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RETINOBLASTOMA GENETIC SCREENING UNIT

RNA ANALYSIS FOR INTERPRETATION OF SPLICE MUTATIONS IN RETINOBLASTOMA

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- Retinoblastoma: tumour of the developing retina
- Incidence: 1/15, 000 to 1/20,000
- 2-3% of all childhood cancers
- RB1 gene mutation screening in new patients: SSCP/HDX, dosage, methylation analysis
- Linkage analysis and carrier testing for previously characterised families

RB1 Gene Mutations

- Cytogenetically detectable 8%
- **Abnormalities detected at the molecular level** **>90%**

Molecular level

- Substitutions 60%
- Small deletions/insertions 20%
- Large rearrangements 10-15%
- Hypermethylation (tumours) 10-15%

RB1 Gene Mutations

- Stop Codon 75%
  frameshift to stop
  nonsense
- **Splicing errors** **20%**
- Missense 5%

RB1 Variants

RNA work is required for unclassified variants –

- changes in splice consensus sequences that are outside the invariant nucleotides
- other intronic changes (deep intronic)
- missense changes
- branch site changes

If there is no evidence for pathogenicity in the literature

RB1 Mutations

RNA work is also conducted

- To gain an insight into the penetrance of splice mutations.

  Some RB1 in-frame changes have reduced penetrance

- To confirm whole exon deletions.

RNA Extraction Methods

- From tumour cells - Tri Reagent (Sigma)
- From whole blood - PAXgene (PreAnaltiX/Qiagen) RNA blood
  - PAXgene RNA blood can be extracted using either the PAX gene extraction kit or using TriReagent

RT-PCR

Using Qiagen ‘OneStep’ kit

- Gene specific primers encompassing exon-exon junctions are used

RB1 splice variants tested so far

RB1 Int	DNA c.	RNA r.	Protein p.	Predicted Change
05	540-10T>A	539_540ins540-8_540-1	I181LfsX8	Create cryptic sa; inclusion of int
06	608-12T>G	607_608ins608-11_608-1	G203VfsX15	Create cryptic sa; inclusion of int
10	1049+2T>C	940_1049del	V314FfsX2	Abolish sd; skip ex 10
17	1696-2A>G	1698_1733del	D566_K577del	Abolish sa; use cryptic sa
19	1961-3C>G	1960_1961ins1961-2_1961-1	V654EfsX5	Create cryptic sa; Inclusion of int

Splice Acceptor Site Mutation

t/c>11 n c/t ag | G

Intron 17 splice acceptor - invariant base mutated:

intron 17 exon 18

tttcatat **ag** | GATTCACCTTTATTTGATCTTATTAAACAATCAA **AG** GACC

|

g

alternative site in exon 18 AG | GAC……

Skip exon 18, or a 36 bp in-frame deletion in exon 18?

A heterozygous A>G abolishes the splice acceptor site in intron 17. A cryptic splice site in exon 18 is utilized leading to an in-frame deletion in the exon (p.D566_K577del).

Splice Site Variant

t/c>11 n c/t ag | G

Intron 05 splice acceptor site altered:

intron 05 exon 06

ctgctttctatt t g tttaat **ag** | GATATCTACTG

|

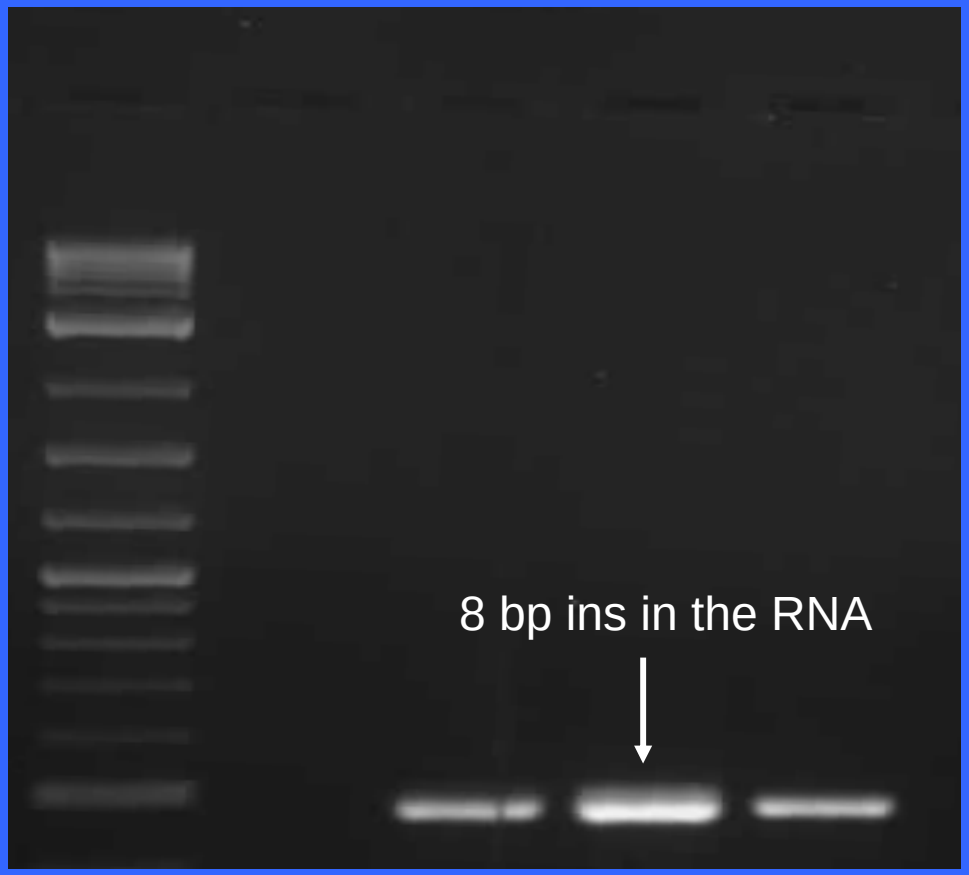
a

New site **ag** | tttaatag 8 bp insertion

Invariant bases are maintained

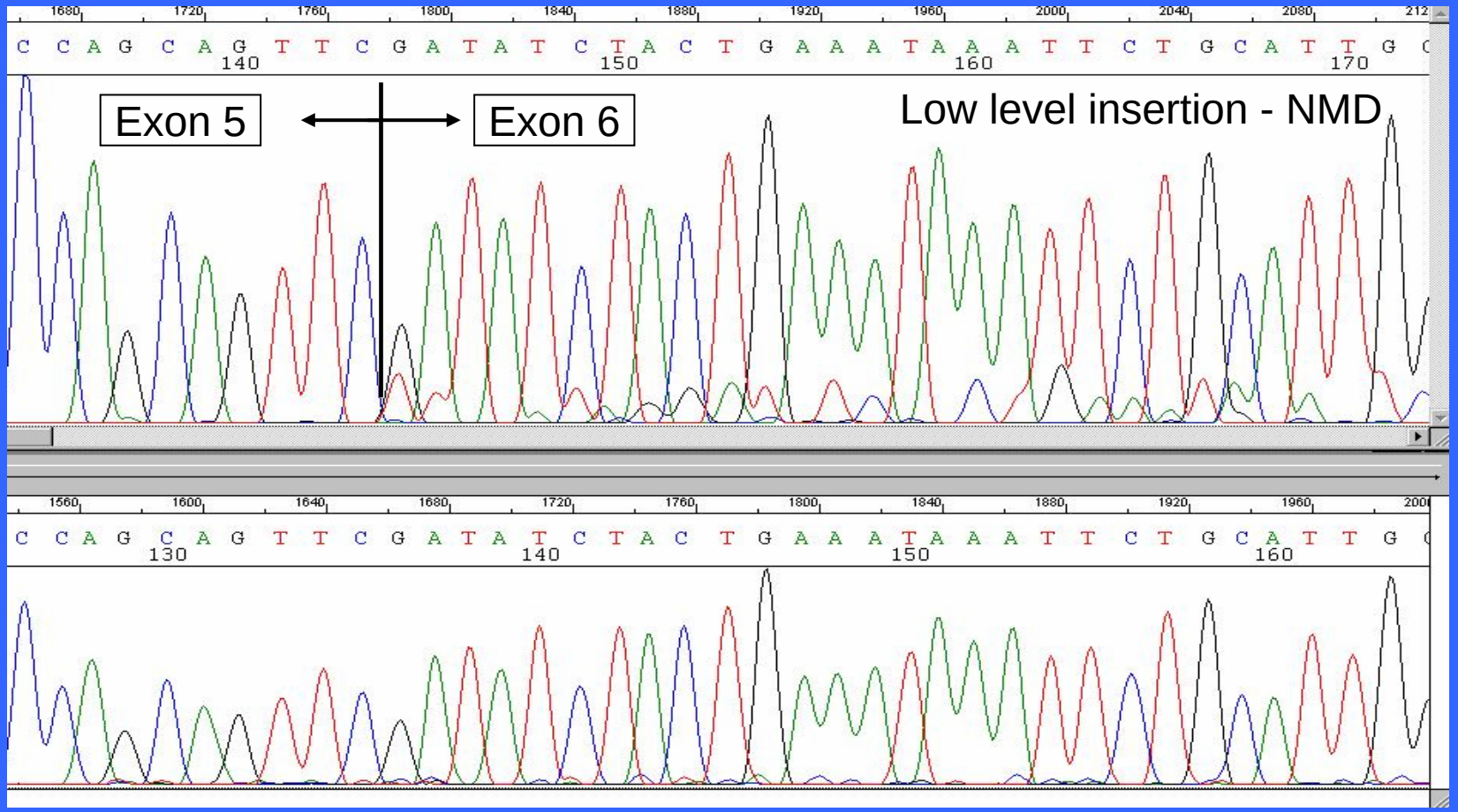
A cryptic splice acceptor created?

A heterozygous T>A in intron 5 creates a cryptic splice acceptor. The inclusion of 8 bp of intronic sequence in exon 6 leads to a Stop Codon (p.I181LfsX8).



Exons 2/3 – 7/8

A heterozygous T>A in intron 5 creates a cryptic splice acceptor. The inclusion of 8 bp of intronic sequence in exon 6 leads to a Stop Codon (p.I181LfsX8).



Splice Site Variant

t/c>11 n c/t ag | G

Intron 19 splice acceptor site altered:

intron 19 exon 20

ttttctta ttccc a **c ag** | TGTATCGG

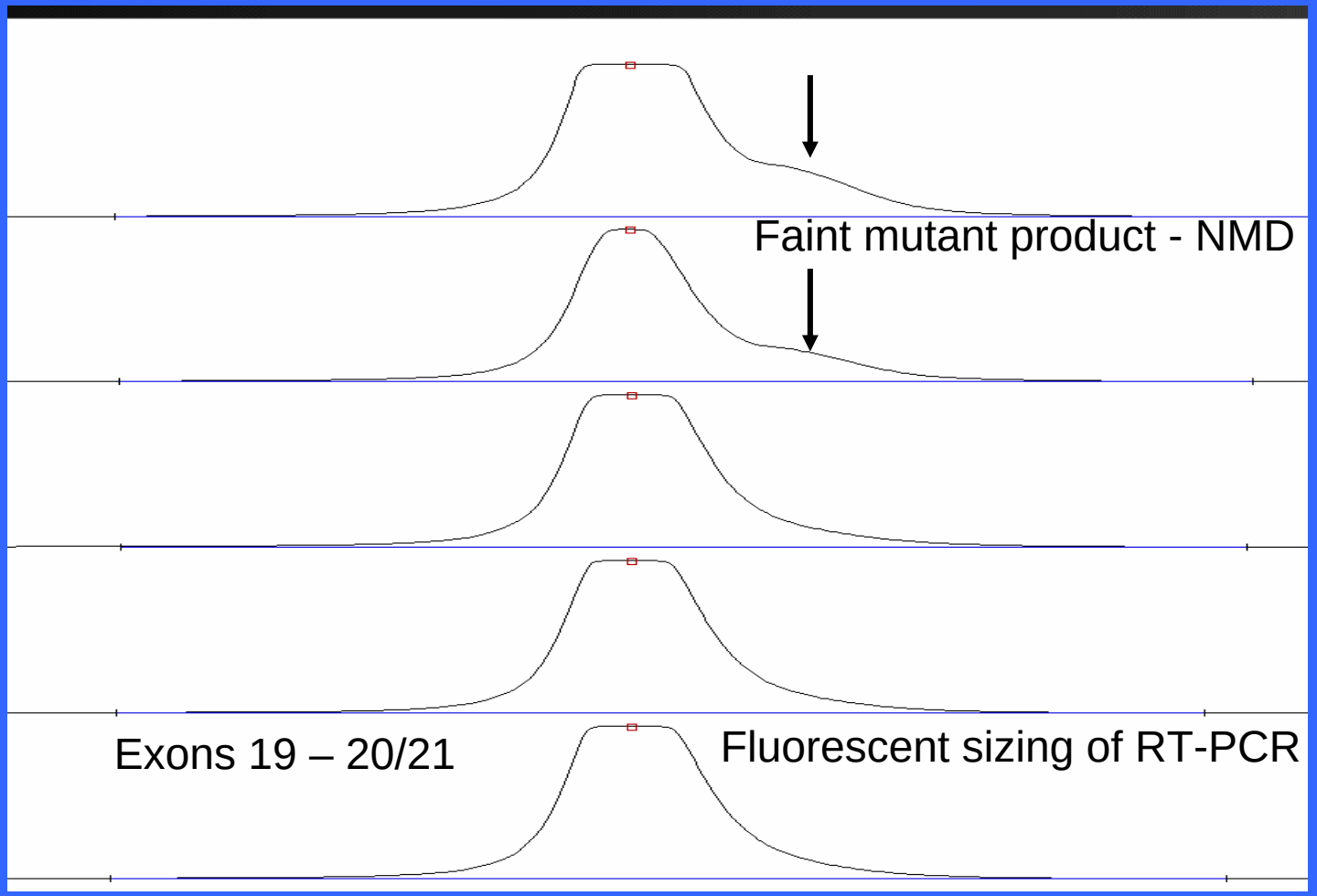
|

g

New site **ag** | ag 2 bp insertion from intron

Reduced consensus value of the wild-type.
A cryptic splice acceptor created?

A heterozygous C>G creates a cryptic splice acceptor site in intron 19. The inclusion of 2 bp of intronic sequence in exon 20 leads to a Stop Codon (p.V654EfsX5).



Splice Site Mutation

C/A AG | gt a/g agt

Intron 10 splice donor site: an invariant base mutated

investigated two possible outcomes

exon 10 intron 10

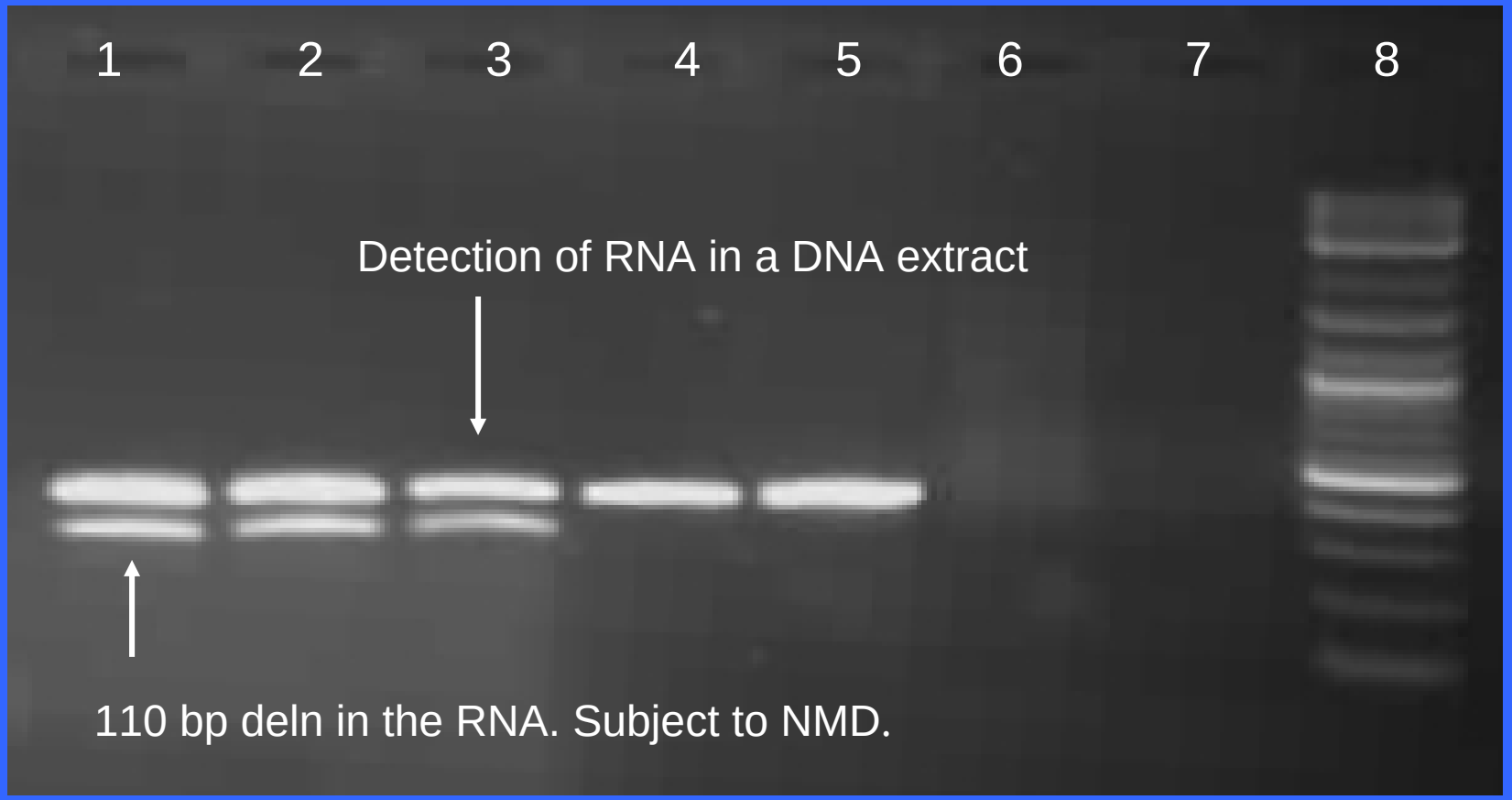
ATA GA C AG | **g t** at tgc aca tg **gt** at atttgat

|

c

1) donor site abolished – exon 10 skipping
2) downstream cryptic site used – in-frame12 bp insertion from intron 10

A heterozygous T>C at the splice donor site in intron 10 leads to exon 10 skipping (p.V314FfsX2). The abnormal product is present at a lower intensity than the normal allele.



RB1 whole exon deletions tested using RT-PCR

- Single exon deletion of exon 12

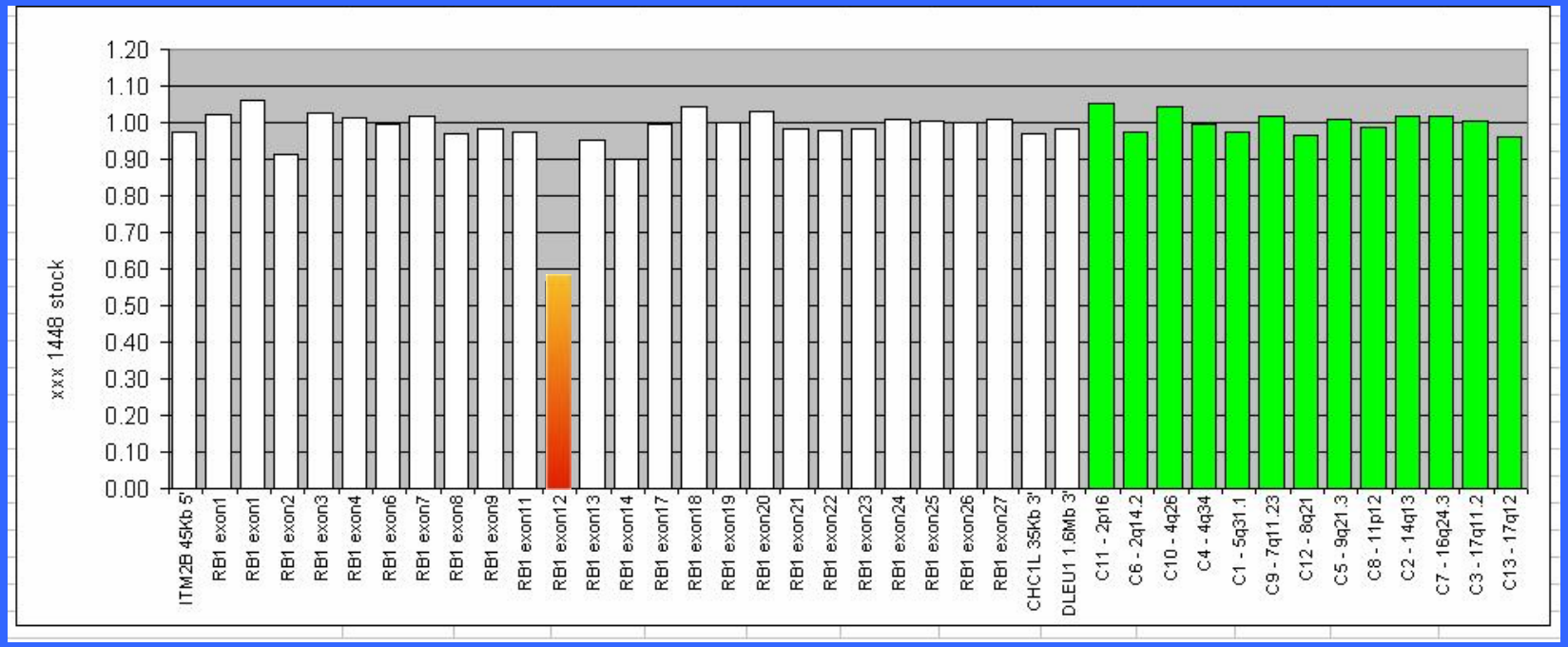
  88bp deleted; out of frame message
- Singe exon deletion of exon 13

  117 bp deleted; in-frame message
- Whole exon deletion of exons 21,22,23

  383bp deleted; out of frame message

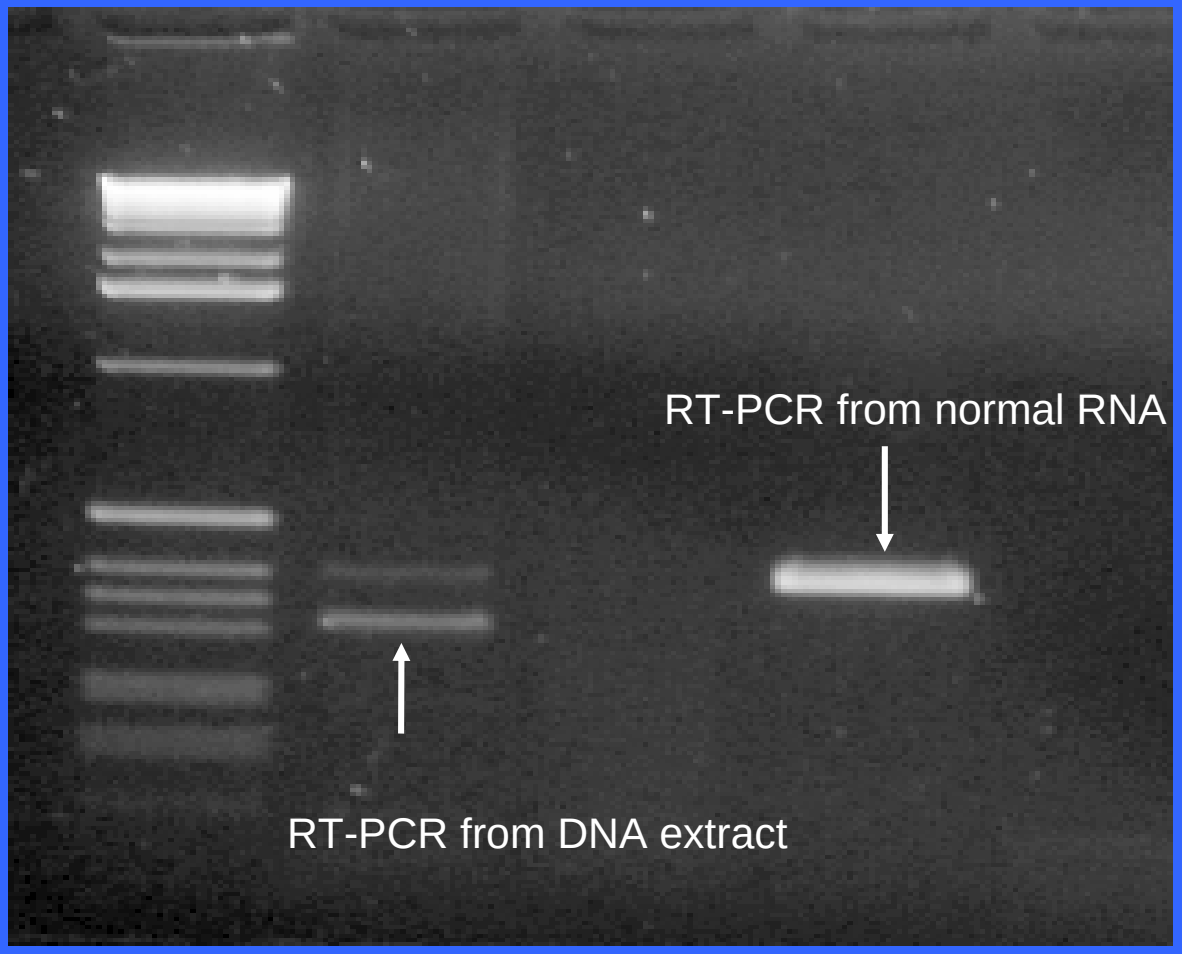
  homozygously deleted in tumour

A heterozygous deletion of exon 12 detected by QF-PCR and MLPA . Breakpoints identified by Long Range PCR and sequencing of genomic DNA.



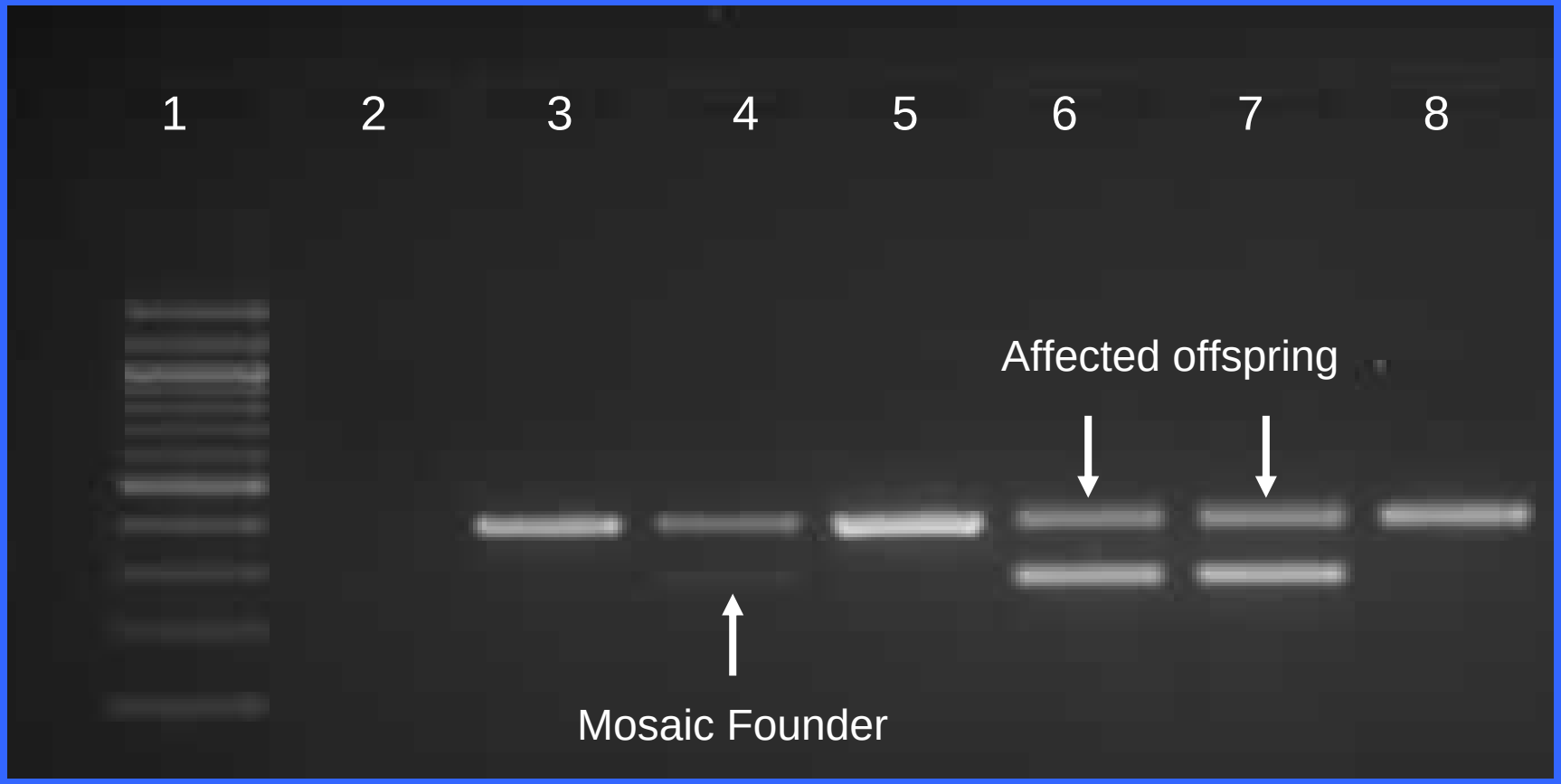
RB1 MLPA dosage analysis

Out of frame loss of 88 bp from RNA (lost exon 12) confirmed by RT-PCR and sequencing (p.V378AfsX3). RNA detectable in a Qiagen Midkit DNA extract.

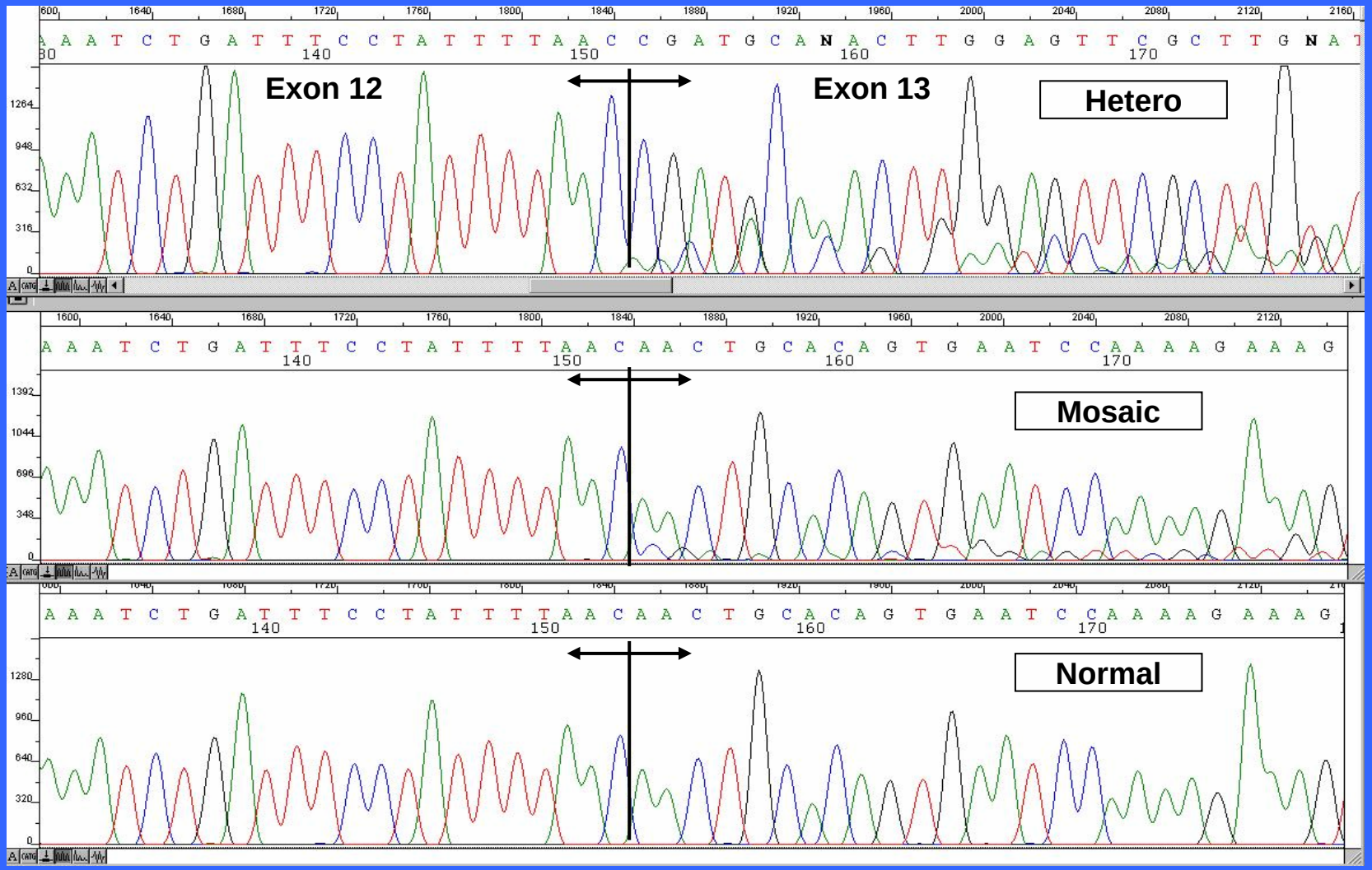


Exons 10/11 – 15/16

Single exon 13 deletion – detected by in-house QF-PCR but missed by commercial MLPA kit. Confirmed in-frame loss of 117 bp from RNA by RT-PCR and sequencing (p.N406_Q444del).



Single exon 13 deletion. Confirmed in-frame loss of 117 bp from RNA (p.N406_Q444del).



Future RNA work

- missense changes to see if they affect splicing
- various intronic unclasssified variants
- a suspected branch point mutation
- a 3’RB1 gene deletion in a tumour

Splice Sites

Intron ————**AG-** E X O N **-GT** ——Intron

- Splice Acceptor

  T/C>11 N C/T **AG** | G

- Splice Donor

  C/A AG | **GT** A/G AGT

- Branch Point

  TNCT A/G A C