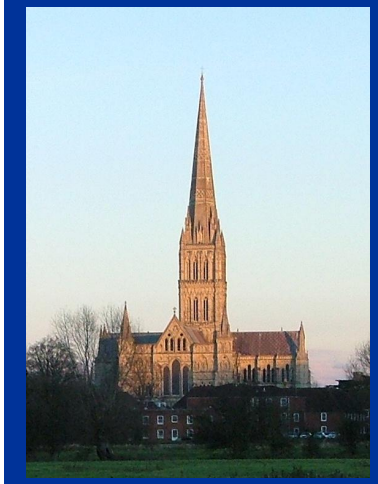
Potential applications of high resolution melt curve analysis for genetic diagnostics



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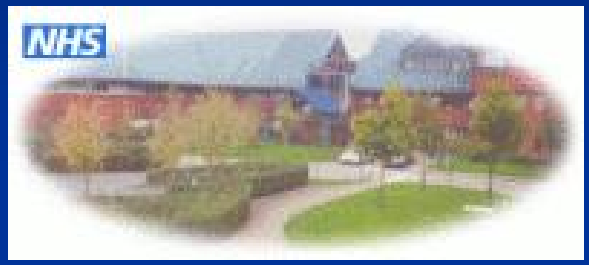
Salisbury

UK

UK National Genetics Reference Laboratories

- Established in 2002 by the Department of Health (UK)
- Two laboratories based in Manchester and Salisbury (Wessex)



- Aim to evaluate technologies and systems that are close to service and assess their applicability to genetic testing within the National Health Service
- Other functions of the laboratories include:

  - Horizon Scanning and Technology Assessment
  - Developing new Quality Assessment Systems
  - Developing reference and control reagents

Outline of talk

- What is High Resolution Melt curve analysis (HRM)?
- Potential applications in genetic diagnostics
  - Mutation scanning
  - Methylation analysis
  - Detection of somatic mutations

What is High Resolution Melt Curve analysis?

- **Simple, cost effective post PCR technique for high throughput mutation scanning, genotyping and methylation profiling**

- **Uses standard PCR reagents and double stranded DNA binding dyes**

- **Closed tube method:**

  - no post PCR handling and no separation step

- **Historically HRM limited due to technical constraints**

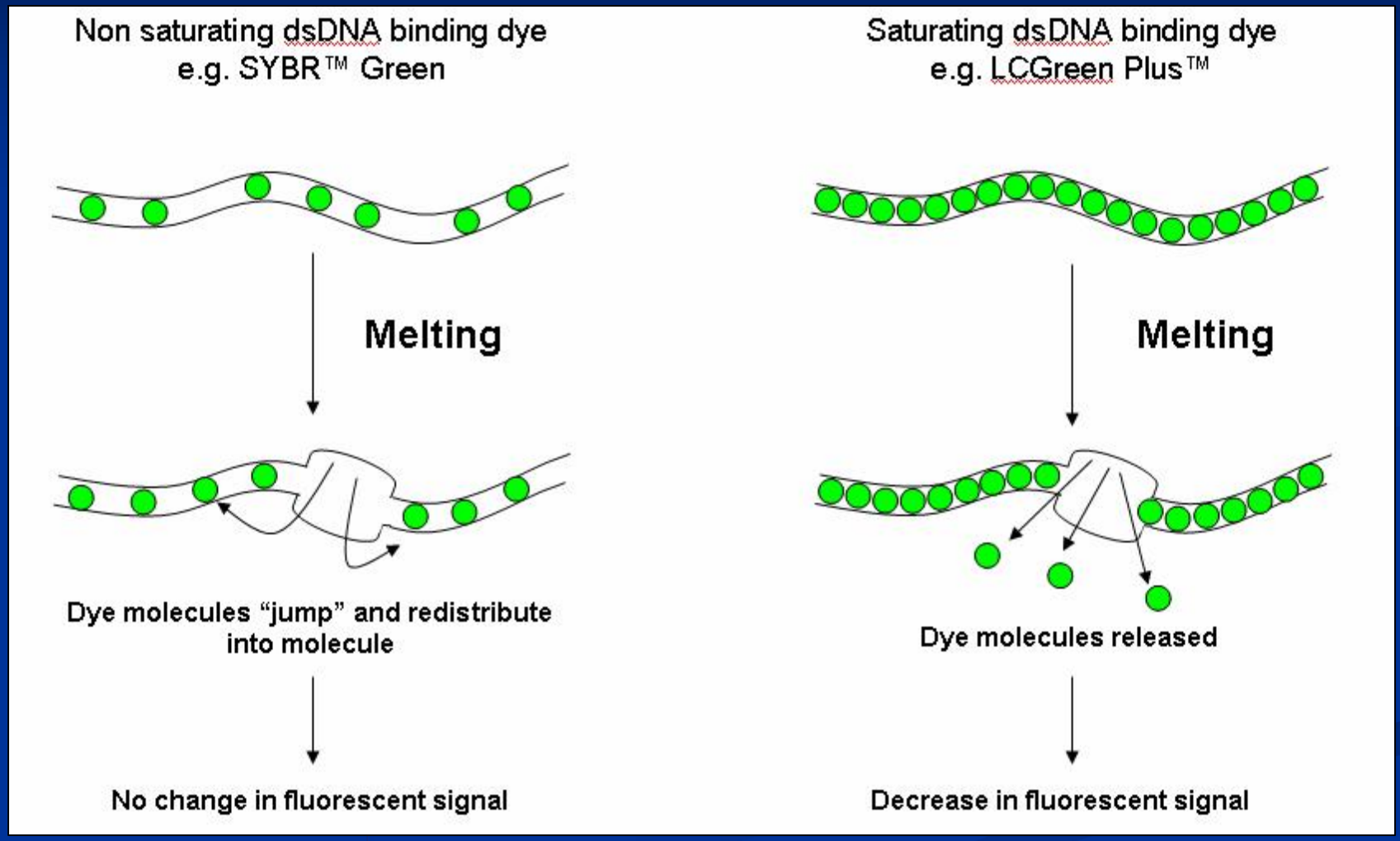
  - data acquisition

  - sensitivity of instrumentation

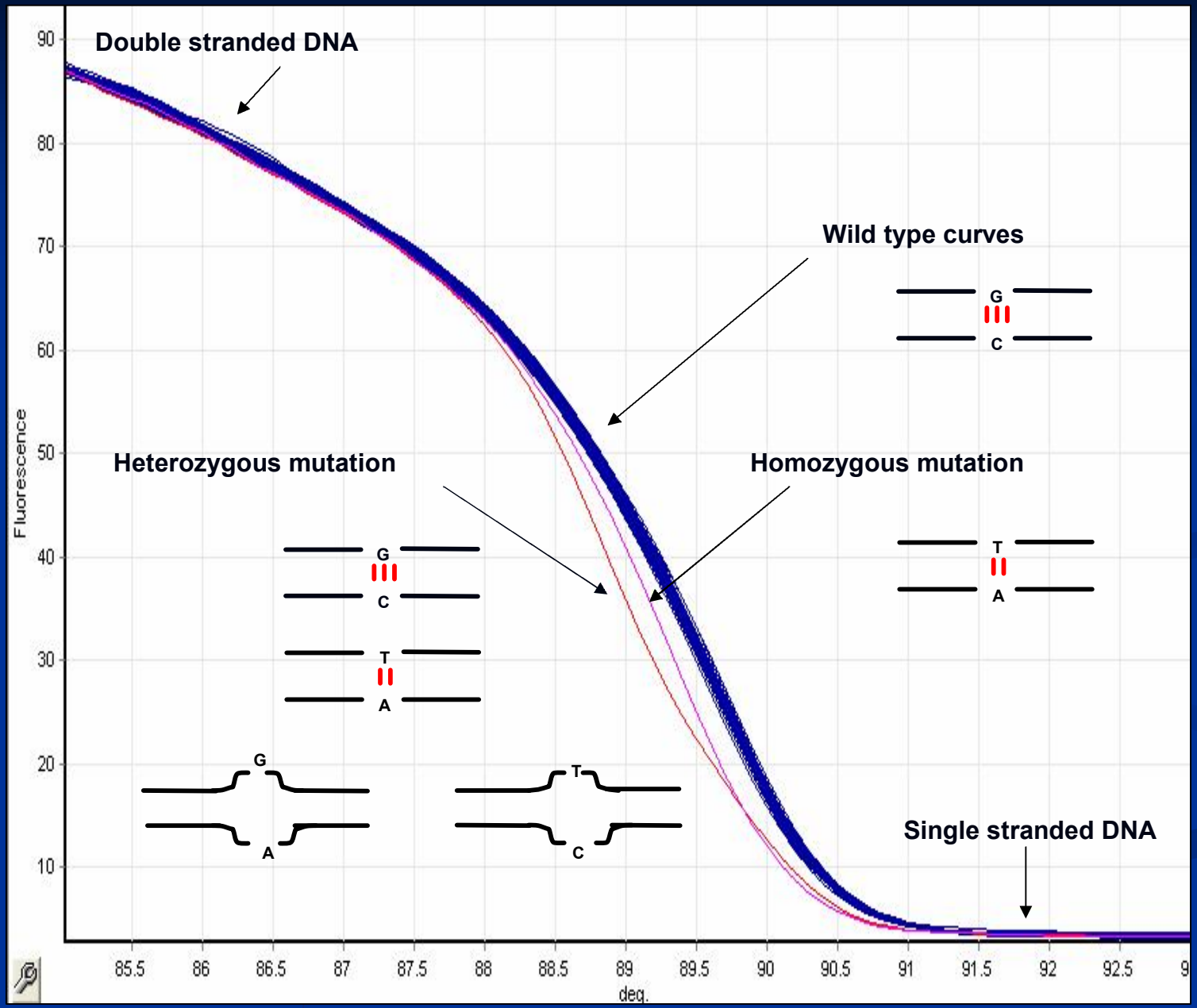
  - inadequacies of fluorescent chemistry

- **Promising method of mutation scanning with sensitivity comparable current techniques**

High Resolution Melt Curve Analysis



High Resolution Melt Curve Analysis



Mutation Scanning

Mutation Scanning

- Detecting ‘unknown’ sequence variation at any position within an amplicon:

  e.g. single base substitutions (point mutations)

  deletions

  insertions

- In the UK the results of mutation scanning of large genes are now required to be reported within 6-8 weeks of sample receipt

- Over half of all genetic test performed in the UK involve mutation scanning for ‘private’ mutations e.g. hereditary breast cancer and colorectal cancer, Marfans etc

- Use of a ‘pre-screening technique’ compared to direct sequencing has the potential to greatly reduce costs of these genetic tests and improve reporting times

Evaluation protocol January – March 2006

- 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 and monitored using real time PCR
- Identical amplicons analysed using HRM on three machine platforms:

• HR-1	(Idaho Technology)
• 384 well LightScanner	(Idaho Technology)
• RotorGene 6000	(Corbett Research)

Evaluation results (March 2006)

Tested total of 624 samples (including controls) in eleven amplicons

Analysed: 212 mutated samples (105 unique mutations)

393 wild type samples

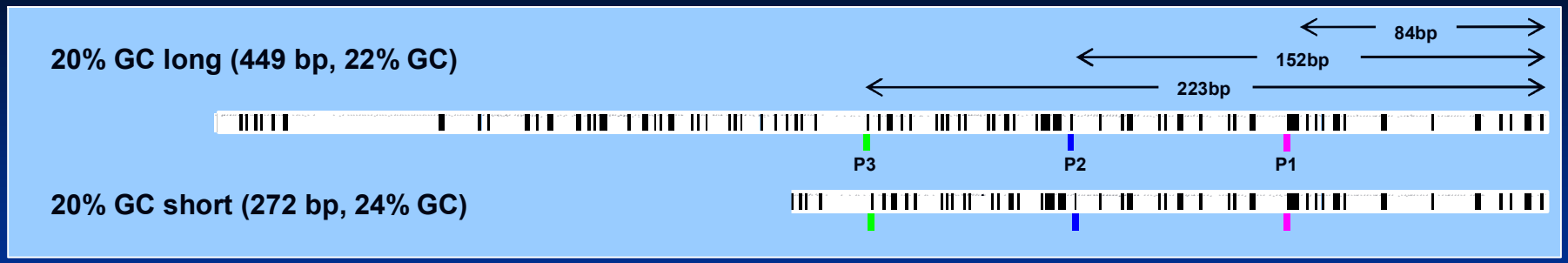
	Sensitivity	Specificity
RotorGene 6000	100.0	95.3
HR-1	98.4	95.0
LightScanner 384 well (High)	99.0	88.0

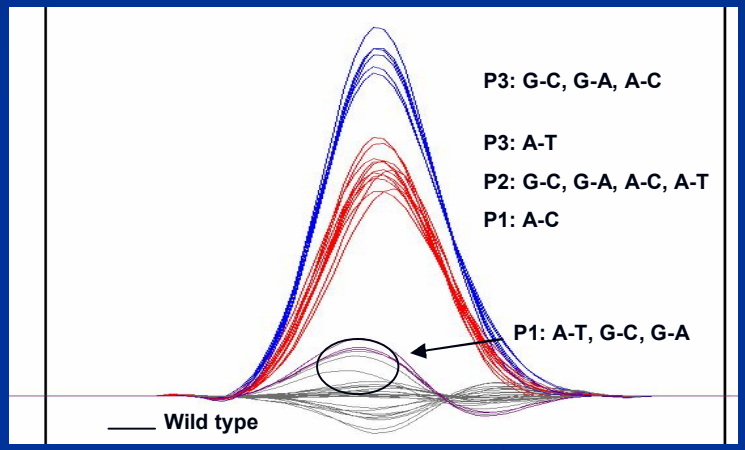
HRM evaluation currently being undertaken by EuroGentest:

http://www.eurogentest.org/

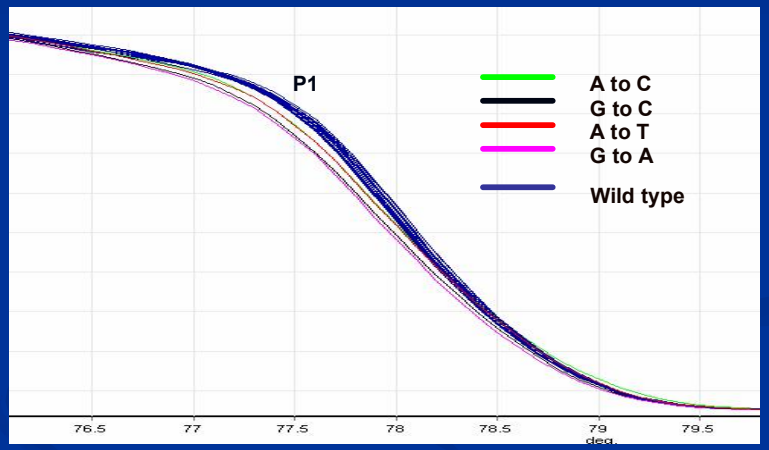
Factors affecting sensitivity and specificity

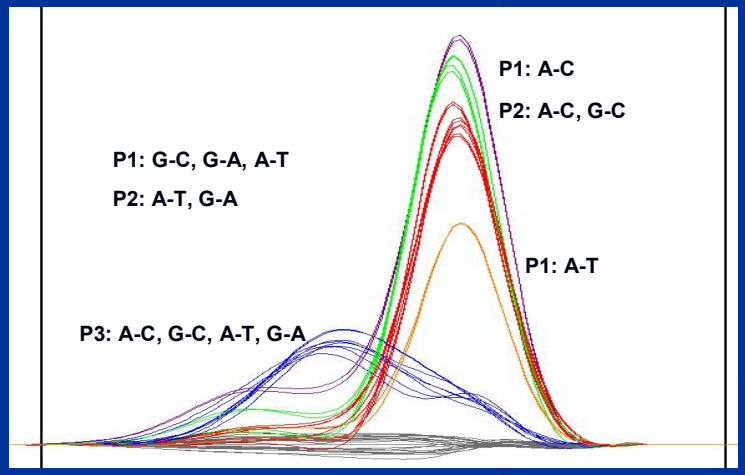
Length of amplicon: Shorter is better



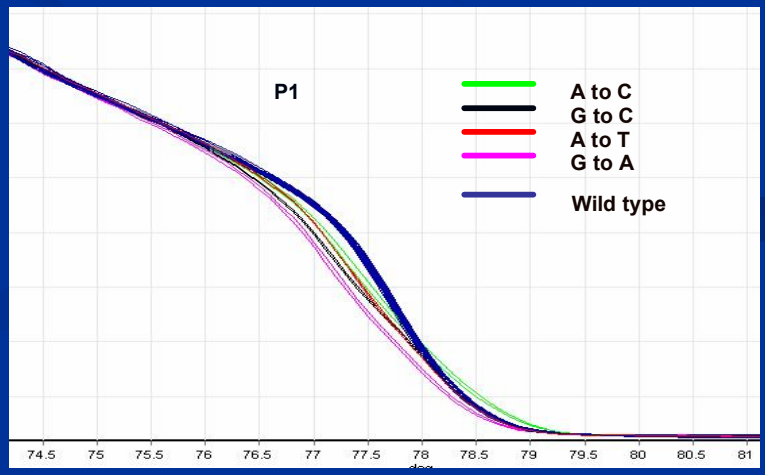


Long



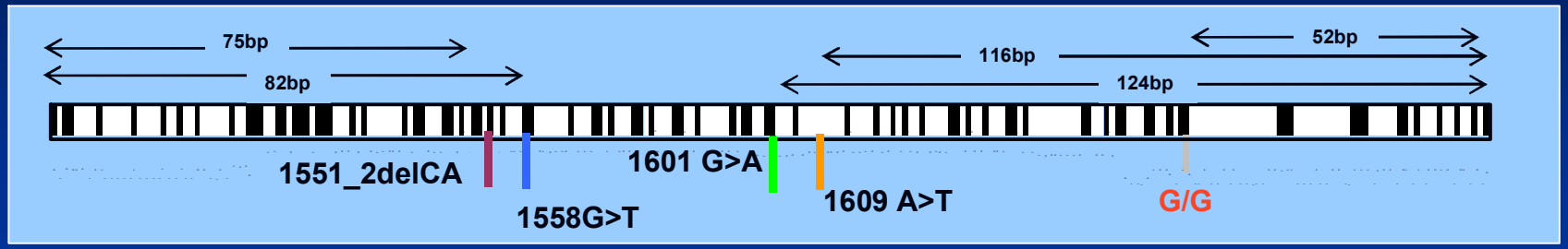


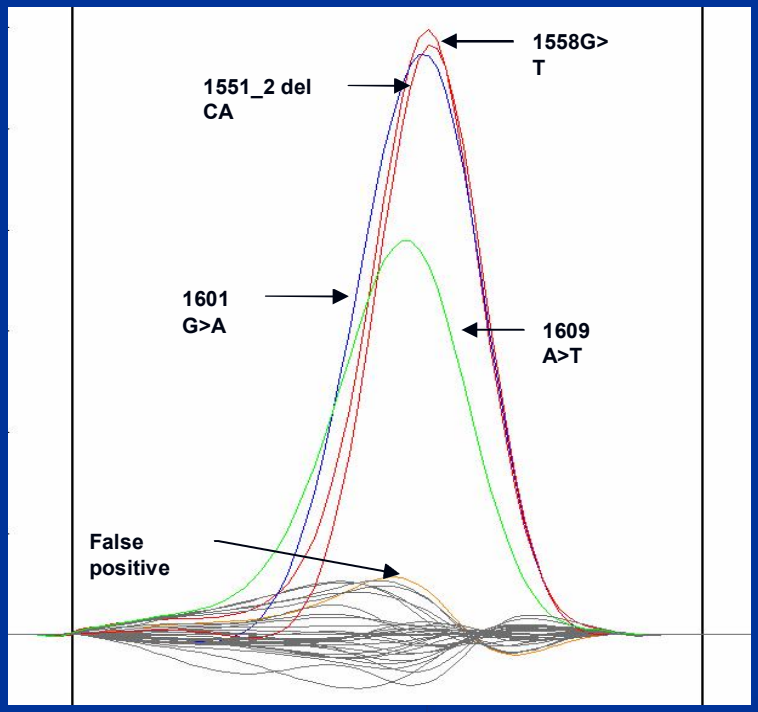
Short

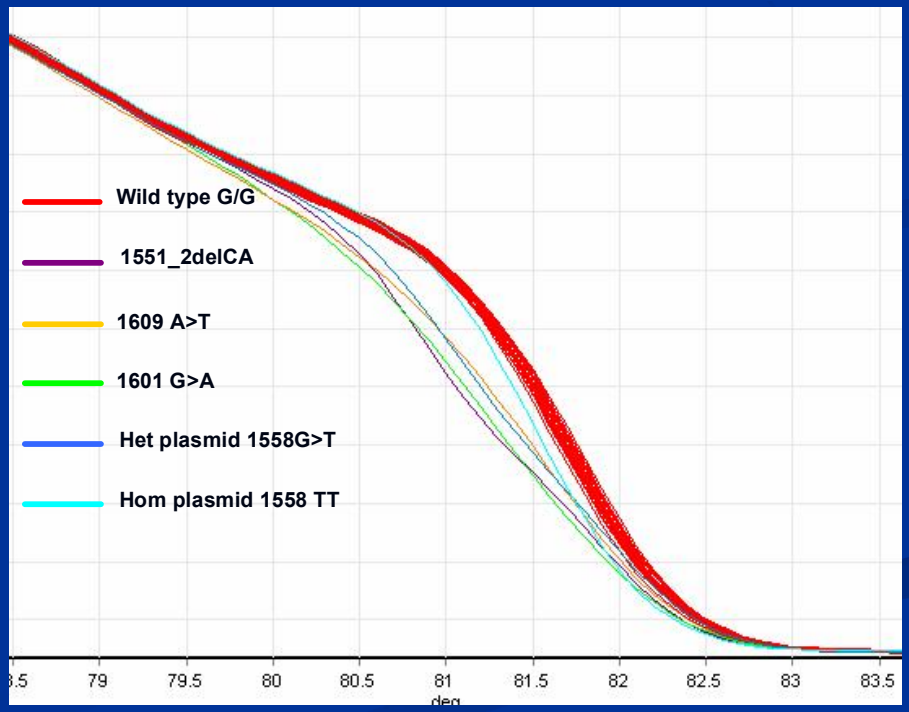


Position in amplicon – no obvious effect

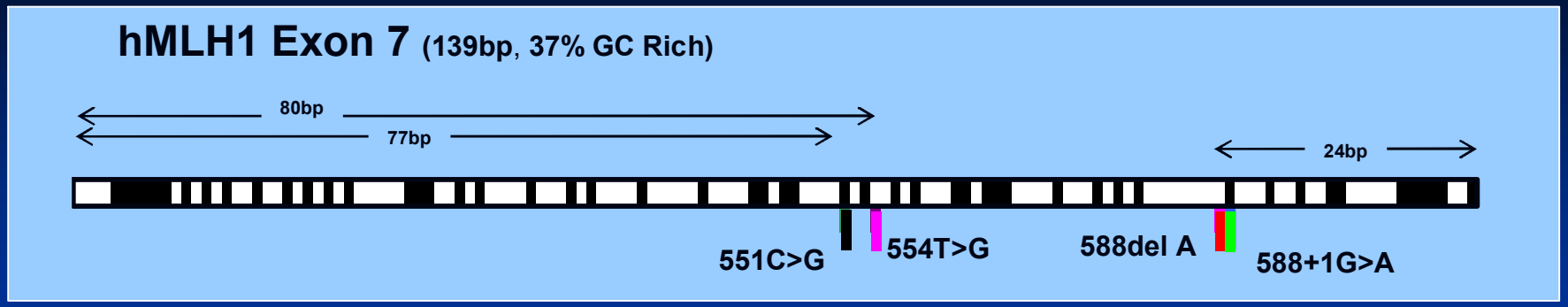
hMSH2 Exon 10 (249bp, 34% GC Rich)

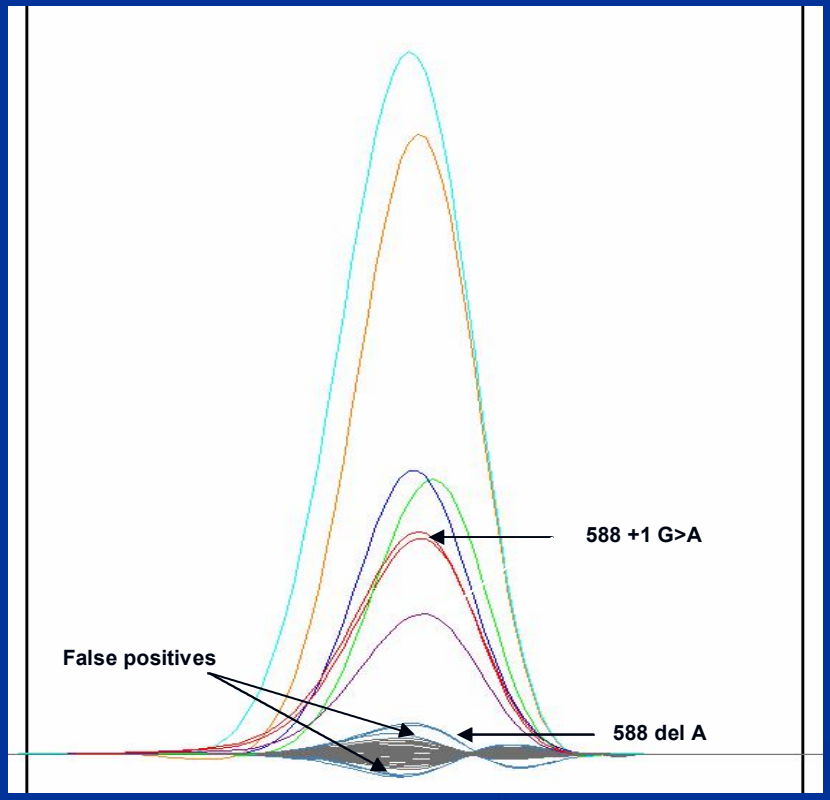


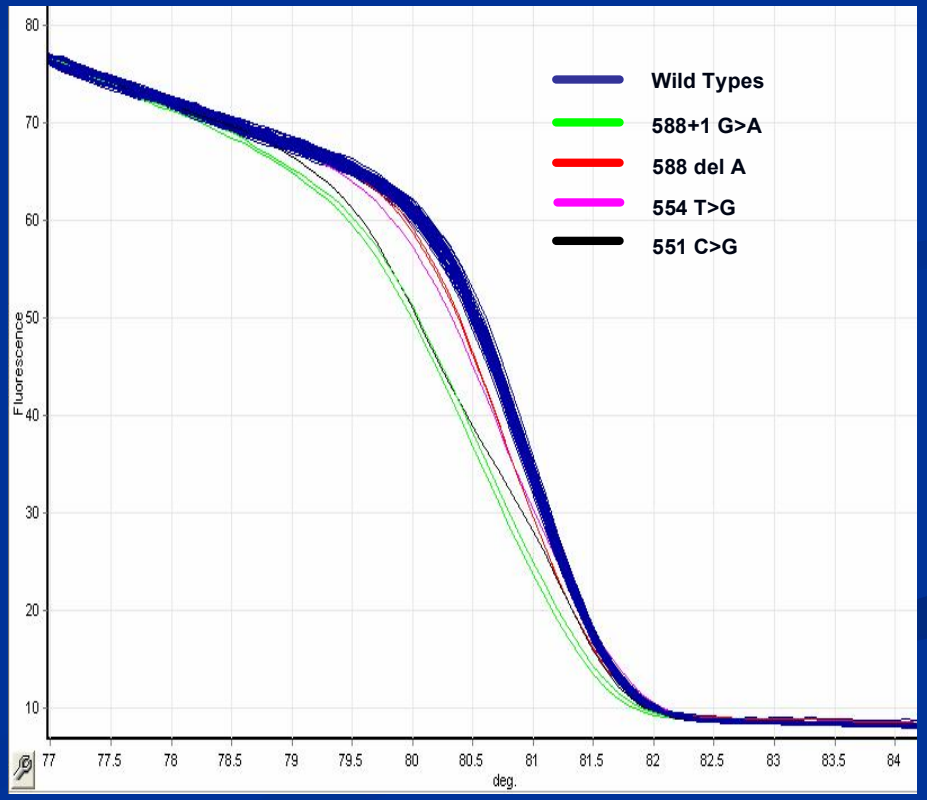




Local sequence context / type of mutation







Methylation Profiling

Prader Willi and Angelman Syndromes

- Two clinically distinct neurodevelopmental disorders (1 : 15 – 20,000)
- Caused by deficiency of specific parental contributions at an imprinted domain at 15q11.2-13

PWS Caused by loss of the paternal (unmethylated) contribution

- Paternal deletion (~70%)
- Maternal UPD (~30% cases)
- Mutation in the imprinting region causing abnormal methylation (<2%)

Phenotype:
infantile hypotonia
mild to moderate mental retardation
hypogonadism
hyperphagia with obesity
short stature and obsessive-compulsive behaviour

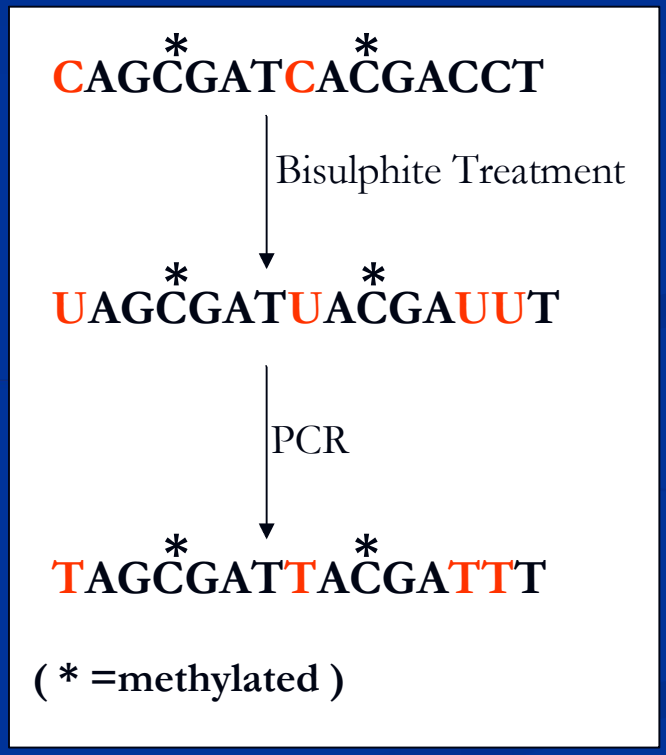
AS Caused by loss maternal (methylated) contribution

- Maternal deletion (~70%)
- Paternal UPD (~5% cases)
- Mutation in the imprinting region causing abnormal methylation (~5%)

Phenotype:
developmental delay, functionally severe
speech impairment, none or minimal use of words;
movement or balance disorder,
behavioral uniqueness: frequent laughter/smiling; apparent happy demeanor;
easily excitable personality, often with hand flapping movements

Bisulphite Treatment

- Bisulphite treatment causes ummethylated Cytosines to convert to Uracil while methylated cytosines remain unchanged.



NORMAL

AGGGAGTTGGGATTTTTGTATTGYGGTAAATAAGTAYGTTTGYGYGGTYGTAGAGGTAGGTTGGYGYGTATG
TTTAGGYGGGGATGTGTGYGAAGTTTGTYGTTGTTGTAGYGAGTTTGGYGTAGAGTGGAGYGGTYGTYGGAG
ATGTTTGAYGTATTTGTTTGAGGAGYGGTTAGTGAYGYGATGGAGYGGGTAAGGTTAGTTGTGTYGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

PWS

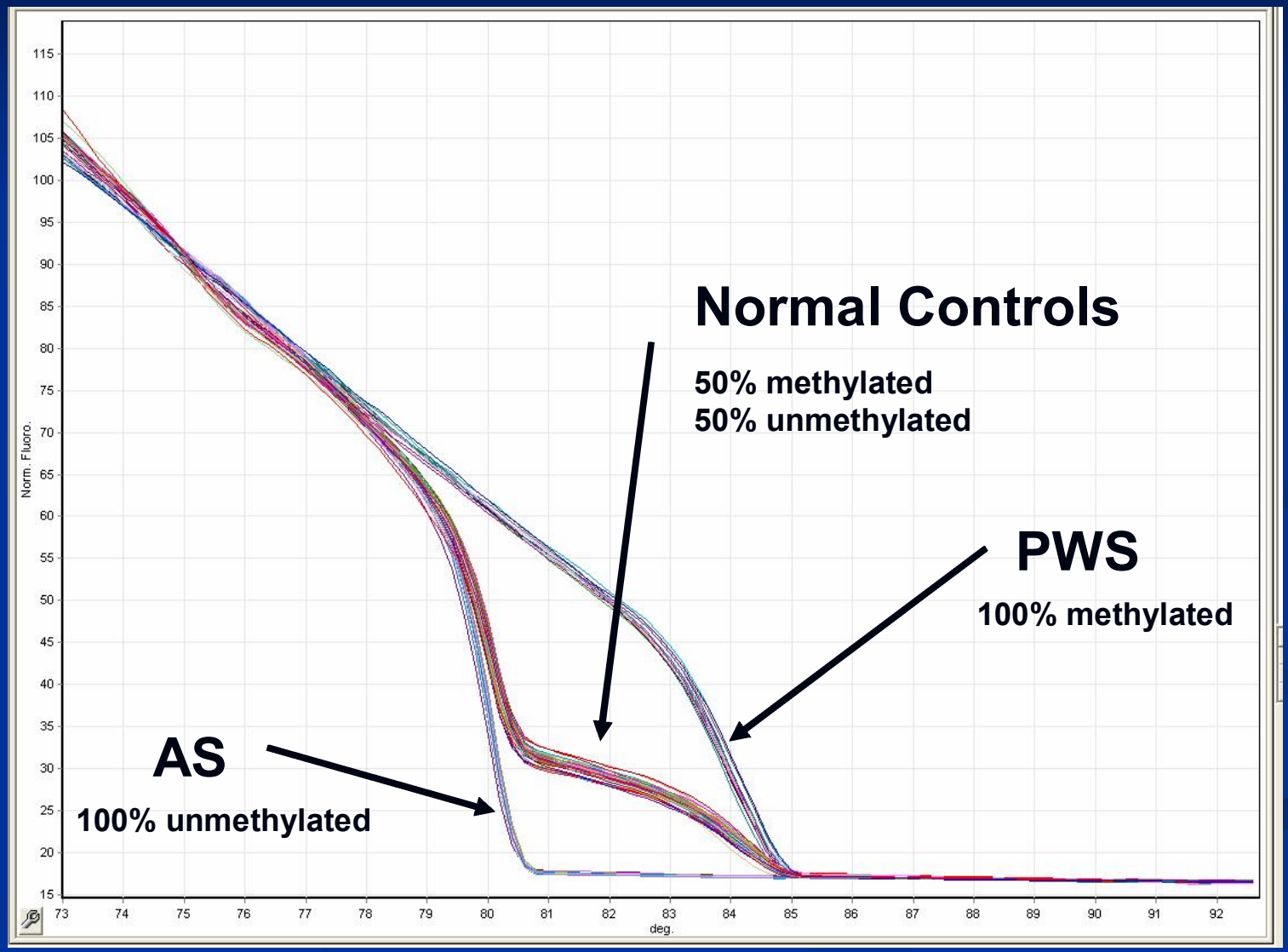
AGGGAGTTGGGATTTTTGTATTGCGGTAAATAAGTACGTTTGCGCGGTCGTAGAGGTAGGTTGGCGCGTATG
TTTAGGCGGGGATGTGTGCGAAGTTTGTCGTTGTTGTAGCGAGTTTGGCGTAGAGTGGAGCGGTCGTCGGAG
ATGTTTGACGTATTTGTTTGAGGAGCGGTTAGTGACGCGATGGAGCGGGTAAGGTTAGTTGTGTCGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

AS

AGGGAGTTGGGATTTTTGTATTGTGGTAAATAAGTATGTTTGTGTGGTTGTAGAGGTAGGTTGGTGTGTATG
TTTAGGTGGGGATGTGTGTGAAGTTTGTTGTTGTTGTAGTGAGTTTGGTGTAGAGTGGAGTGGTTGTTTGGAG
ATGTTTGATGTATTTGTTTGAGGAGTGGTTAGTGATGTGATGGAGTGGGTAAGGTTAGTTGTGTTGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

Promoter region of SNRPN: 21 CpG sites can vary

HRM for diagnosis of PWS / AS

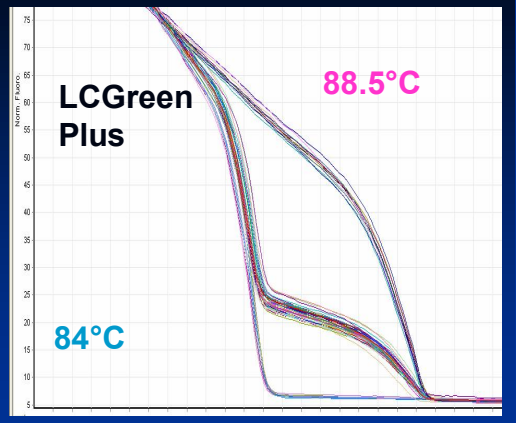


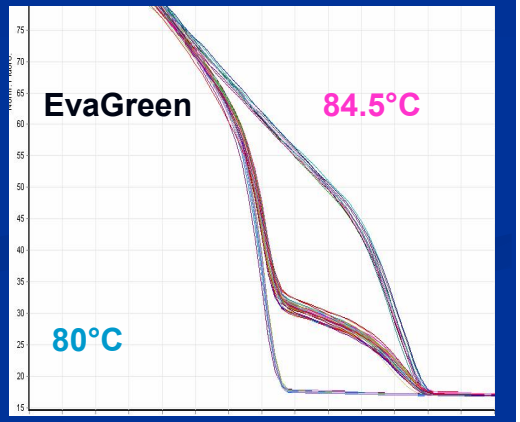
dsDNA binding dyes

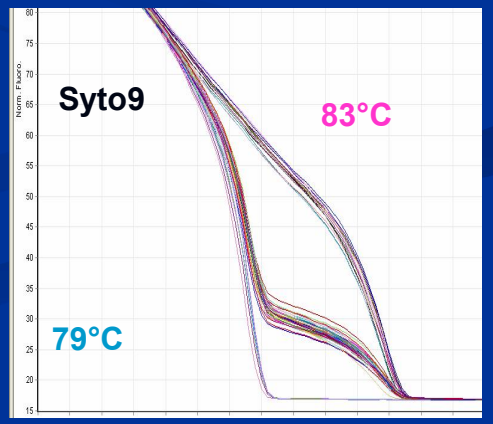
Analysed 166 bisulphite treated DNA samples:

39 PWS, 31 AS, 96 Normal Controls

Dye	Correctly classified with automated calling at > 80% confidence*
LCGreen Plus	95 %
EvaGreen	98 %
Syto9	95 %

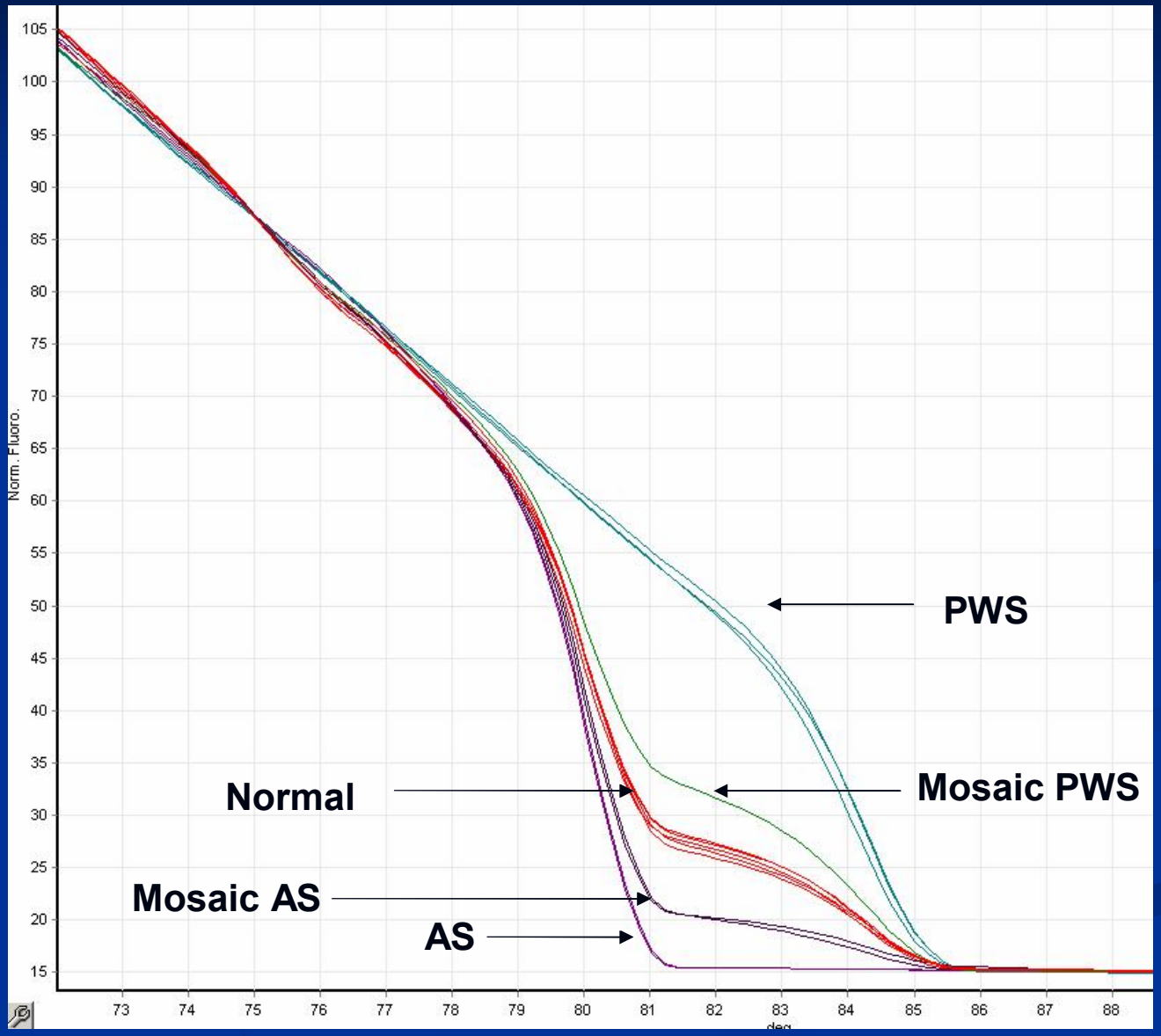






- * 1 AS and 1 PWS patient also had mutations and were included if correctly scored as variants.
- Data from automated calling concordant with MS-PCR assay.
- Remaining 2 – 5% could be correctly classified by eye.

Detection of mosaicism

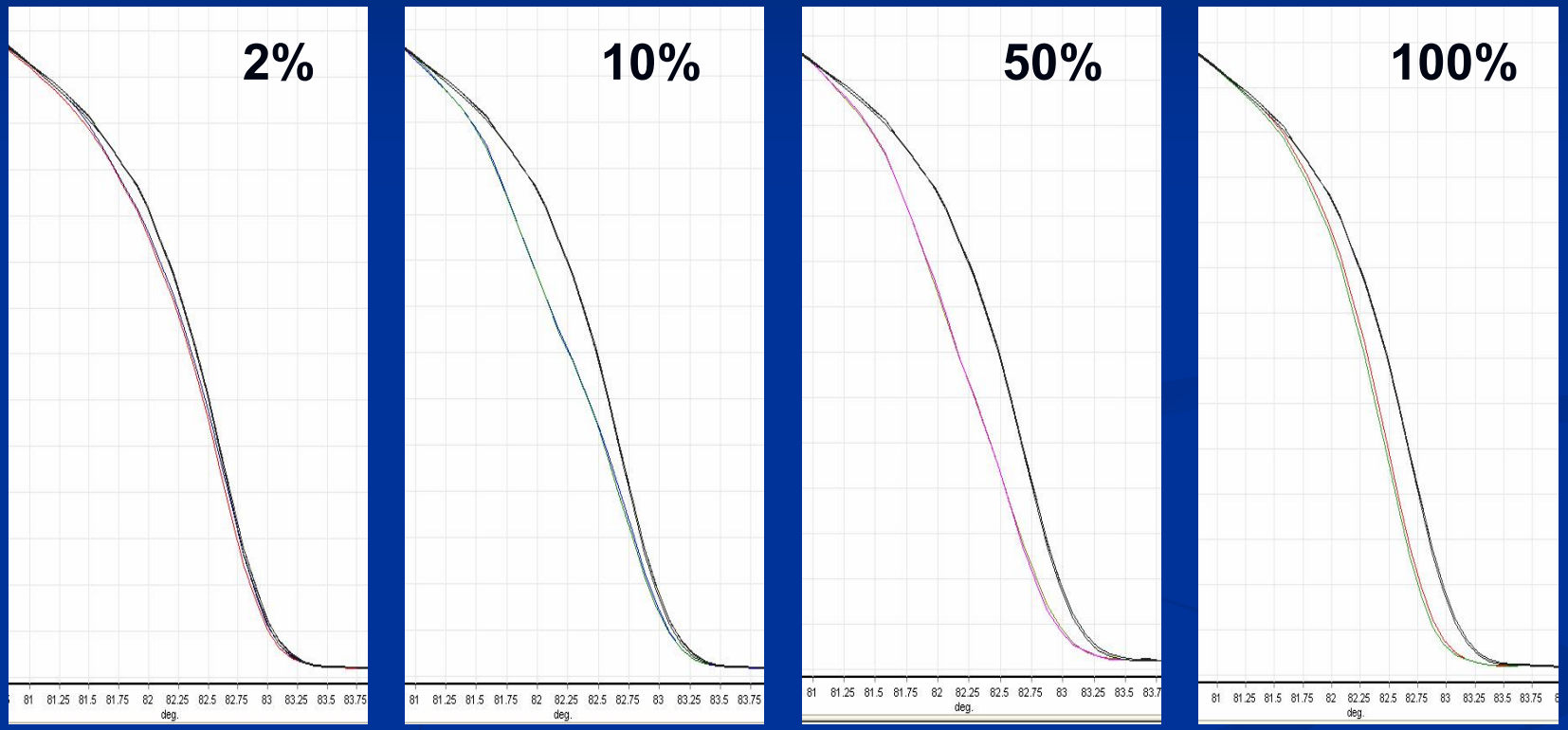


Detection of Acquired Mutations

Detection of acquired / somatic mutations

- Human myeloproliferative disorders form a range of clonal haematological diseases
- The molecular pathogenesis of these disorders is unknown, but tyrosine kinases have been implicated in several related disorders
- Recently a high proportion of patients with myeloproliferative disorders have been found to carry a dominant gain-of-function mutation of JAK2
- JAK2 V617F is a somatic mutation present in hematopoietic cells
- Detection of this acquired mutation is likely to have a major impact on the way patients with MPD are diagnosed

Detection of acquired JAK2 V617F mutation



Summary

- HRM is a simple and cost effective post-PCR technique which can be used for high throughput mutation scanning (constitutional and some acquired), methylation profiling and genotyping

- Requires the use of only PCR reagents and dsDNA binding dyes e.g. LCGreen® Plus, EvaGreen, Syto9

- Requires no post-PCR handling and no separation step

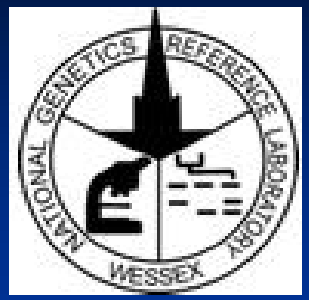
- HRM has a mutation detection sensitivity and specificity which is comparable to currently available pre-screening techniques although PCR optimisation is essential

- Capable of detecting some homozygous mutations

- Has potential to be used to screen polymorphic exons

- HRM had the potential to be integrated into clinical diagnostic pre-screening strategies to facilitate large genes to be screened and reported within the 6-8 weeks recommended in the UK Genetics White Paper

Acknowledgements



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