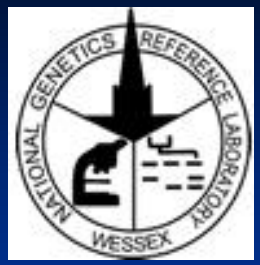


Evaluation of High Resolution Melt Curve Analysis for Mutation Scanning

Helen White

National Genetics Reference Lab (Wessex), Salisbury

NGRL (Wessex) Evaluation protocol

- PCR optimised with LC Green Plus
- 11 different amplicons analysed; 7 plasmid based, 4 genomic DNA
  - Size range: 139bp – 449bp
  - GC content: 22% - 79%
  - Types of mutation: All possible heteroduplex types
    ins C, ins AA
    del A, del C, del CA
- Amplicons amplified using RotorGene 6000 in 20ul reactions
- Amplification efficiency monitored using real time PCR
- Same amplicons analysed using HRM
- Machines evaluated:
  - HR-1 (Idaho Technology)
  - 384 well LightScanner (Idaho Techonology)
  - Rotor Gene 6000 (Corbett Research)

HR-1™ Instrument (Idaho Technology)

Single sample (glass capillary tubes)

Temp Control +/- 0.05 °C

5-20 µl Capacity

35 samples per hour with a 0.3°C ramp rate (after amplification)

HRM only

LightScanner™ Instrument (Idaho Technology)

Standard 96 or 384 microtiter plate

Temp Control +/- 0.1 °C

5-20 µl Capacity

15 minutes per run (after amplification)

HRM only

Rotor-Gene™ 6000 (Corbett Life Science)

36 / 72/ 100 well rotor format

Thermal uniformity ±0.01°C, Resolution ±0.02°C,

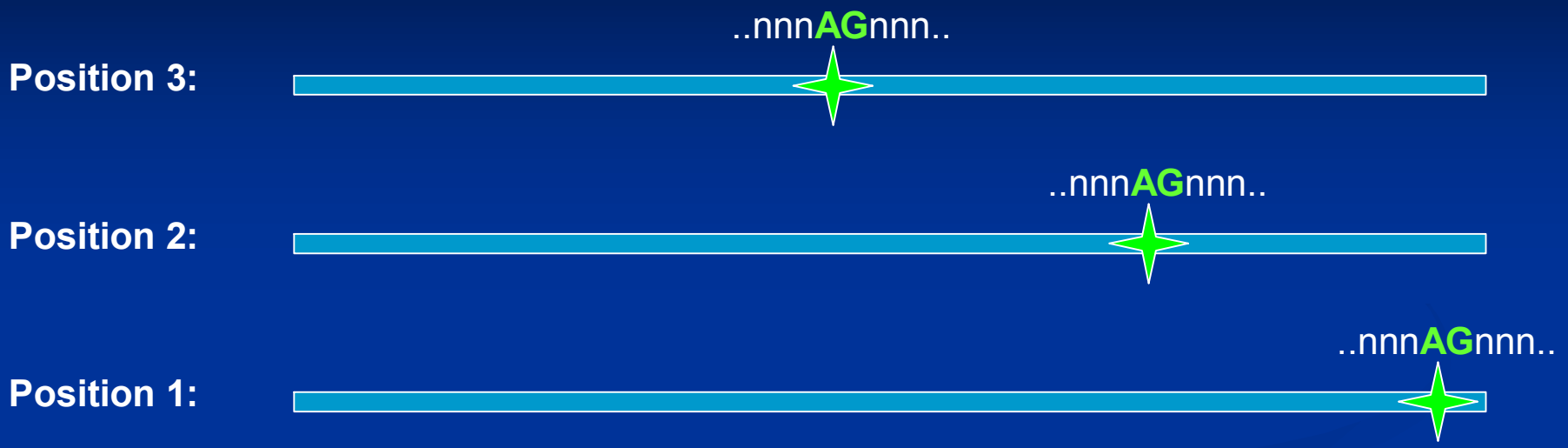
HRM data acquisition (read) rate: 20 reads for each 0.02°C increment

5-20 µl Capacity

15 minutes per run (after amplification)

HRM, real time PCR and allelic discrimination (5 colours)

Generic Mutation Detection Reference Reagents

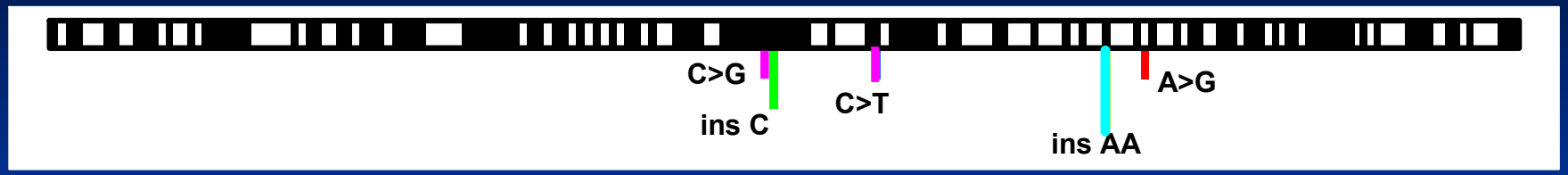


Mutation created	Sequence generated	Heteroduplex produced
A > C	nnnCGnnn	C:T & G:A
A > T	nnnTGnnn	T:T & A:A
G > A	nnnAAnnn	A:C & G:T
G > C	nnnACnnn	C:C & G:G

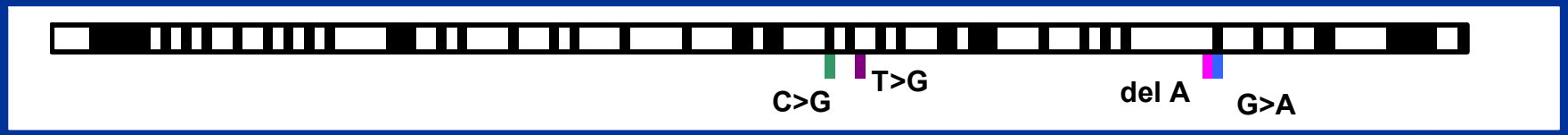
Amplicons and Mutations Analysed I

Amplicons and Mutations Analysed II

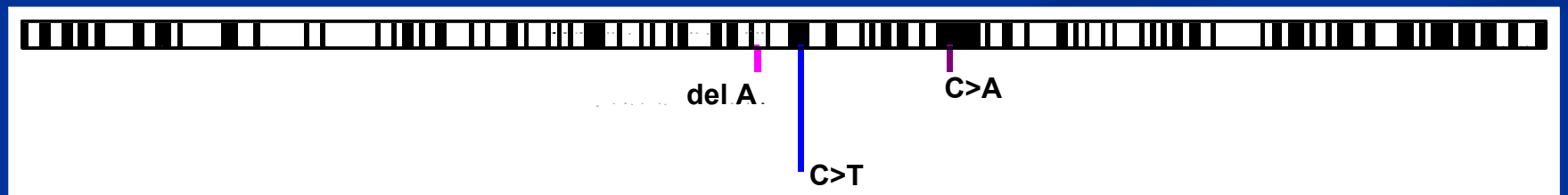
hMLH1 Exon 1 (193bp, 57% GC Rich)



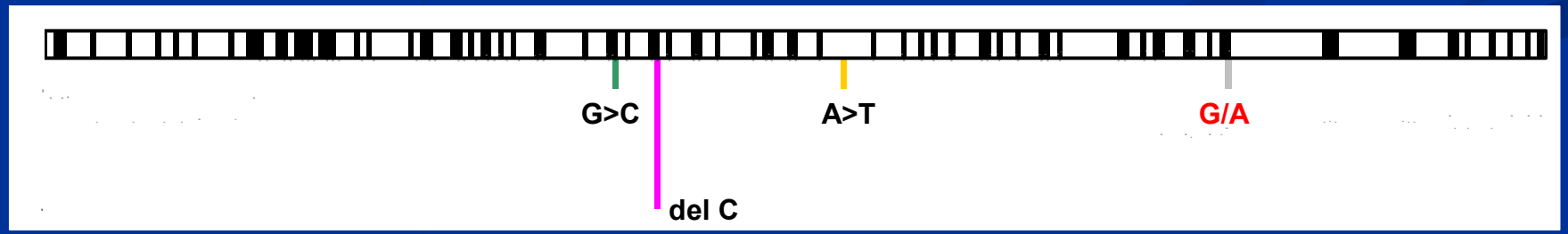
hMLH1 Exon 7 (139bp, 37% GC Rich)



hMLH1 Exon 13 (277bp, 44% GC Rich)



hMSH2 Exon 10 (249bp, 34% GC Rich)



Samples

Plasmid based samples (47 samples total)

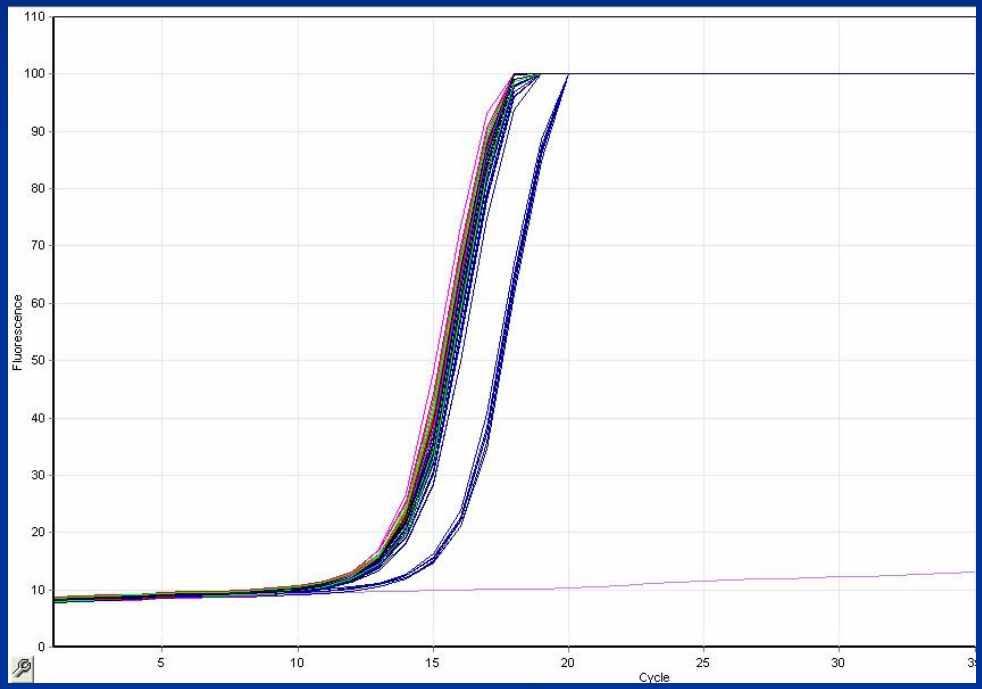
- 24 mutated samples (duplicates of each mutation at each position)
- 23 wild type samples
- 1 NTC
- Randomised differently for each GC content

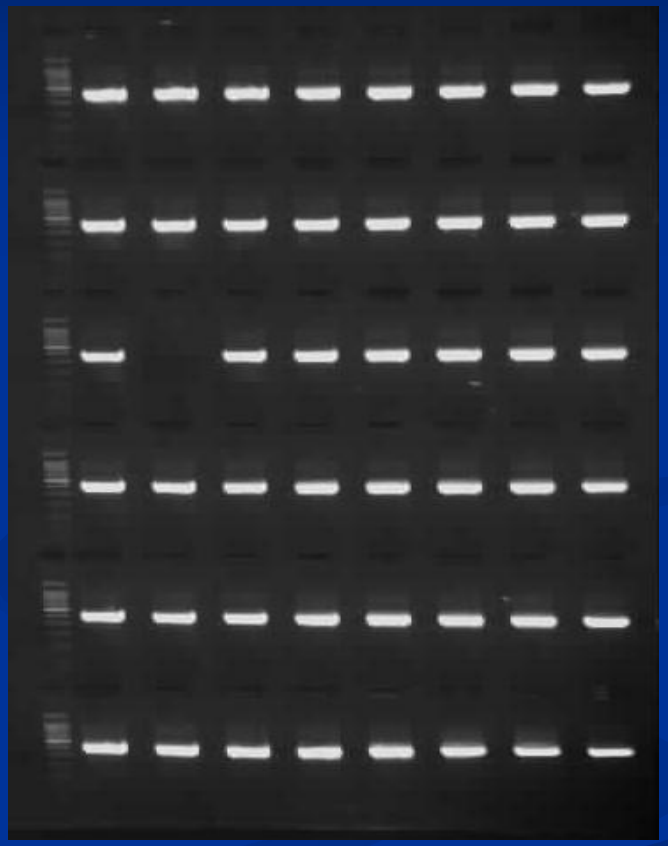
Genomic DNA samples (70 samples total)

- 35 known HNPCC mutation carriers for exons tested
- 32 anonymised normal controls
- 1 wild type plasmid contol
- 1 hetrozygous plasmid control
- 1 homozygous plasmid control
- 2 NTC
- Randomised

ALL AMPLICONS WERE VERIFIED BY SEQUENCING

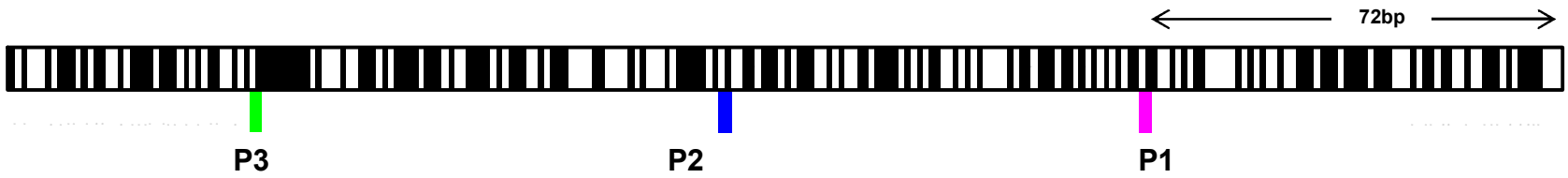
40%GC Long Amplification Plot and Agarose gel

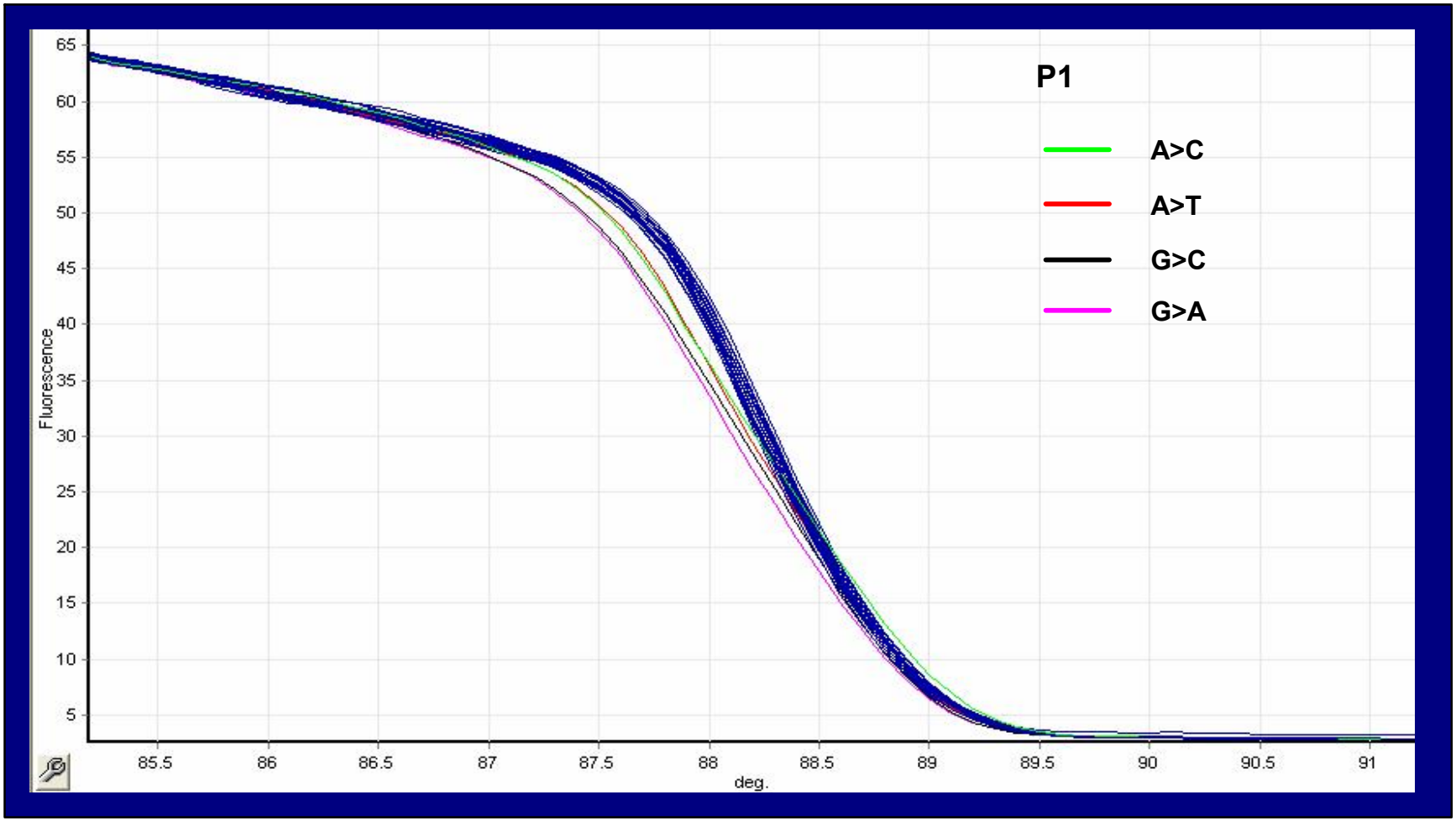




60% GC short

(260 bp, 56%GC rich)





Data Analysis

HR-1 and RotorGene 6000

Samples analysed manually by 2 operators

LightScanner

Samples analysed using software provided:

High Sensitivity
Normal Sensitivity

Data unblinded and true and false positive and negatives recorded

Sensitivity and Specificity

Sensitivity = true positive / (true positive + false negative)

Specificity = true negative / (true negative + false positive)

	RotorGene 6000		HR-1		LightScanner 384 well			
					High Sensitivity		Normal Sensitivity	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
20L	100	82.6	89.6	97.8	87.5	100	75	100
20S	100	100	100	91.3	100	100	100	100
40L	100	100	95.8	97.8	100	100	75	100
40S	100	100	95.8	97.8	100	100	71	100
60L	100	90.9	95.8	84.4	-	-	-	-
60S	100	100	100	97.7	100	95.7	69.6	100
80S	100	87	100	93.5	100	87	98.5	87
hMLH1 x1	100	96.7	100	94.2	100	80	80	90
hMLH1 x7	100	100	100	95.2	100	90.5	71.4	96.8
hMLH1 x13	100	98.2	100	85.5	100	96.4	50	100
hMSH2 x10 Combined	100	96.4	100	96.3	100	80	100	90.9

False Negative Results

HR-1

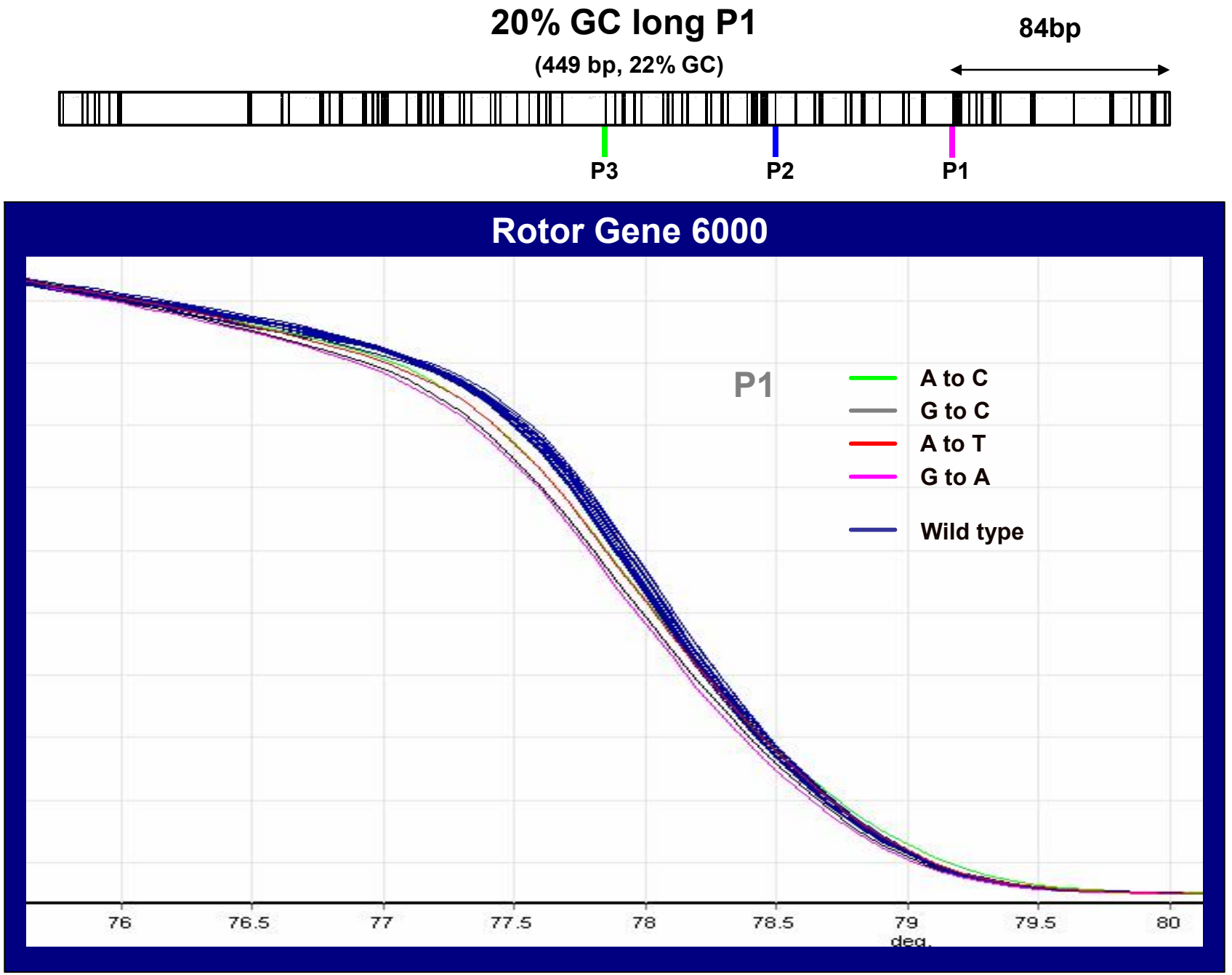
20L	Position 1	A to T	G to C	G to A
40L	Position 3	A to T		
40S	Position 3	G to A	G to C	

LightScanner (Analysed with High Sensitivity)

20L	Position 1	A to T	G to C	G to A

Rotor Gene 6000

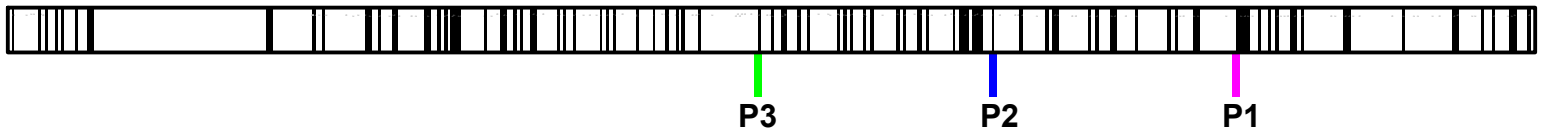
No false negatives


20% GC long P1
(449 bp, 22% GC)
84bp
P3
P2
P1
Rotor Gene 6000
P1
A to C
G to C
A to T
G to A
Wild type
76
76.5
77
77.5
78
78.5
79
79.5
80
deg.

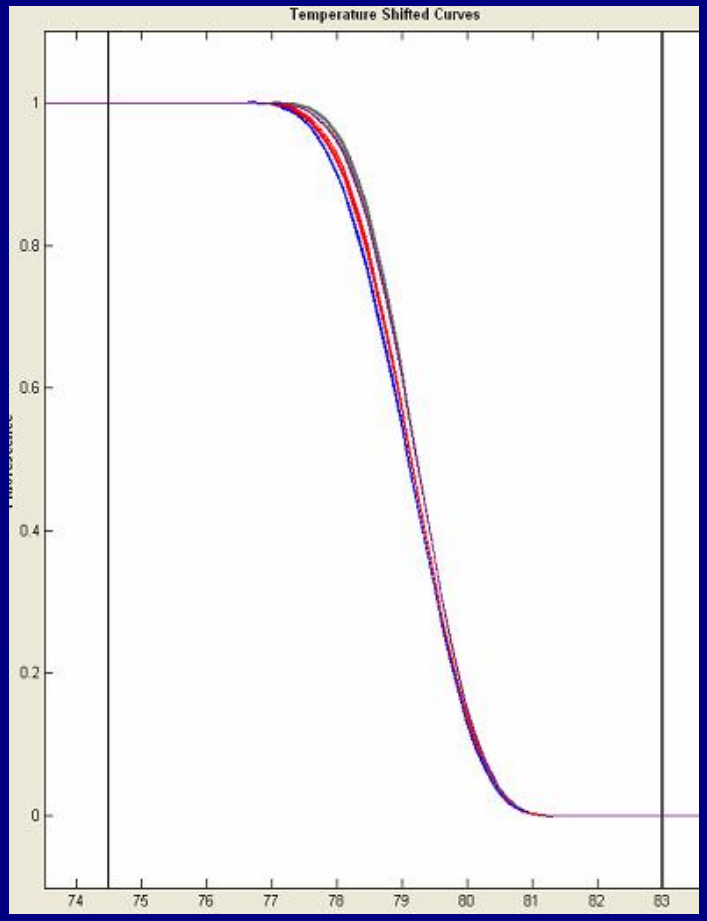
20% GC long

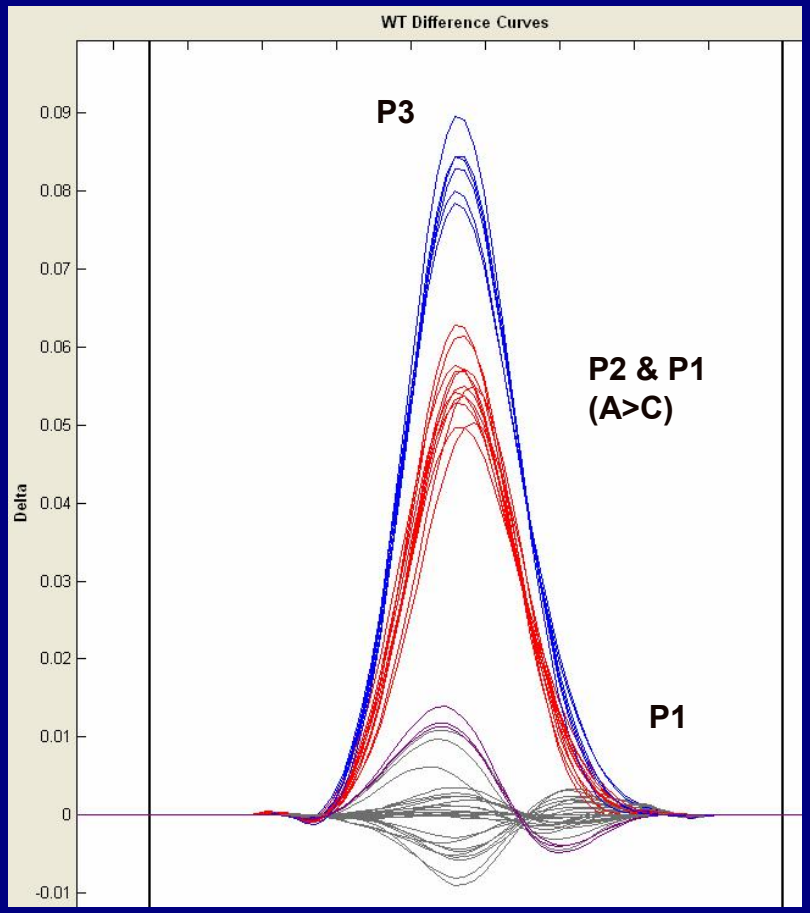
(449 bp, 22% GC)

84bp



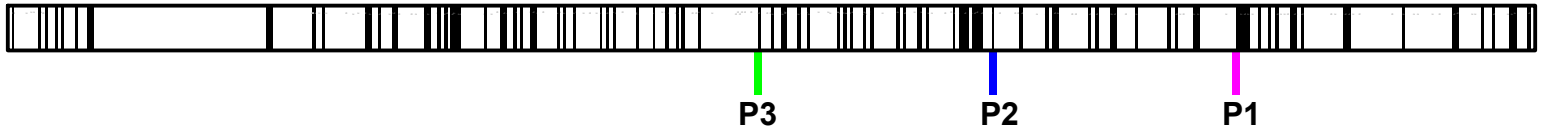
LightScanner 384 well



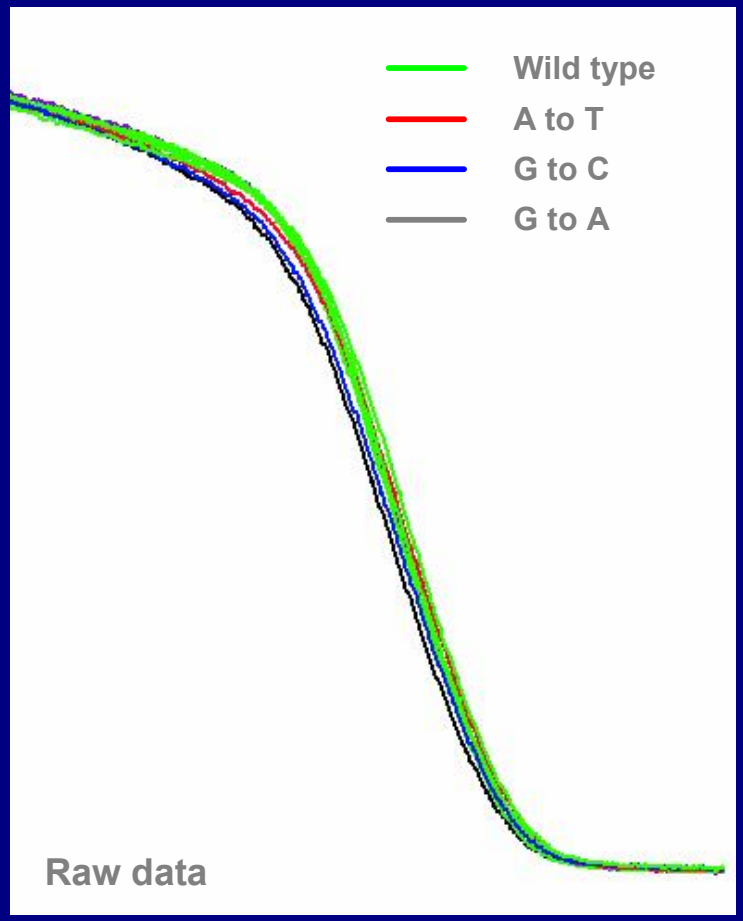


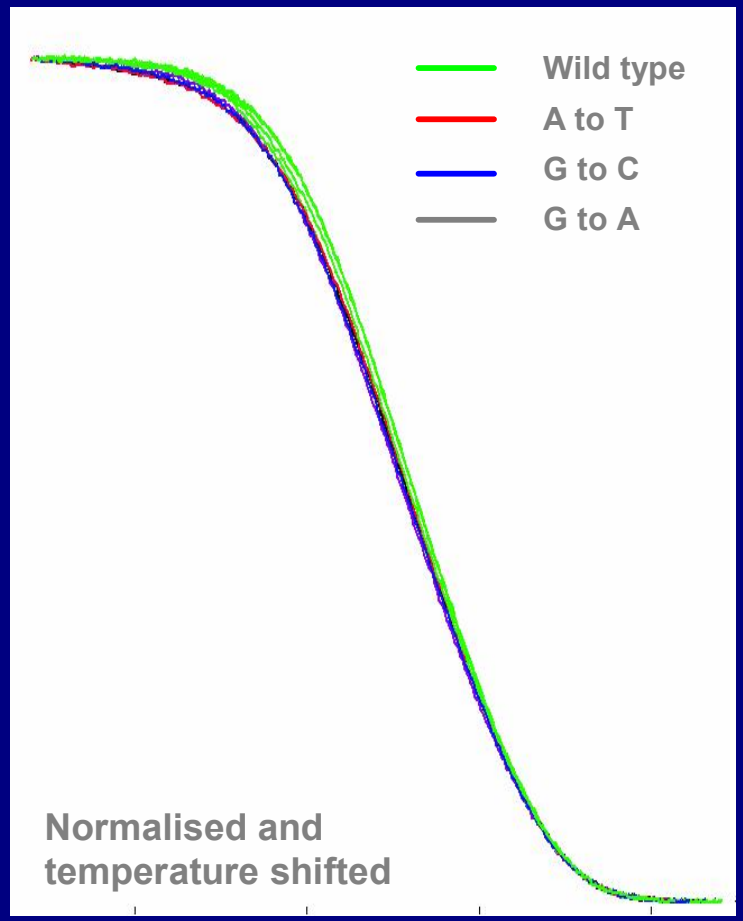
20% GC long

(449 bp, 22% GC)



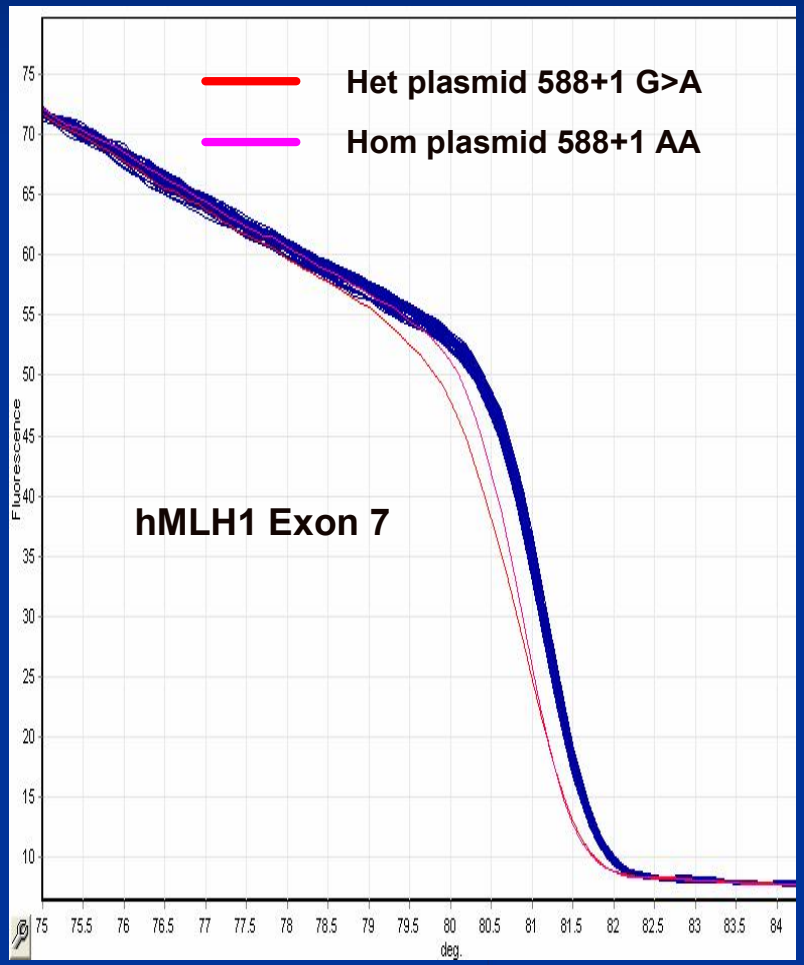
HR-1



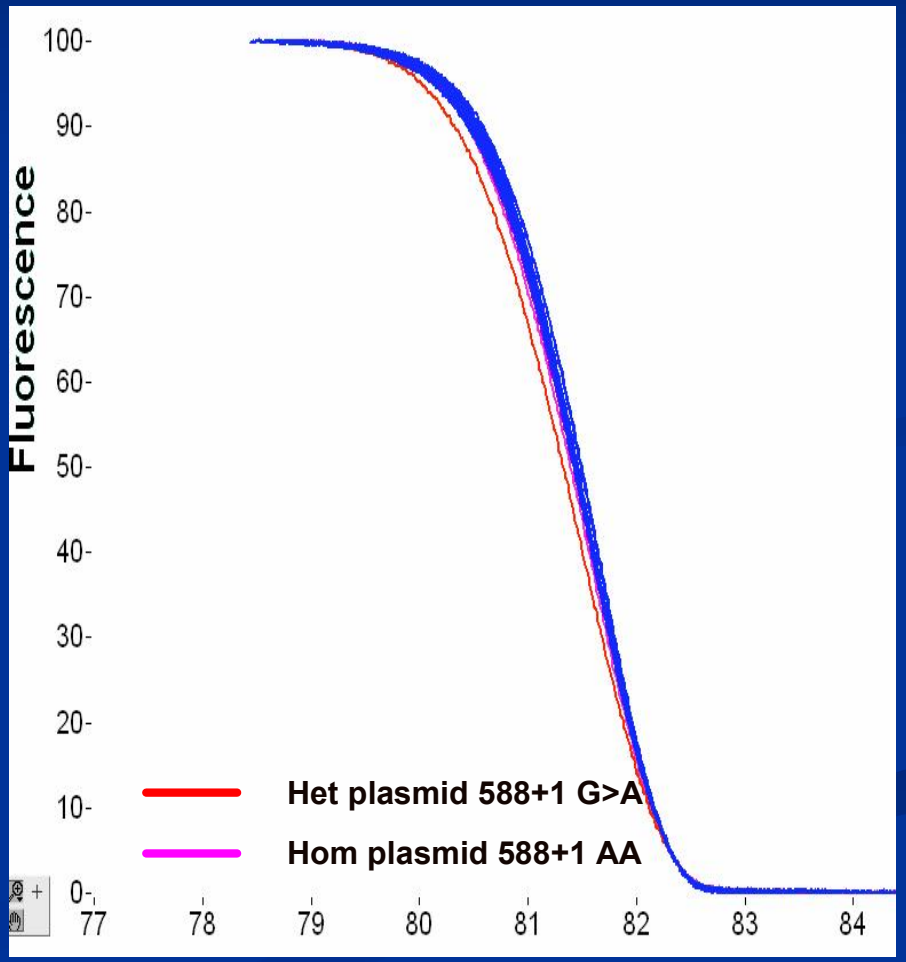


Detection of Homozygous mutations

RotorGene 6000

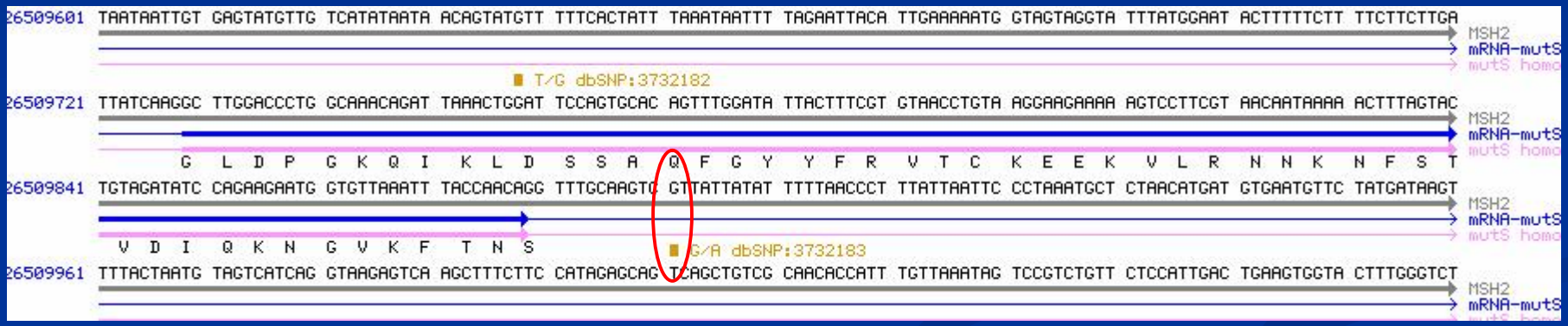


HR-1



Detection of pathogenic mutations in a polymorphic exon

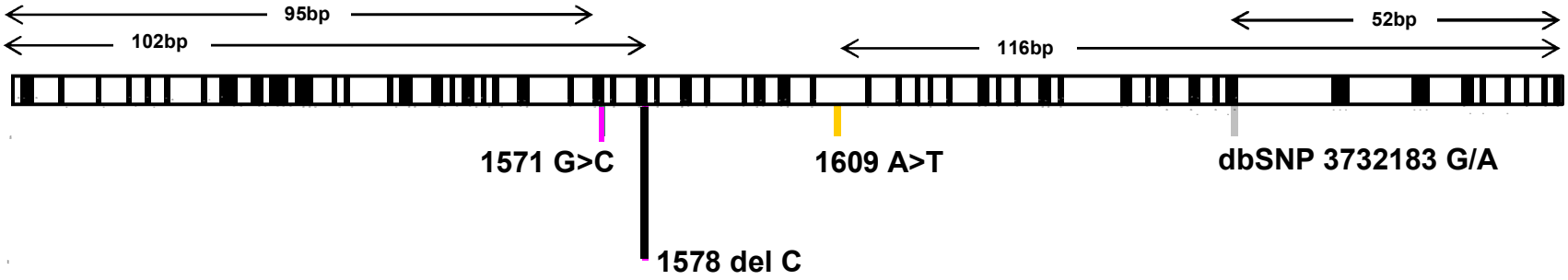
Example: Screening MSH2 Exon 10

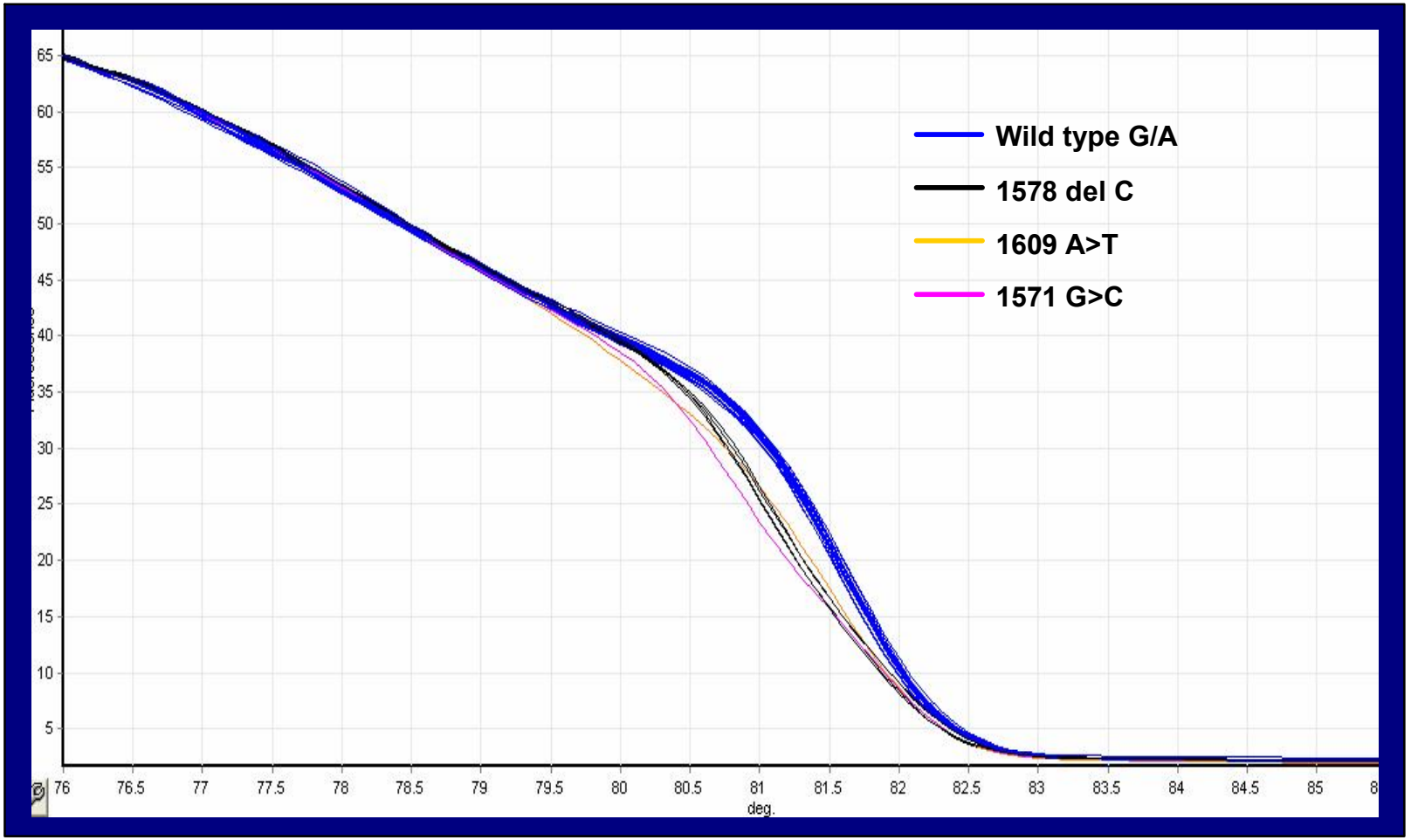


Genotype frequency (HapMap European):

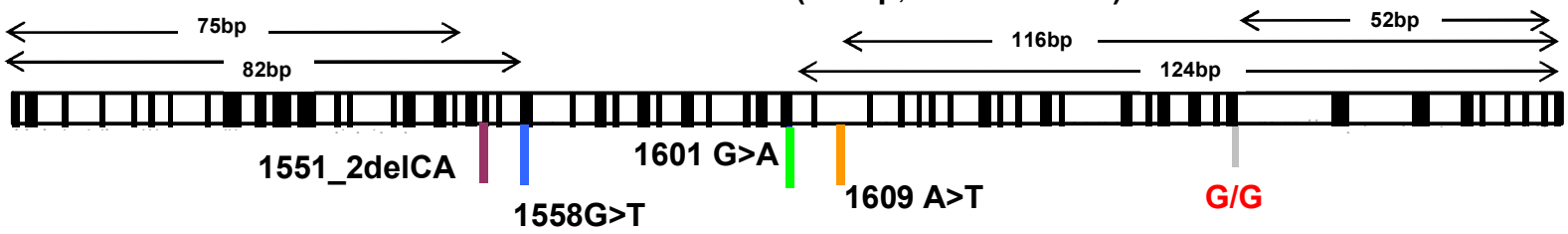
AA	0.13
GA	0.42
GG	0.45

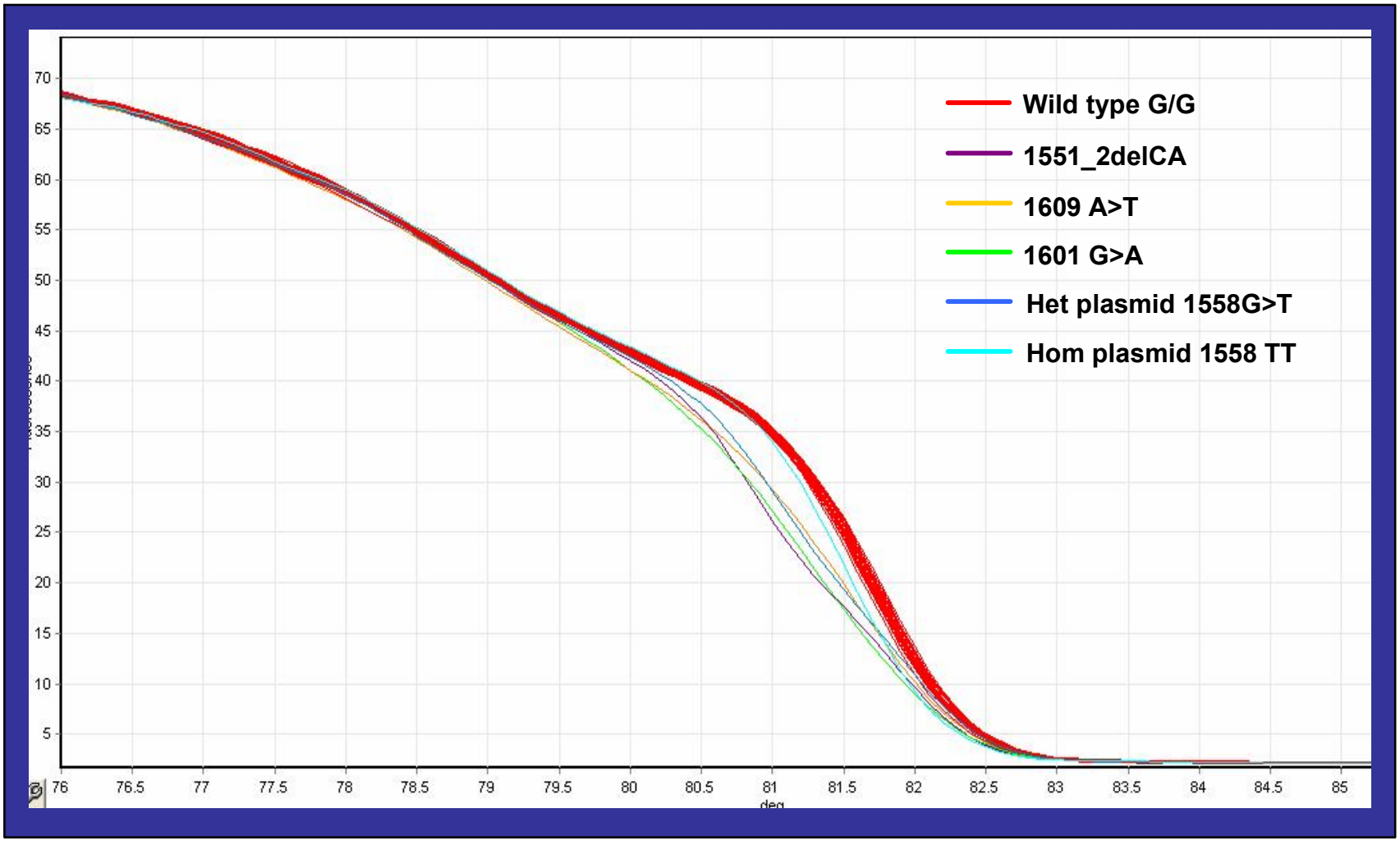
hMSH2 Exon 10 (249bp, 34% GC Rich)





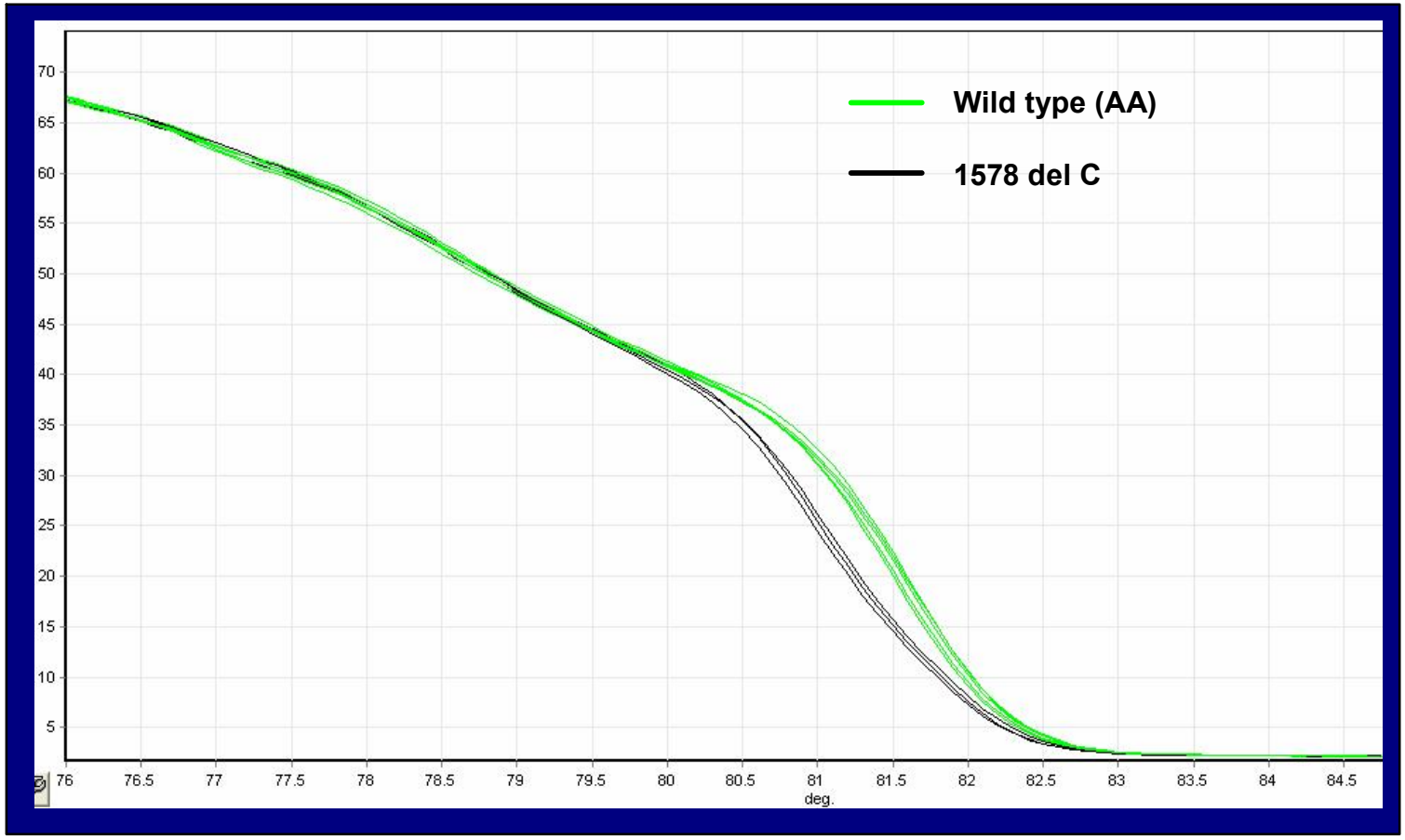
hMSH2 Exon 10 (249bp, 34% GC Rich)





hMSH2 Exon 10

(249bp, 34% GC)



Summary I

Sensitivity and Specificity of mutation detection for each platform were comparable:

	Sensitivity	Specificity
RotorGene 6000	100.0	95.3
HR-1	98.4	95.0
LightScanner 384 well (High)	99.0	88.0
LightScanner 384 well (Normal)	83.9	95.3

Factors effecting sensitivity and specificity:

- Amplicon design and optimisation
- Length of fragment
- Position of mutation in fragment
- Local sequence context

Summary II

- Simple and cost effective post-PCR technique which can be used for high throughput mutation scanning and genotyping
- Requires the use of only PCR reagents and the dsDNA binding dye LCGreen® Plus or equivalent
- Requires no post-PCR handling and no separation step - improves analysis time
- HRM has a mutation detection sensitivity and specificity which is comparable to currently available pre-screening techniques
- Capable of detecting some homozygous mutations
- Can be used to screen polymorphic exons
- HRM could be easy integrated into clinical diagnostic pre-screening strategies and has the potential to allow large genes to be screened and reported within the 6-8 weeks recommended in the White Paper.

Future Work

- Detection of homozygous / hemizygous mutations
- Simultaneous SNP detection and mutation scanning
- Software developments
- dsDNA binding dyes
- Amplicon design
- Batching of different amplicons
- Other applications *e.g.* methylation analysis

Acknowledgements

NGRL (Wessex)	Gemma Potts Chris Mattocks Ian Day
Corbett Research	Lawrence Murphy Greg Nowak
Cadama Medical	David Harris
Idaho Technology	Jason McKinney
CRUK Mutation detection facility	Graham Taylor Claire Taylor