Analysis of Genes with Pseudogenes: Balancing what you get, what you don't get, and what you need.

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EGL Genetics

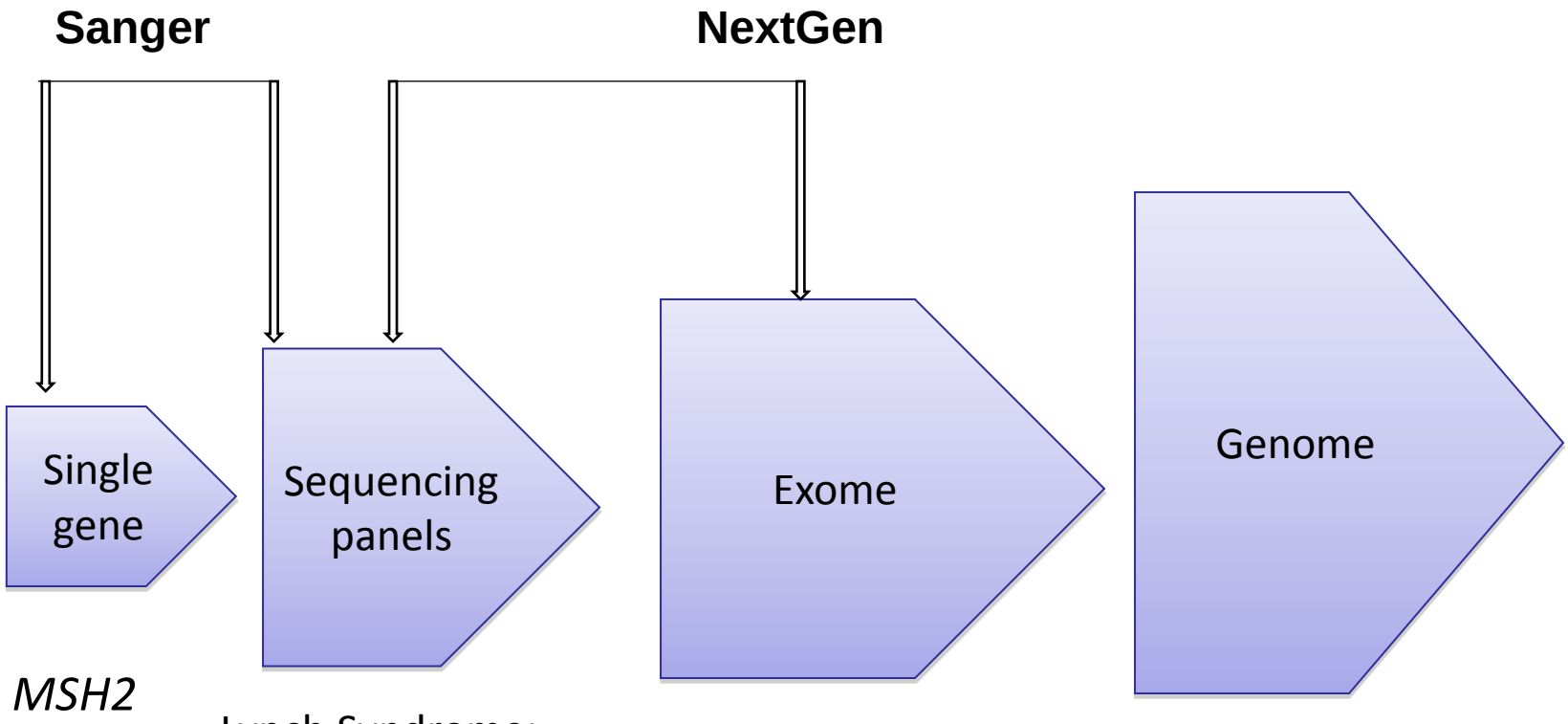
Disclosures

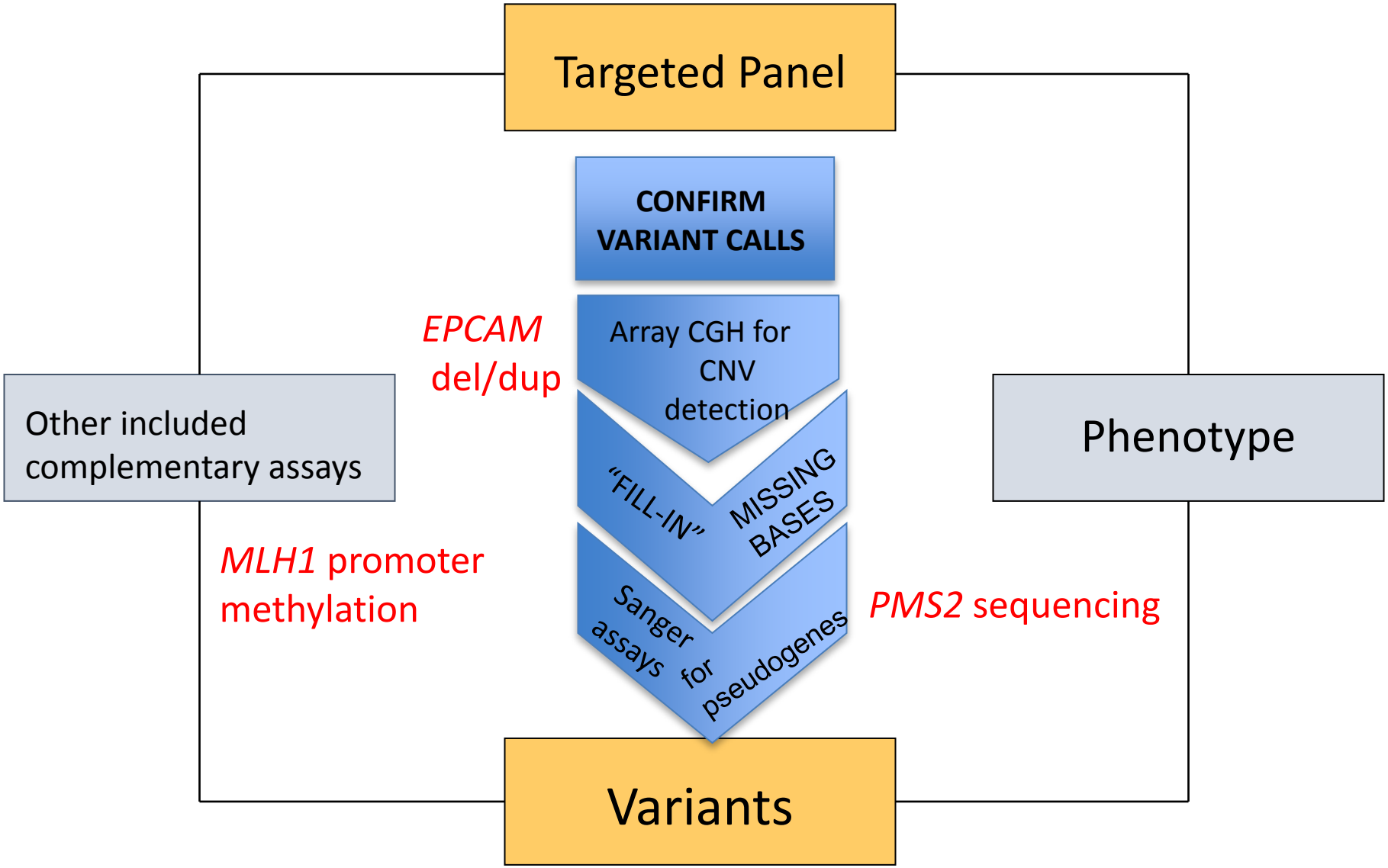
Lora Bean is a laboratory director at EGL, a laboratory that does fee for service testing.

Objectives

- Present the benefits and limitations of NGS
- Briefly review the nature and origin of pseudogenes
- Discuss the difficulties pseudogenes present for molecular diagnostics
- Present methods to interrogate clinically relevant genes with pseudogenes.

Choosing the Right Clinical Test





XLID/Autism panel: *FMR1*, *FMR2*, Biochemical assays

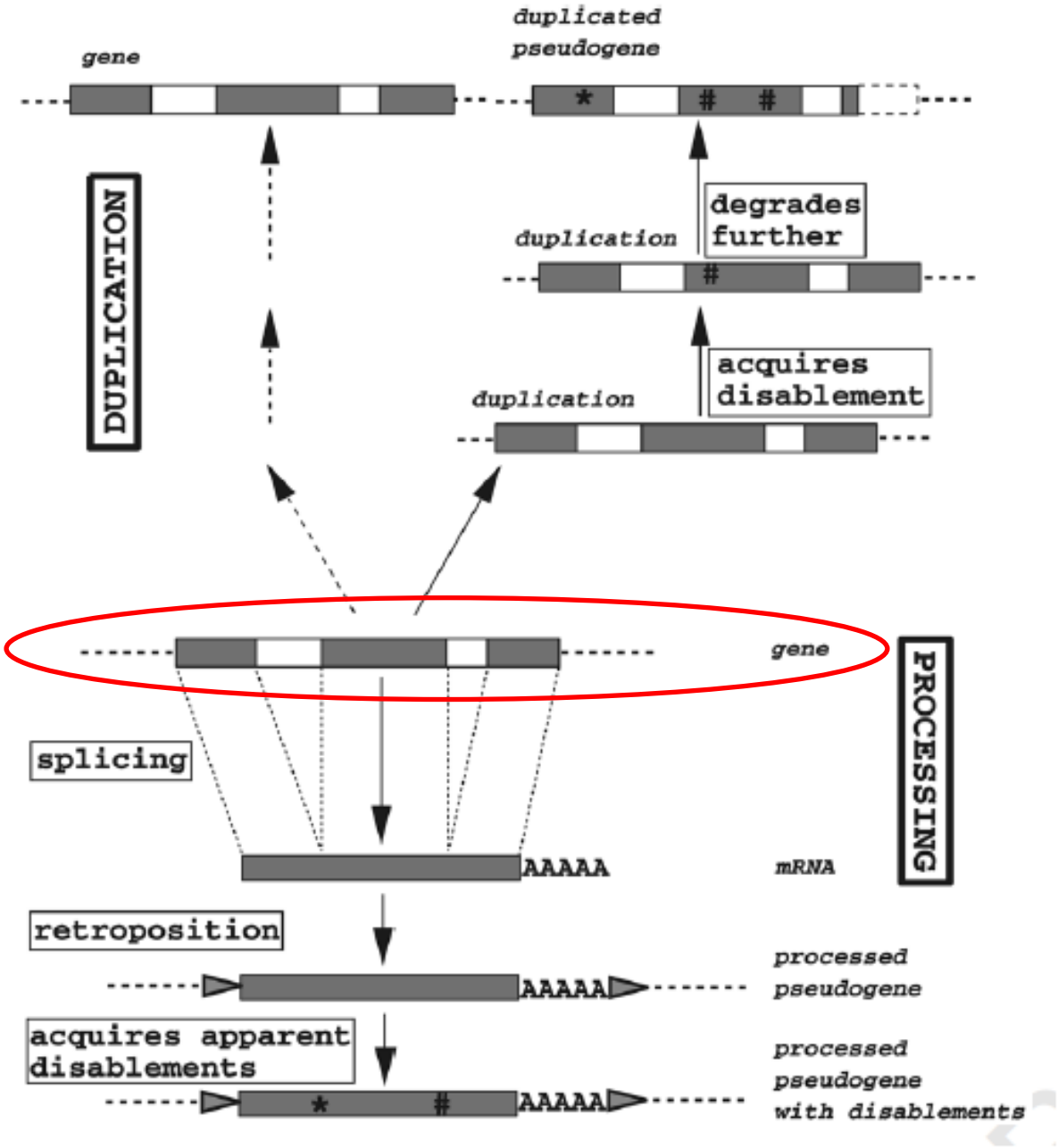
Short stature panel: Russell Silver (H19/Lit1 methylation), UPD7

Lynch syndrome: *EPCAM* del/dup, *PMS2* sequencing, *MLH1* promoter methylation

Chin et al, BMC Genetics, 2012, Askree et al, BMC Genetics, 2013, Valencia et al, J Mol Diag, 2013, Valencia et al, PLoS One, 2013

Genes and Pseudogenes

- Gene
  - Original copy
  - Functional (Active gene)
- Pseudogene
  - Derived from active gene
    - Duplication
    - Retrotransposition (Processed pseudogene)
  - Non-functional



Harrison and Gerstein 2002 https://www.ncbi.nlm.nih.gov/pubmed/12083509

Techniques Used in Molecular Genetics

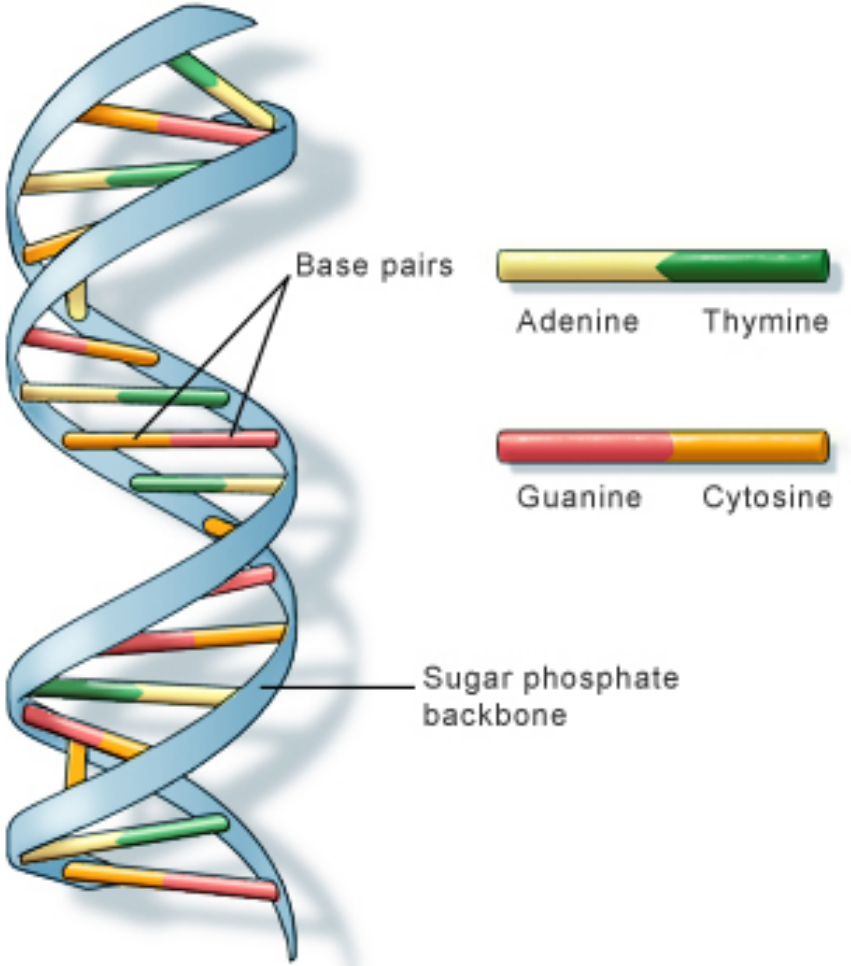
Sequencing

- Sanger
- Next-generation sequencing

Deletion / Duplication analysis

- Arrays
-Southern blotting
- MPLA

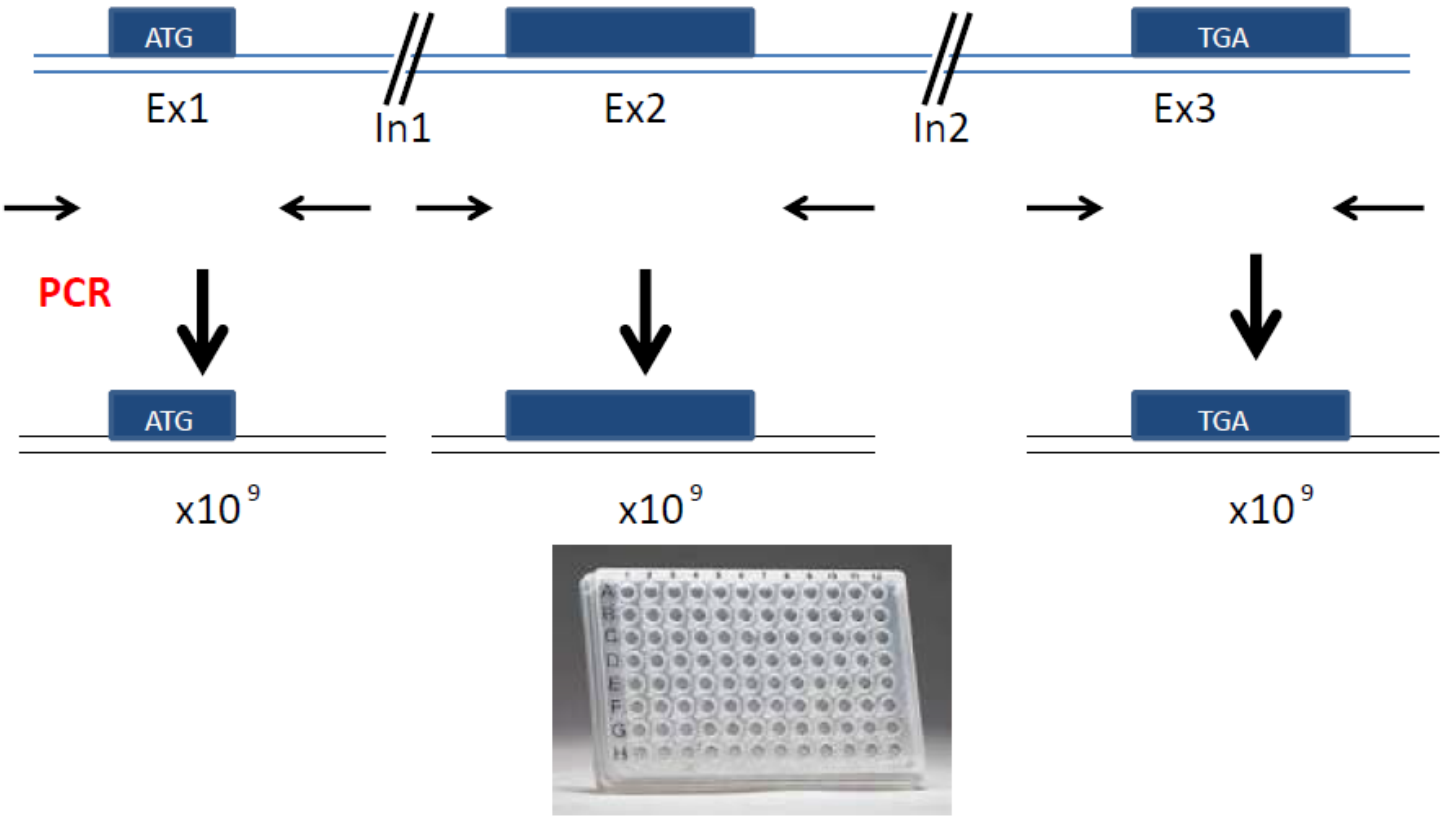
Properties of DNA



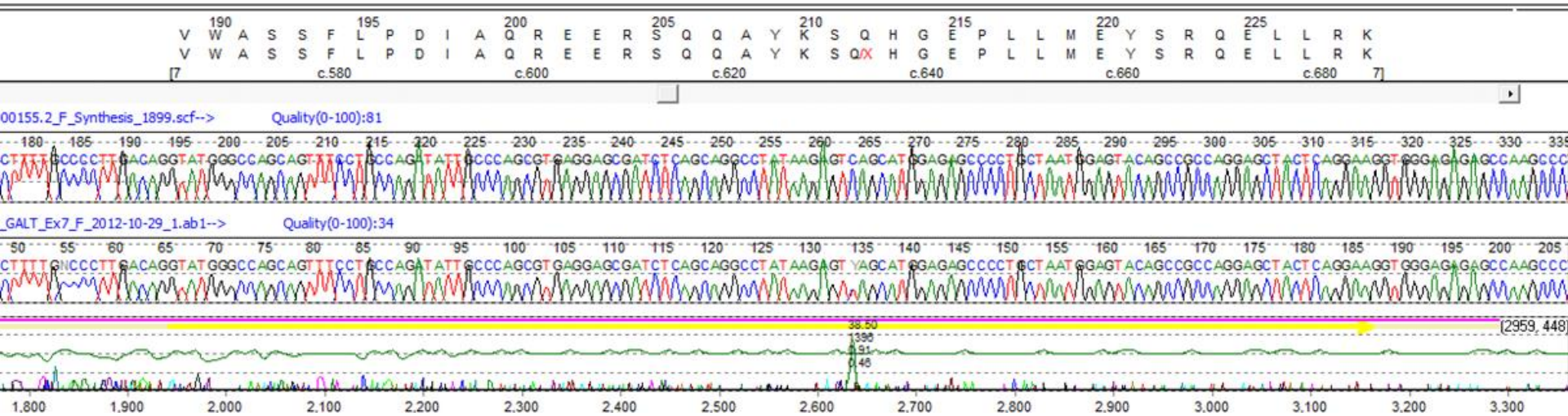
- Double stranded
- Denatures with heat
- Complementary basepairing
- Primers and enzymes to replicate
- Large
- Negatively charged

Sanger (di-deoxy termination) sequencing

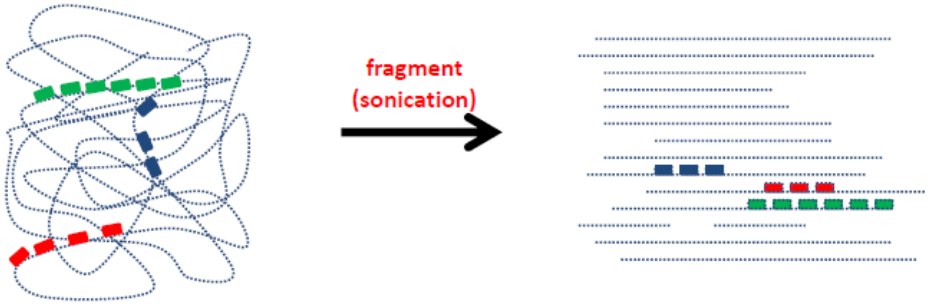
Step 1: PCR amplification of target segments of genome (amplicons)

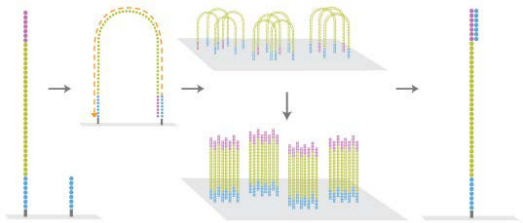


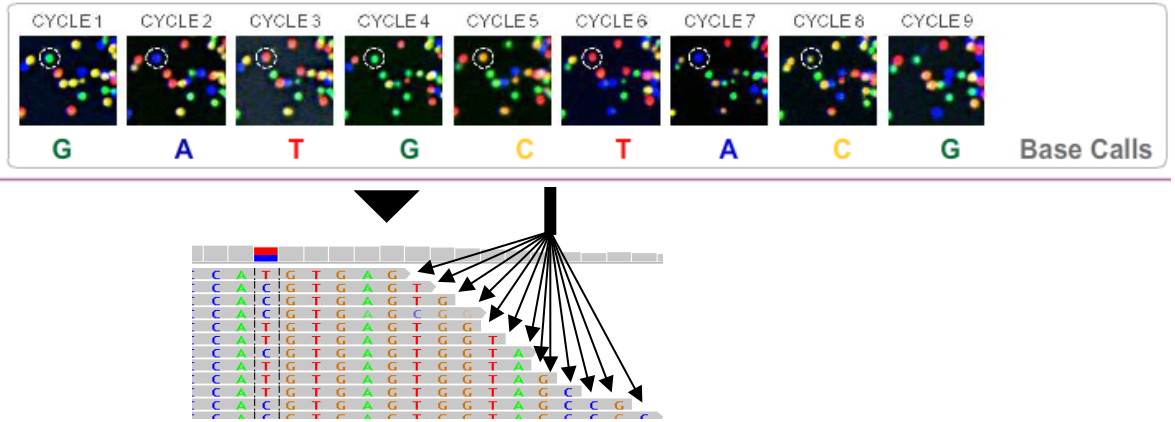
Sanger Sequencing



Next Generation Sequencing







Genomic DNA

↓

Fragment
Randomly shear DNA + end repair + size select

↓

"Barcode"

Amplification

Capture

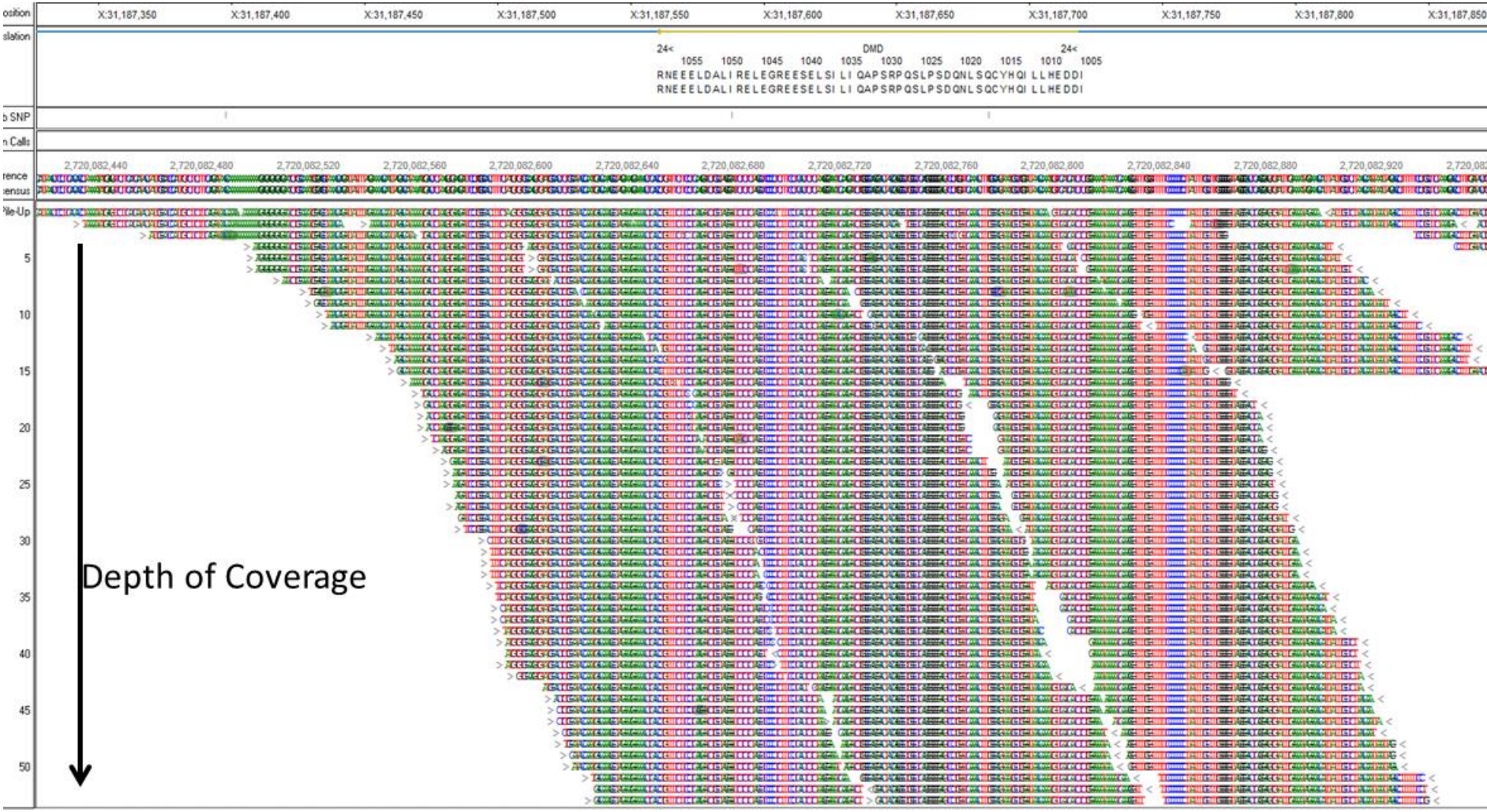
↓

NextGen Sequencing

↓

Raw Data

NGS “Reads”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X:31,187,700
X:31,187,750
X:31,187,800
X:31,187,850
DMD
1055 1050 1045 1040 1035 1030 1025 1020 1015 1010 1005
RNEEELDALIRELEGREESELSILIQAPSRPQSLPSDQNLSQCYHQILLHEDDI
RNEEELDALIRELEGREESELSILIQAPSRPQSLPSDQNLSQCYHQILLHEDDI
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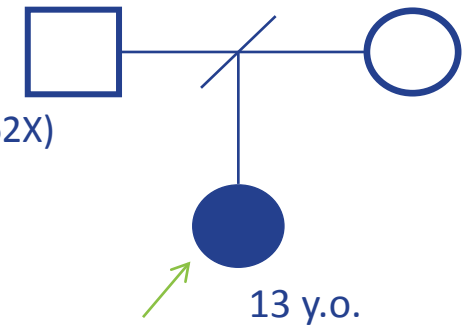
2,720,082,880
2,720,082,920
5
10
15
20
25
30
35
40
45
50
Depth of Coverage

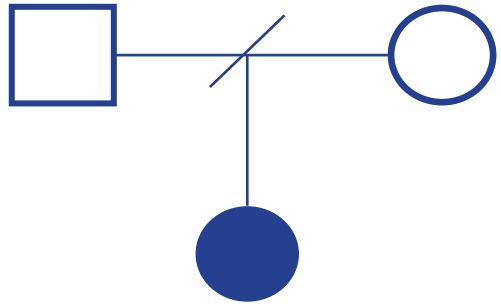
Pseudogenes in NGS Analysis: A Cautionary Tale

Exome Sequencing – A suspected case of Schachman-Diamond syndrome plus

- Shwachman-Diamond syndrome
  - Previous testing found only one pathogenic variant in the *SBDS* gene (c.183_184delTAinsCT (p.K62X)
- Short stature
- Failure to thrive
- Hypothyroidism, vitamin D deficiency
- Pancreatic insufficiency
- Bone mineral density abnormalities, sparse hair, maldentition
- Intestinal lymphangiectasia with protein losing enteropathy
- Neutropenia
- Multiple bacterial infections
- History of lymphopenia with combined cellular and humoral immune deficiency
- Dilated vasculature, collateral artery formation, stenosis of blood vasculature



RAG1: c.256_257delAA/c.322C>T (p.R108X)



c.256_257delAA/
c.322C>T (p.R108X)

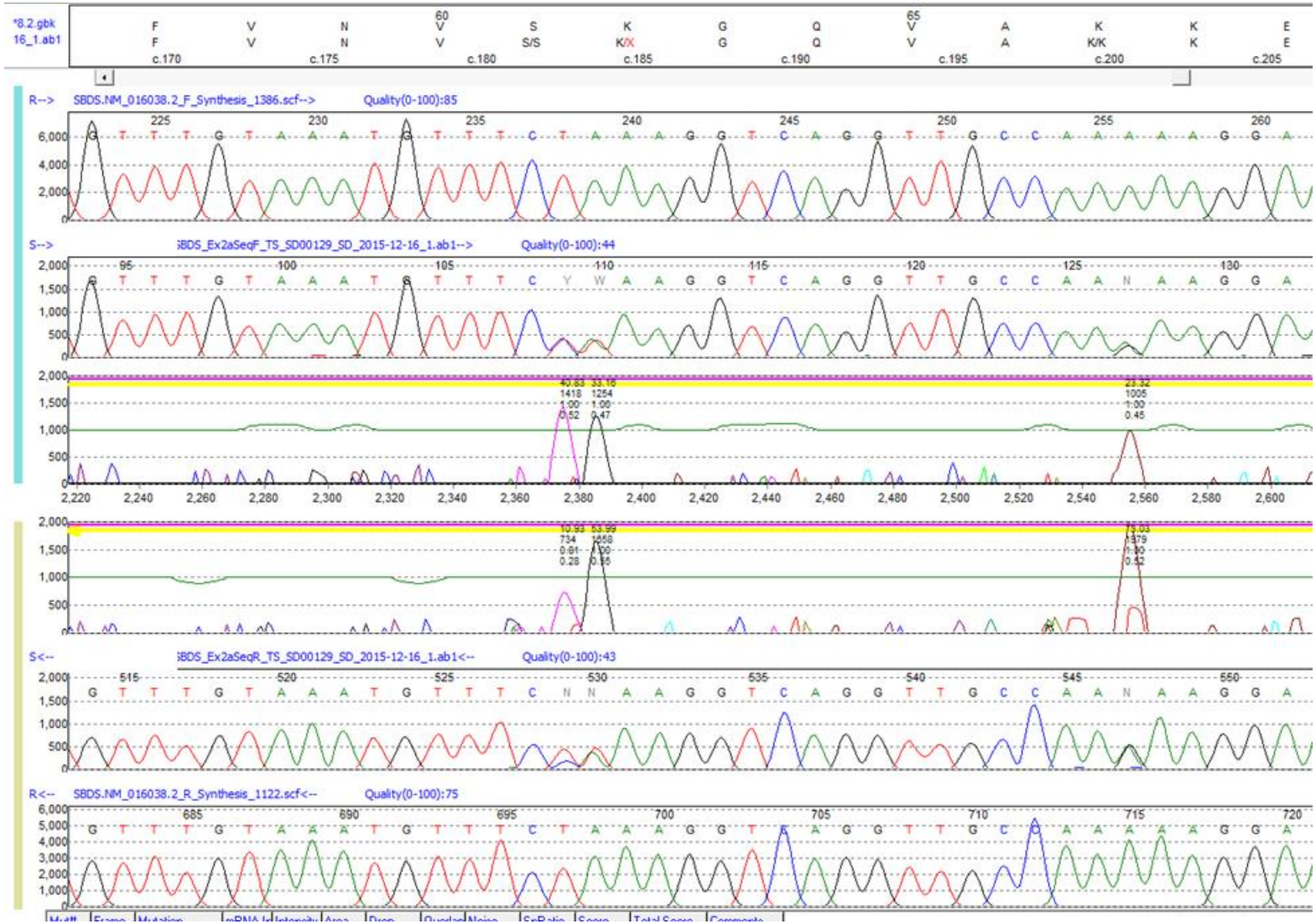
- **c.256_257delAA: Pathogenic**
  - Previously reported in the literature in individuals with severe combined immunodeficiency
  - Of a type expected to cause disease
- **c.322C>T (p.R108X): Pathogenic**
  - Previously reported in the literature in individuals with severe combined immunodeficiency
  - Of a type expected to cause disease

But what about the *SBDS* variant?

- Previous sequencing of the *SBDS* gene performed at Duke and at GeneDx identified a heterozygous c.183_184delTAinsCT (p.K62X) pathogenic variant.
- Deletion/duplication analysis for the *SBDS* gene performed at Prevention Genetics was negative.

NGS reads for pseudogene (*SBDSP1*)

Sanger - exon 2 - *SBDS* gene



Sanger Confirmation

Gene	Accession Number	Exon	Nucleotide	AA	Proband	Mother
RAG1	NM_000448.2	2	c.256_257delAA	FS	Heterozygous	Negative
RAG1	NM_000448.2	2	c.322C>CT	p.R108XR	Heterozygous	Heterozygous
SBDS	NM_016038.2	2	c.183_184delTAinsCT	p.K62X	Heterozygous	Negative
SBDS	NM_016038.2	2	c.201A>AG	p.K67KK	Heterozygous	Negative

Find the differences!

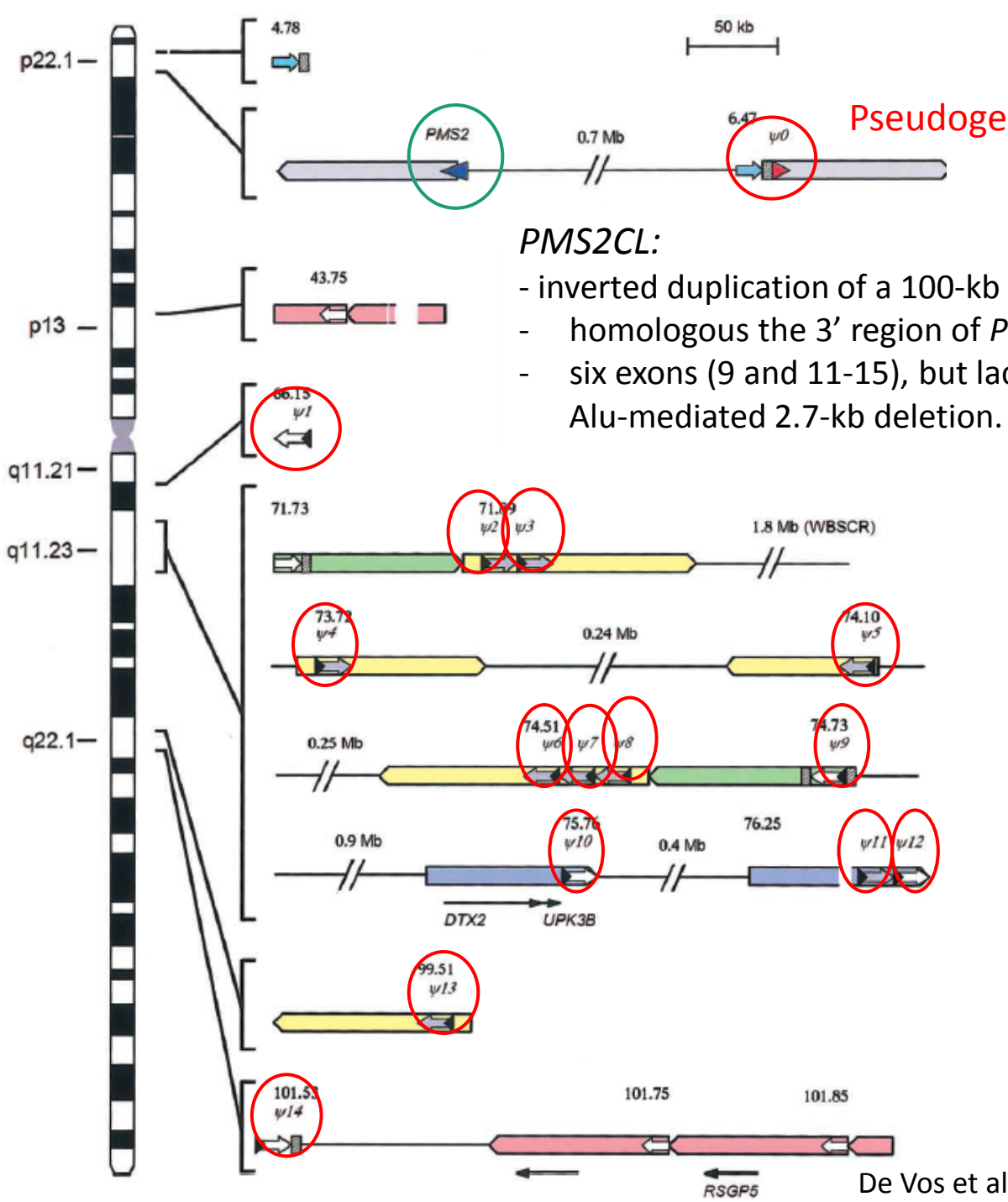
Gene

Pseudogene

PMS2

Lynch syndrome

Gene	Proportion of Lynch Syndrome	Proportion of Pathogenic Variants Detectable by this Method	
		Sequence analysis	Gene-targeted deletion/duplication analysis
MLH1	50%	90-95%	5-10%
MSH2	40%	~80%	~20%
MSH6	7%-10%	>95%	Rare
PMS2	<5%	~80%	~20%
EPCAM	~1%-3%	None	All



Pseudogene exons 9-15

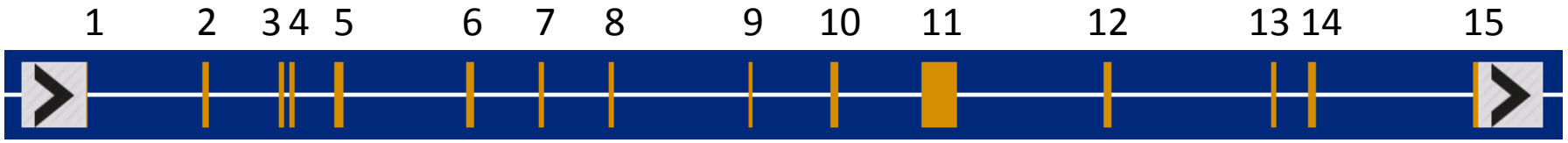
PMS2CL:

- inverted duplication of a 100-kb repeat element
- homologous the 3' region of *PMS2*
- six exons (9 and 11-15), but lacks exon 10 due to an Alu-mediated 2.7-kb deletion.

Pseudogenes exons 1-5

De Vos et al., Am J Hum Genet. 2004 May;74(5):954-64.

PMS2

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                                  |         |         |         |         |         |
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chr7_74714278       CTTTGTTTCTTGTAACTGATTTCTTTAGTACAGAACCTGCTAAGGCCATCAAACCTATTG
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chr7_99929836       CTTTGTTTCTTGTAACTGATTTCTTTAGTACAGAACCTGCTAAGGCCATCAAACCTATTG
chr7_6045296active  ATTTGTTTCTTGTAACTGATTTCTCTAGTACAGAACCTGCTAAGGCCATCAAACCTATTG
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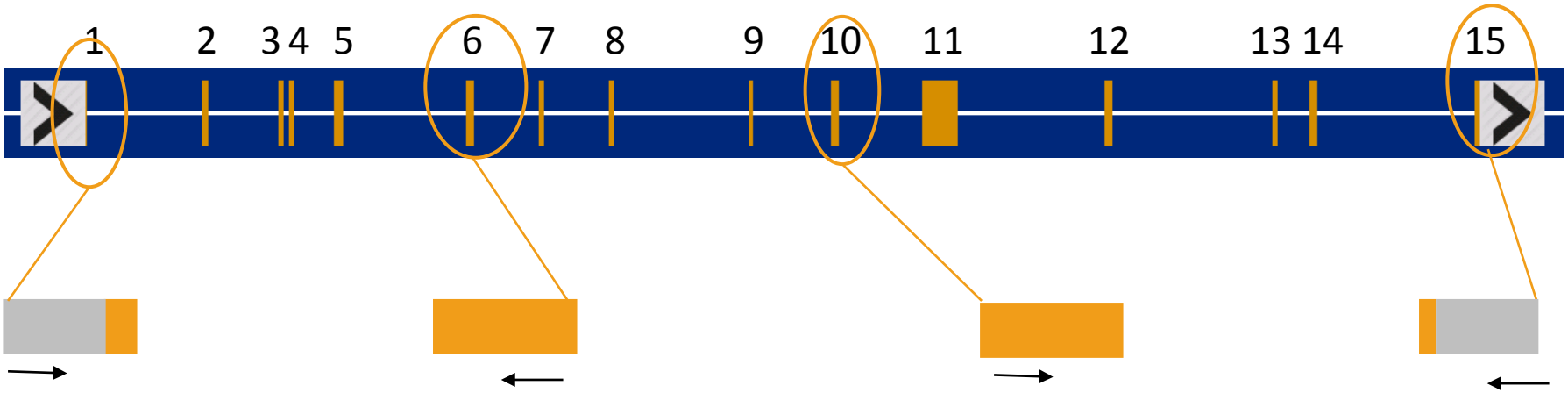
Intron 1 Exon 2

How can we deal with repetitive regions?

Sequencing

Long-range PCR



Long range F ~10 kb Long range R

Long range F ~18 kb Long range R

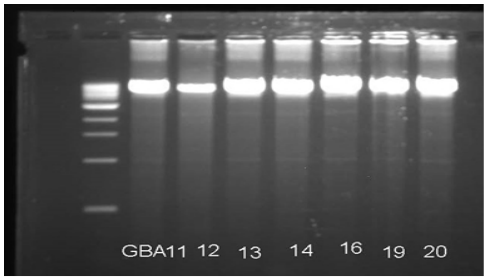
Isolate PCR product and use as template for exon-specific primers

Isolate PCR product and use as template for exon-specific primers

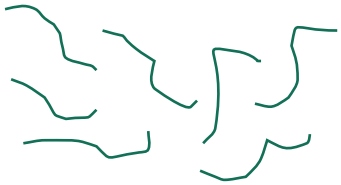
Sanger sequencing

Sanger sequencing

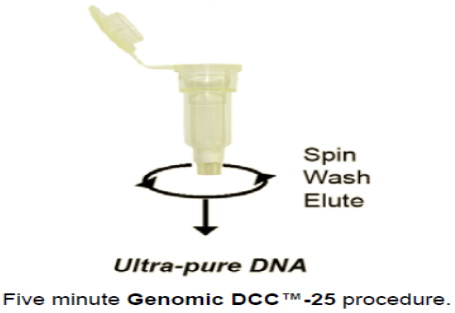
LR-PCR_ NeoPrep_Miseq



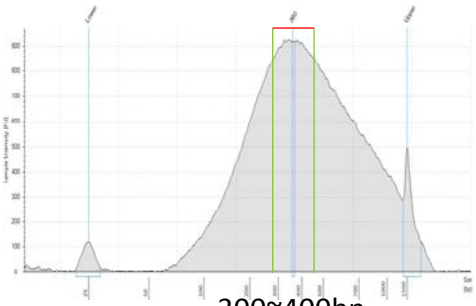
1.LR-PCR



2. NEB Enzymatic fragmentaton



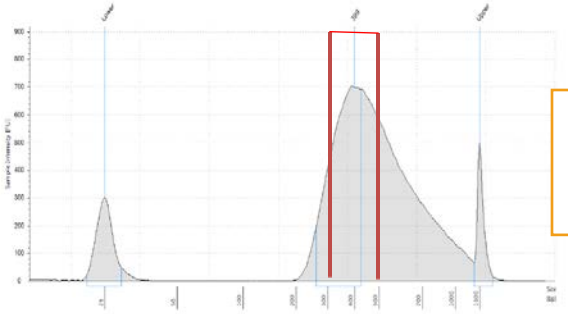
3. Zymo Purification



300~400bp

4. Tape station ~ 350bp

5. NeoPrep



350~450bp

6. Library Tape Station

7. Miseq

Long-range PCR (Sanger or NGS)

- Benefits
  - Amplify unique sequence
  - Interrogate regions of interest
- Limitations
  - Working with amplified product is a contamination risk
  - Gene conversion events may not be detected
  - Labor intensive

How can we deal with repetitive regions?

Deletion / Duplication

Gene Deletion / Duplication Testing

Quantitative Assay (e.g. Quantitative PCR, MLPA):

Benefits:

Many small regions (exons) can be tested

Accurate

Limitations: Labor intensive

Significant QA/QC investment

Single base pair changes can interfere with probe binding

Southern blotting:

Benefit:

Technically simple, can be performed in most laboratories

Limitations:

High cost, labor intensive

Low resolution: may miss small deletions or duplications

Array-based technologies:

Benefits:

Highly accurate

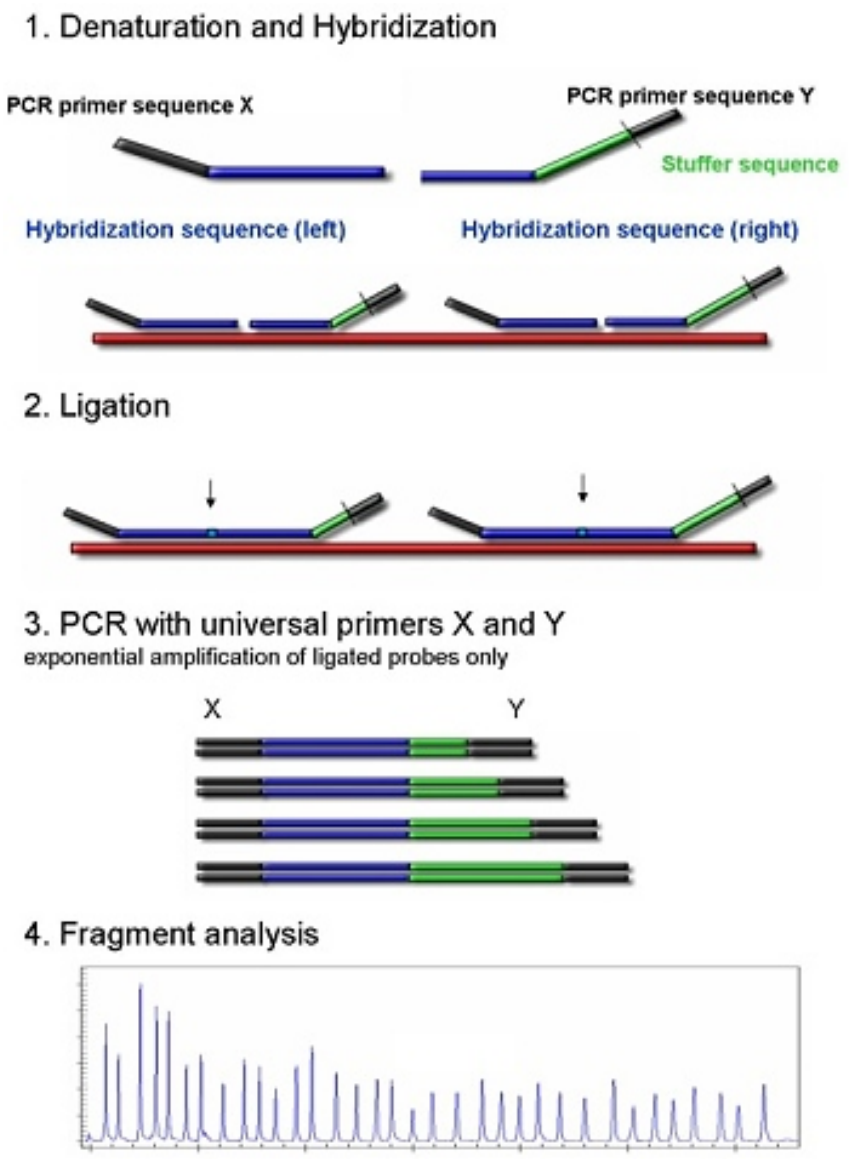
High resolution

Limitations:

Technically sophisticated and significant platform investment required

Probes must be in unique regions

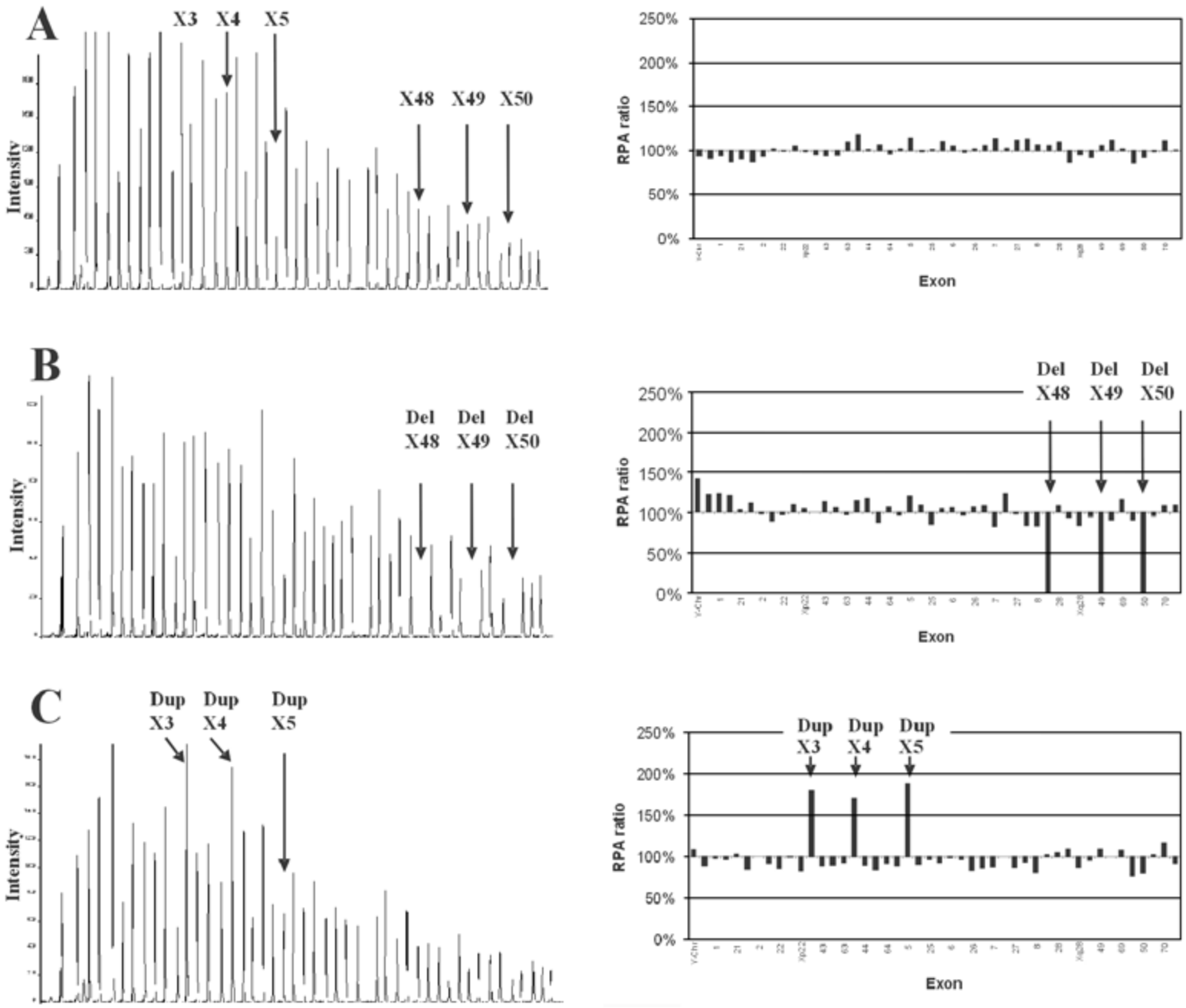
Multiplex Ligation-dependent Probe Amplification (MLPA)



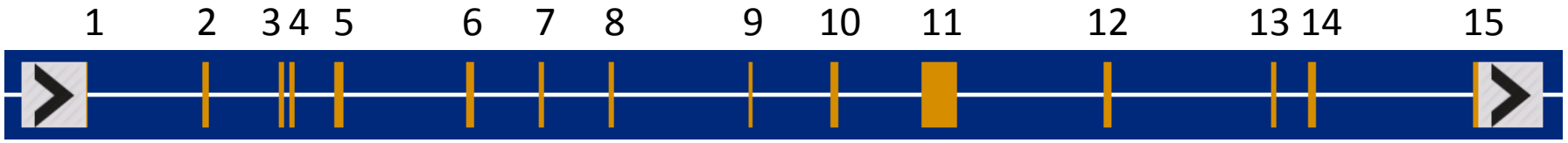
1 – 2 probes per exon

Capillary electrophoresis

www.MLPA.com


A
X3
X4
X5
X48
X49
X50
Intensity
RPA ratio
250%
200%
150%
100%
50%
0%
Exon
B
Del
X48
Del
X49
Del
X50
Intensity
RPA ratio
Exon
C
Dup
X3
Dup
X4
Dup
X5
Intensity
RPA ratio
Exon

PMS2




```
                                  70        80        90       100       110       120
                                  |         |         |         |         |         |
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chr7_99929836       CTTTGTTTCTTGTAACTGATTTCTTTAGTACAGAACCTGCTAAGGCCATCAAACCTATTG
chr7_6045296active  ATTTGTTTCTTGTAACTGATTTCTCTAGTACAGAACCTGCTAAGGCCATCAAACCTATTG
```

Intron 1 Exon 2

Table 1. SALSA MLPA P008-C1 PMS2 probemix

Length (nt)	SALSA MLPA probe	Chromosomal position Reference	PMS2	PMS2CL
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA			
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation			
100	X-fragment: Specific for the X chromosome			
105	Y-fragment: Specific for the Y chromosome			
128	Reference probe 00797-L00093	5q31		
133	**PMS2 probe** 14452-L00900		Exon 11	
140	**PMS2 probe** 14448-L16160		Exon 9	
146	**PMS2 probe** 07935-L16148		Exon 1	
154	Reference probe 02417-L04306	6p21		
160	Reference probe 08583-L08584	17q23		
165	**PMS2 probe** 14453-L16164		Exon 11 (SNP)	
171	**PMS2 probe** 14453-L16165			Exon 11 (SNP)
177	Reference probe 04359-L03779	7p13		
184	**PMS2 probe** 01176-L16620		Exon 2	
190	**PMS2 probe** 15768-L18167		**Exon 14**	**Exon 14**
196	Reference probe 07510-L07172	14q24		
202	**PMS2 probe** 14458-L16176		Exon 14 (SNP)	
208	**PMS2 probe** 14458-L16177			Exon 14 (SNP)
214	**PMS2 probe** 14456-L16511		Exon 13 (SNP)	
220	**PMS2 probe** 14456-L16512			Exon 13 (SNP)
226	Reference probe 07083-L06712	11p13		
232	**PMS2 probe** 14445-L16154		Exon 5	
238	**PMS2 probe** 14455-L16168		Intron 12 (SNP)	
244	**PMS2 probe** 14455-L16169			Intron 12 (SNP)
250	**PMS2 probe** 01180-L16157		Exon 6	
261	**PMS2 probe** 15767-L17448		**Exon 13**	**Exon 13**
268	Reference probe 19040-L09299	3q29		
276	**PMS2 probe** 01181-L16158		Exon 7	
283	**PMS2 probe** 15769-L17786		**Exon 12**	**Exon 12**
292	Reference probe 11087-L11770	2p24		
299	**PMS2 probe** 01182-L16159		Exon 8	
310 *	**PMS2 probe** 19910-L26895		Exon 3	
319	**PMS2 probe** 01184-L00745		Exon 10	
328	Reference probe 08543-L08544	3q24		
339	**PMS2 probe** 07934-L16147		Exon 1	
349	**PMS2 probe** 14460-L04046		Exon 15 (SNP)	
356	**PMS2 probe** 14460-L16180			Exon 15 (SNP)
364	**PMS2 probe** 14451-L16163		Exon 11	
373	Reference probe 02528-L01959	17q11		
382	**PMS2 probe** 15293-L17051		**Exon 14**	**Exon 14**
390 *	**PMS2 probe** 19915-L26898		Exon 3	
400	**PMS2 probe** 14441-L16150		Exon 2	
409	**PMS2 probe** 01189-L00750		**Exon 15**	**Exon 15**
418 *	**PMS2 probe** 19906-L26893		Exon 4	
427	Reference probe 06029-L05485	11p13		
436	**PMS2 probe** 14447-L16623		Exon 6	
445	**PMS2 probe** 14449-L16622		Exon 9	
454	**PMS2 probe** 14446-L16621		Exon 5	
463	**PMS2 probe** 14450-L16162		Exon 10	
472	Reference probe 15978-L18133	8q12		
483	Reference probe 08480-L08491	10p12		

SNP-specific Probes hybridizing to either *PMS2* or *PMS2CL*

Universal Probes hybridizing to both *PMS2* and *PMS2CL*

SNP-specific Probes hybridizing to either *PMS2* or *PMS2CL*

SNP-specific Probes hybridizing to either *PMS2* or *PMS2CL*

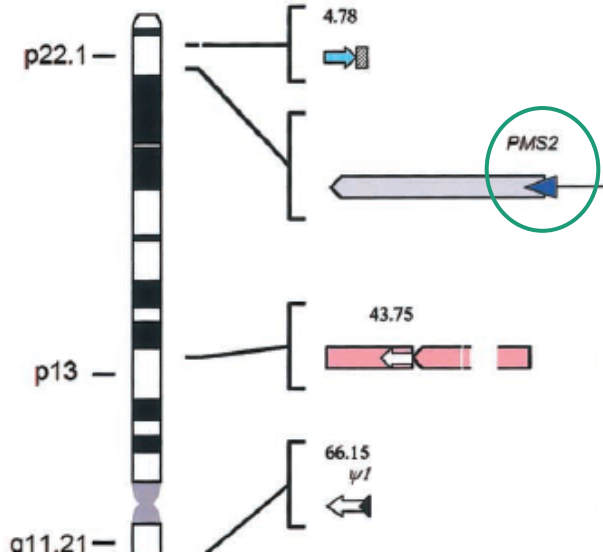
Universal Probes hybridizing to both *PMS2* and *PMS2CL*

Universal Probes hybridizing to both *PMS2* and *PMS2CL*

SNP-specific Probes hybridizing to either *PMS2* or *PMS2CL*

Universal Probes hybridizing to both *PMS2* and *PMS2CL*

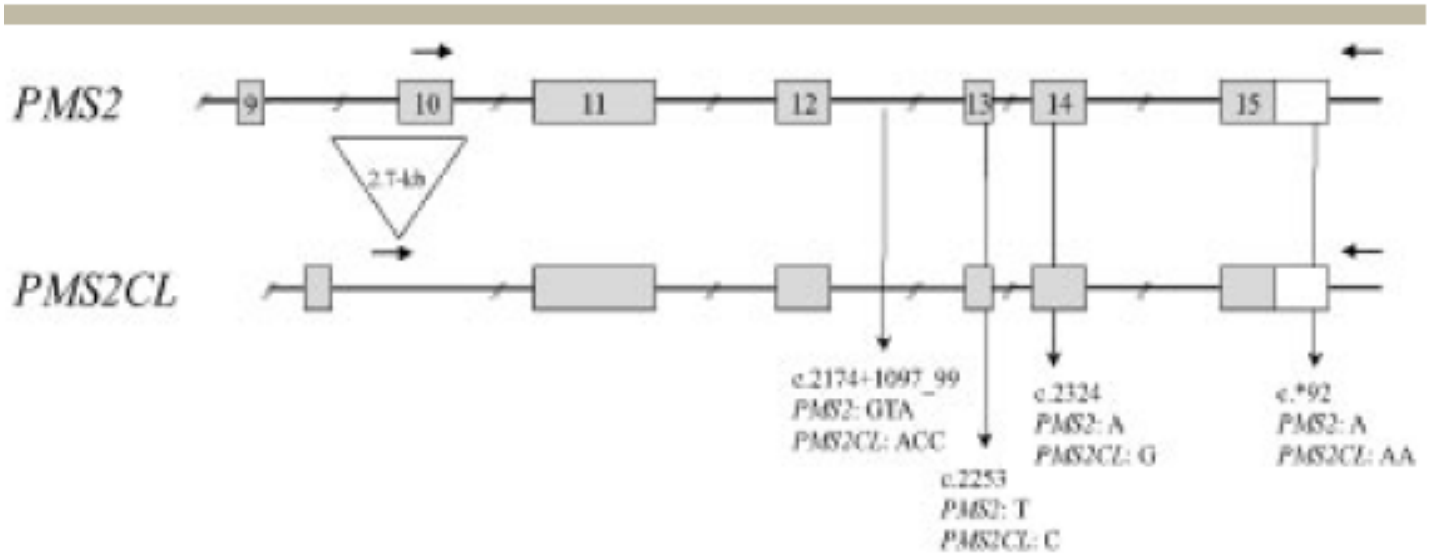
Universal Probes hybridizing to both *PMS2* and *PMS2CL*



Pseudogene exons 9-15

PMS2CL:

- inverted duplication of a 100-kb repeat element
- homologous the 3’ region of *PMS2*
- six exons (9 and 11-15), but lacks exon 10 due to an Alu-mediated 2.7-kb deletion.



PMS2 copy number by MLPA

- Reference:
  - 2 copies *PMS2*
  - 2 copies *PMS2CL*

The reference has two copies of each

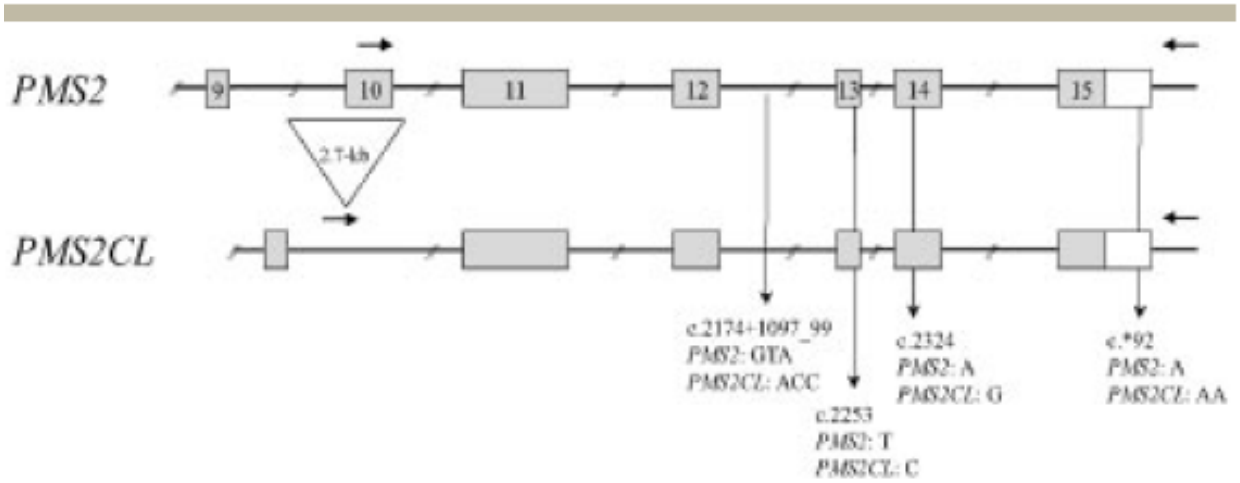
- *PMS2*- and *PMS2CL*-specific probes relative ratio:
  - 0= 0 copy
  - 0.5= 1 copy
  - 1= 2 copies
  - 1.5= 3 copies
  - 2= 4 copies

Compare patient to reference!

- *PMS2*- and *PMS2CL* universal probes relative ratio:
  - 0= 0 copy
  - 0.25= 1 copy
  - 0.5= 2 copies
  - 0.75= 3 copies
  - 1= 4 copies
  - 1.25= 5 copies
  - 1.5= 6 copies.
- Ten SNP-specific probes targeting exon 11-15 detect a polymorphic sequence: copy numbers for these probes in normal individuals can be 0, 1, 2, 3 or 4. The combined copy number of the two probes of one pair of SNP probes should be 4 in normal individuals.

LR-PCR Sequencing

- Recombination with crossover and/or gene-conversion between *PMS2* and *PMS2CL:*
  - hybrid *PMS2* alleles that contain *PMS2CL-derived* sequences
  - hybrid *PMS2CL* alleles with sequences derived from *PMS2*.
- *PMS2* or *PMS2CL* long-range PCR to amplify exons 11-15



Vaughn et al., 2011

	MLPA ratios			Possible genotypes[d]		Corresponding sequencing results[d]		
	Both[a]	PSV-G[b]	PSV-P[c]	*PMS2*	*PMS2CL*	*PMS2*	*PMS2CL*	*PMS2* deletion
A1	1	1	1	GG	PP			No
A2				GP	GP			No
A3				PP	GG			No
A4	1	0.5	1.5	GP	PP	No deletions present in the sample; sequencing not necessary		No
A5				PP	GP			No
A6	1	1.5	0.5	GG	GP			No
A7				GP	GG			No
A8	1	0	2	PP	PP			No
A9	1	2	0	GG	GG			No

Probes complimentary to the *PMS2* reference sequence are designated as "PSV-G"

Probes complimentary to the *PMS2CL* reference sequence are designated as "PSV-P"

Probes that bind both the gene and pseudogene are designated as "Both"

"G" corresponding to the *PMS2* reference sequence

"P" corresponding to the *PMS2CL* reference sequence

BUT - Recombination with crossover and/or gene-conversion between *PMS2* and *PMS2CL* produces hybrid *PMS2* alleles that contain *PMS2CL-derived* sequences as well as hybrid *PMS2CL* alleles with sequences derived from *PMS2*

	MLPA ratios			Possible genotypes[d]		Corresponding sequencing results[d]		
	Both[a]	PSV-G[b]	PSV-P[c]	*PMS2*	*PMS2CL*	*PMS2*	*PMS2CL*	*PMS2* deletion
B1	0.75	0.5	1	G_	PP	G	P	Yes
B2				GP	P_	GP	P	No
B3				PP	G_	P	G	No
B4				P_	GP	P	GP	Yes
B5	0.75	1	0.5	GG	P_	G	P	No
B6				G_	GP	G	GP	Yes
B7				GP	G_	GP	G	No
B8				P_	GG	P	G	Yes

Probes complimentary to the *PMS2* reference sequence are designated as "PSV-G"

Probes complimentary to the *PMS2CL* reference sequence are designated as "PSV-P"

Probes that bind both the gene and pseudogene are designated as "Both"

"G" corresponding to the *PMS2* reference sequence

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BUT - Recombination with crossover and/or gene-conversion between *PMS2* and *PMS2CL* produces hybrid *PMS2* alleles that contain *PMS2CL-derived* sequences as well as hybrid *PMS2CL* alleles with sequences derived from *PMS2*

	MLPA ratios			Possible genotypes[d]		Corresponding sequencing results[d]		
	Both[a]	PSV-G[b]	PSV-P[c]	*PMS2*	*PMS2CL*	*PMS2*	*PMS2CL*	*PMS2* deletion
B9	0.75	0	1.5	PP	P_	Cannot differentiate by sequencing		Family studies required to determine whether deletion is in *PMS2* or *PMS2CL*
B10				P_	PP			
B11	0.75	1.5	0	GG	G_	Cannot differentiate by sequencing		
B12				G_	GG			

Probes complimentary to the *PMS2* reference sequence are designated as "PSV-G"

Probes complimentary to the *PMS2CL* reference sequence are designated as "PSV-P"

Probes that bind both the gene and pseudogene are designated as "Both"

"G" corresponding to the *PMS2* reference sequence

"P" corresponding to the *PMS2CL* reference sequence

BUT - Recombination with crossover and/or gene-conversion between *PMS2* and *PMS2CL* produces hybrid *PMS2* alleles that contain *PMS2CL-derived* sequences as well as hybrid *PMS2CL* alleles with sequences derived from *PMS2*

Table 2. Algorithm for Determining *PMS2* Deletions in Exons 12–15

	MLPA ratios			Possible genotypes[d]		Corresponding sequencing results[d]		
	Both[a]	PSV-G[b]	PSV-P[c]	*PMS2*	*PMS2CL*	*PMS2*	*PMS2CL*	*PMS2* deletion
A1	1	1	1	GG	PP			No
A2				GP	GP			No
A3				PP	GG			No
A4	1	0.5	1.5	GP	PP	No deletions present in the sample; sequencing not necessary		No
A5				PP	GP			No
A6	1	1.5	0.5	GG	GP			No
A7				GP	GG			No
A8	1	0	2	PP	PP			No
A9	1	2	0	GG	GG			No
B1	0.75	0.5	1	G_	PP	G	P	Yes
B2				GP	P_	GP	P	No
B3				PP	G_	P	G	No
B4				P_	GP	P	GP	Yes
B5	0.75	1	0.5	GG	P_	G	P	No
B6				G_	GP	G	GP	Yes
B7				GP	G_	GP	G	No
B8				P_	GG	P	G	Yes
B9	0.75	0	1.5	PP	P_	Cannot differentiate by sequencing		Family studies required to determine whether deletion is in *PMS2* or *PMS2CL*
B10				P_	PP			
B11	0.75	1.5	0	GG	G_	Cannot differentiate by sequencing		
B12				G_	GG			
C1	0.5	0.5	0.5	G_	P_	G	P	Yes
C2				GP	__	GP	No amplification	No
C3				P_	G_	P	G	Yes
C4				__	GP	No amplification	GP	Yes (homozygous)
C5	0.5	0	1	PP	__	P	No amplification	No
C6				P_	P_	P	P	Yes
C7				__	PP	No amplification	P	Yes (homozygous)
C8	0.5	1	0	GG	__	G	No amplification	No
C9				G_	G_	G	G	Yes
C10				__	GG	No amplification	G	Yes (homozygous)
D1	0.25	0	0.5	P_	__	P	No amplification	Yes
D2				__	P_	No amplification	P	Yes (homozygous)
D3	0.25	0.5	0	G_	__	G	No amplification	Yes
D4				__	G_	No amplification	G	Yes (homozygous)
E1	0	0	0	__	__	No amplification	No amplification	Yes (homozygous)

Probes complimentary to the *PMS2* reference sequence are designated as "PSV-G"

Probes complimentary to the *PMS2CL* reference sequence are designated as "PSV-P"

Probes that bind both the gene and pseudogene are designated as "Both"

"G" corresponding to the *PMS2* reference sequence

"P" corresponding to the *PMS2CL* reference sequence

Sequencing cannot resolve the location of a deletion if all the alleles harbor the same sequence at each PSV site.

Vaughn et al., 2011

From a clinical laboratory….

PMS2/PMS2CL	Gross Deletion:	EX13_14del **(see COMMENT)**

SUMMARY

INCONCLUSIVE: Variants of Unknown Significance Detected

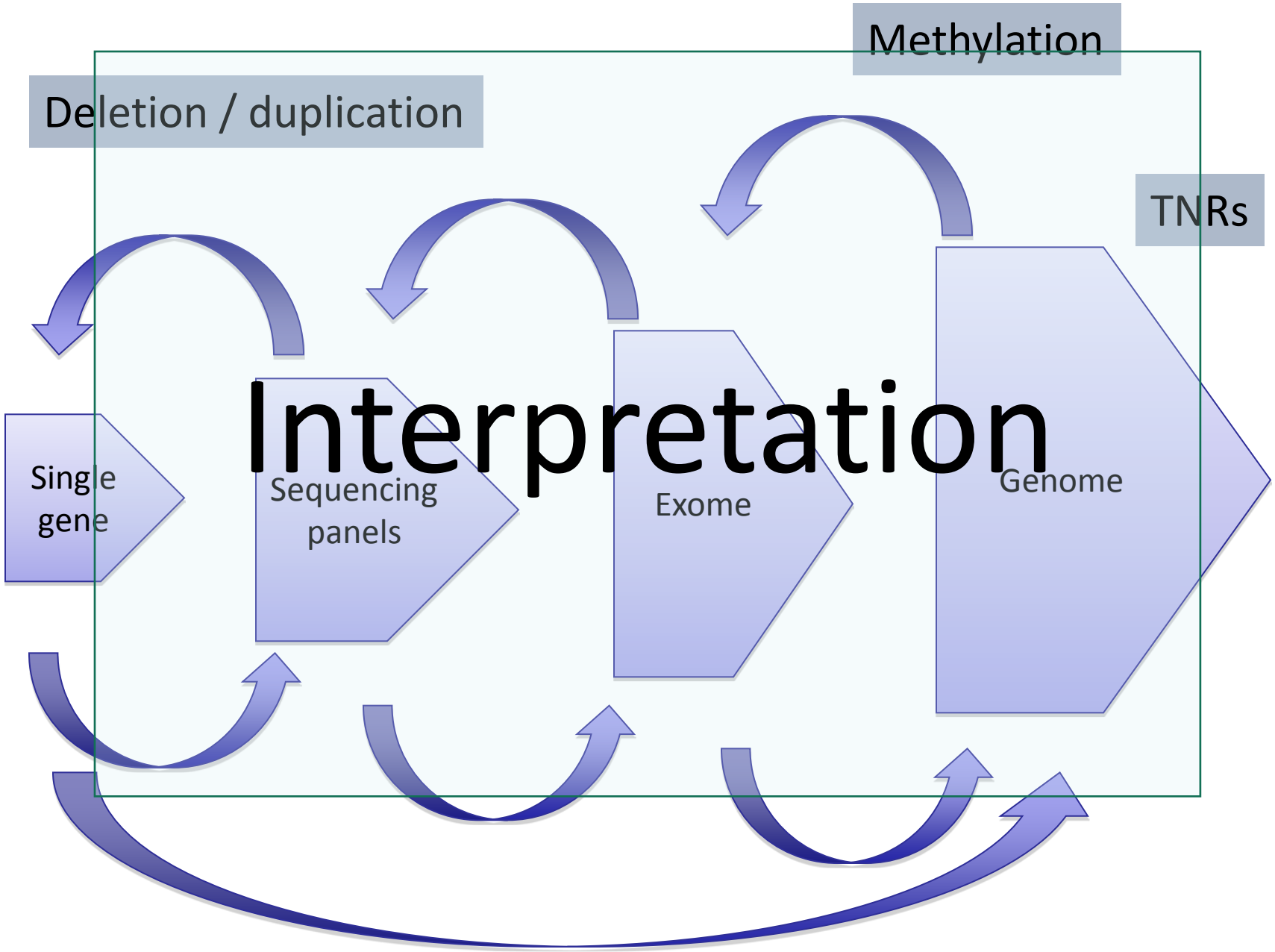
COMMENT: Deletion/duplication analysis of the 3' region of the *PMS2* gene is complicated by significant homology with the *PMS2CL* pseudogene and frequent gene conversion events. Double stranded sequencing of exons 13 and 14 of the pseudogene *PMS2CL* can sometimes be performed to help clarify an individual's result. However, based on the MLPA data, this analysis is not expected to be informative for this patient, and for this reason, it was not performed. Therefore, the possibility that the EX13_14del gross deletion is in the *PMS2CL* pseudogene cannot be ruled out.

Table 3. Patient Results **Vaughn et al., 2011**

Patient no.	Exon	MLPA ratios: *PMS2*-specific[a,b]	MLPA ratios: Both[c,b]	MLPA ratios: PSV-G[d]	MLPA ratios: PSV-P[e]	Sequence results[f,g]: *PMS2*	Sequence results[f,g]: *PMS2CL*	Corresponding rows from algorithm (Table 2)	Result
1	9	0.471, 0.518							*PMS2* deletion, exons 9–15
	10	0.514, 0.536							
	11	0.530, 0.529							
	12		0.776	0.494	1.004	G	P	B1	
	13		0.797	0.495	0.971	G	P	B1	
	14		0.798, 0.752	0.476	0.961	G	P	B1	
	15		0.737	0.541	0.991	G	P	B1	

Gene	Proportion of Lynch Syndrome	Proportion of Pathogenic Variants Detectable by this Method	
		Sequence analysis	Gene-targeted deletion/duplication analysis
MLH1	50%	90-95%	5-10%
MSH2	40%	~80%	~20%
MSH6	7%-10%	>95%	Rare
PMS2	<5%	~80%	~20%
EPCAM	~1%-3%	None	All

Is it worth the limitations?


Methylation
Deletion / duplication
TNRs
Interpretation
Single gene
Sequencing panels
Exome
Genome

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