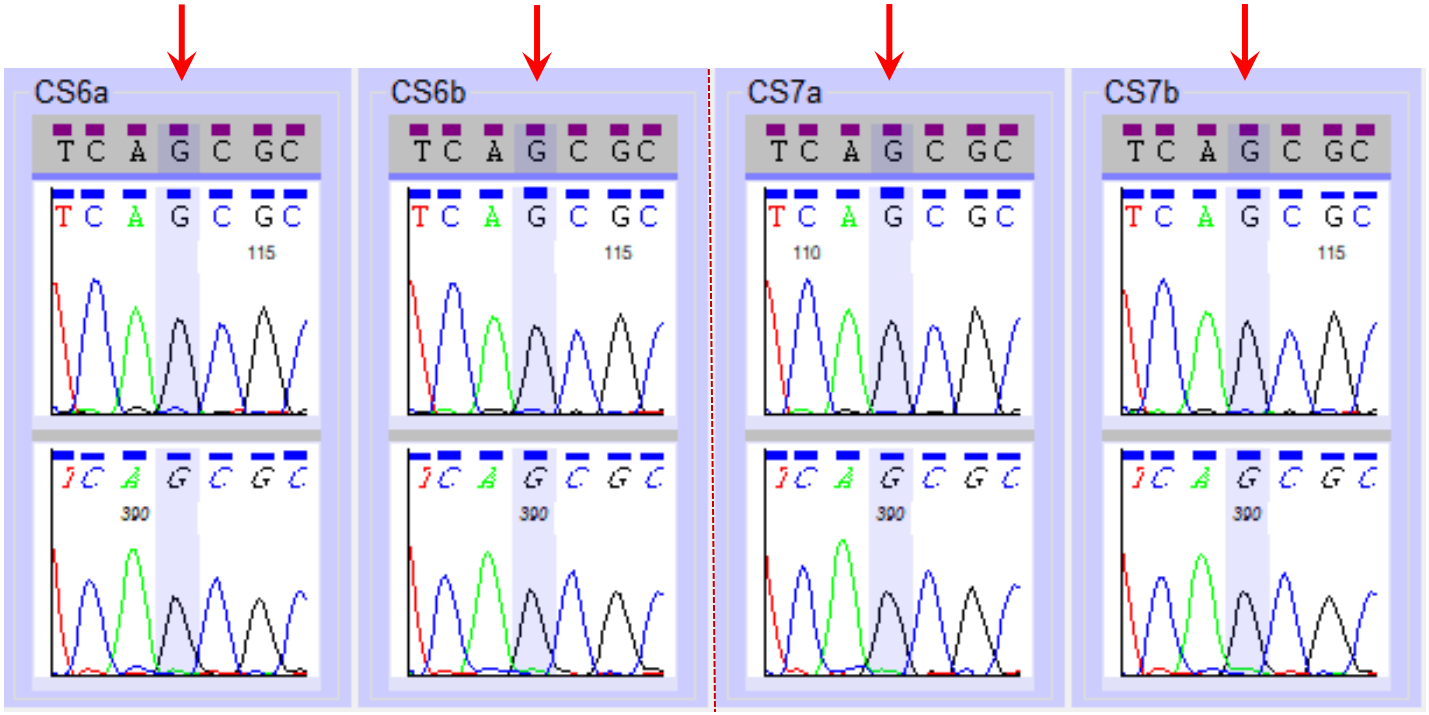


AD-P2 primers set

No Allele dropout, True Homozygous

Genotype retest using alternate primers for variant c.246c>G

Variants	No. of Specimens variant observed	Allele Frequency	Classification
c.246 c>G	2	0.0029	VOUS

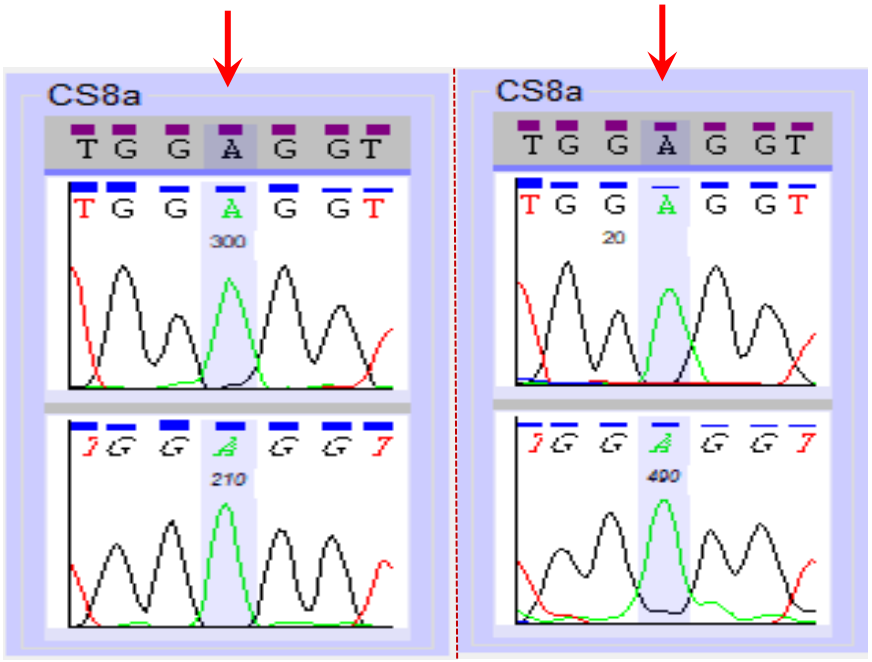


AD-P2 primers set

No Allele dropout,
True Homozygous

Genotype retest using alternate primers for variant c.965t>A

Variants	No. of Specimen/s variant observed	Allele Frequency	Classification
c.965 t>A	1	0.0009	VOUS



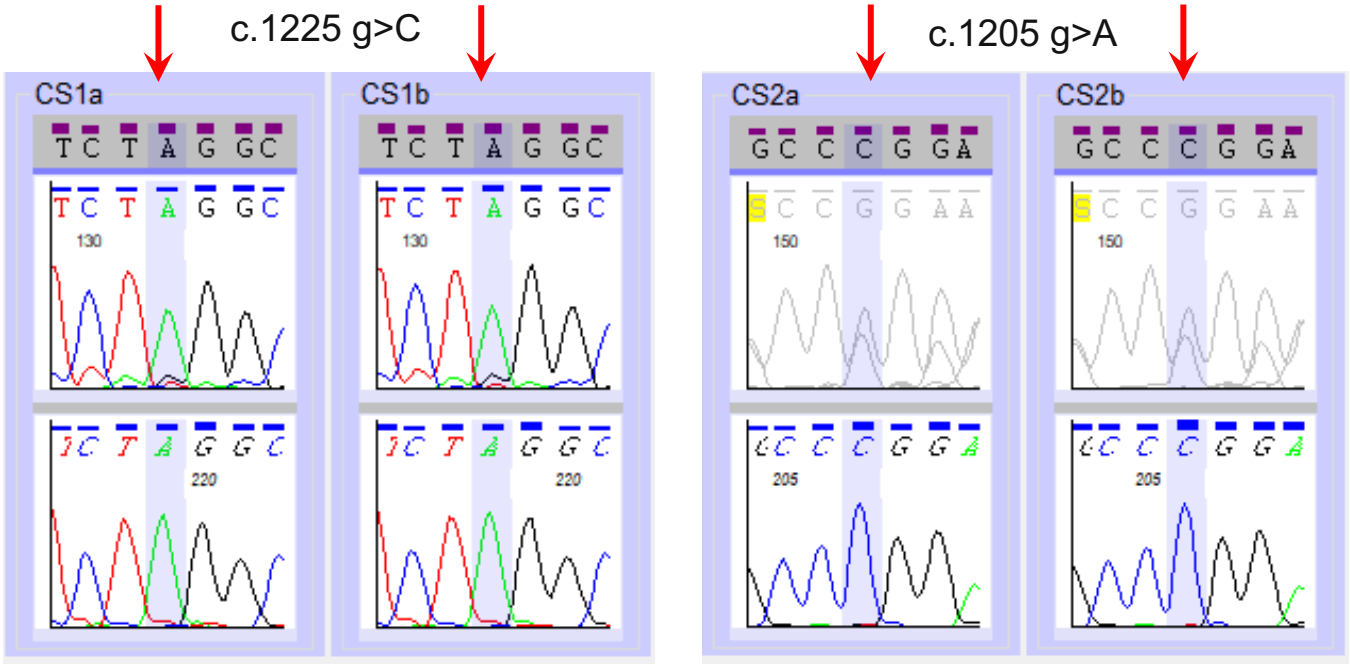
ADM-P7 ADM-P8

AD-P7/P8 primers set

No Allele dropout, True Homozygous

Genotype retest using alternate primers for variants c.1205g>A, and c.1225g>C

Variants	No. of Specimen/s variant observed	Allele Frequency	Classification
c.1205 g>A	1	0.0006	Pathogenic
c.1225 g>C	1	0.0072	VOUS



AD-P9 primers set

No Allele dropout, True Homozygous

Conclusions

- Chance of genotype error due to unidentifiable **allelic drop out is a sinister problem** and may not be prevented even with careful primer design.
- Allele dropout **can occur with sufficient frequency** (1/7=14%) resulting in a potentially important number of **erroneous clinical diagnoses**.
- We have **developed an algorithm** when to retest the genotype/s using the alternate primer set.
- Excising two independent genotyping assays can effectively minimize genotyping artifact, and **this practice should be embraced by Newborn Screening Programs**.
- **NGS inherently utilizes alternate primers**.

Contact

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