High resolution melting: potential applications for genetic diagnostics

Helen White, PhD

National Genetics Reference Lab (Wessex)

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 Melting?

- Potential applications in genetic diagnostics

  - ➤ Mutation scanning

    - Examples of HNPCC screening
    - Data analysis considerations
    - Factors affecting sensitivity and specificity

  - ➤ Methylation analysis

  - ➤ Detection of somatic / acquired mutations

- Assay design considerations

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**

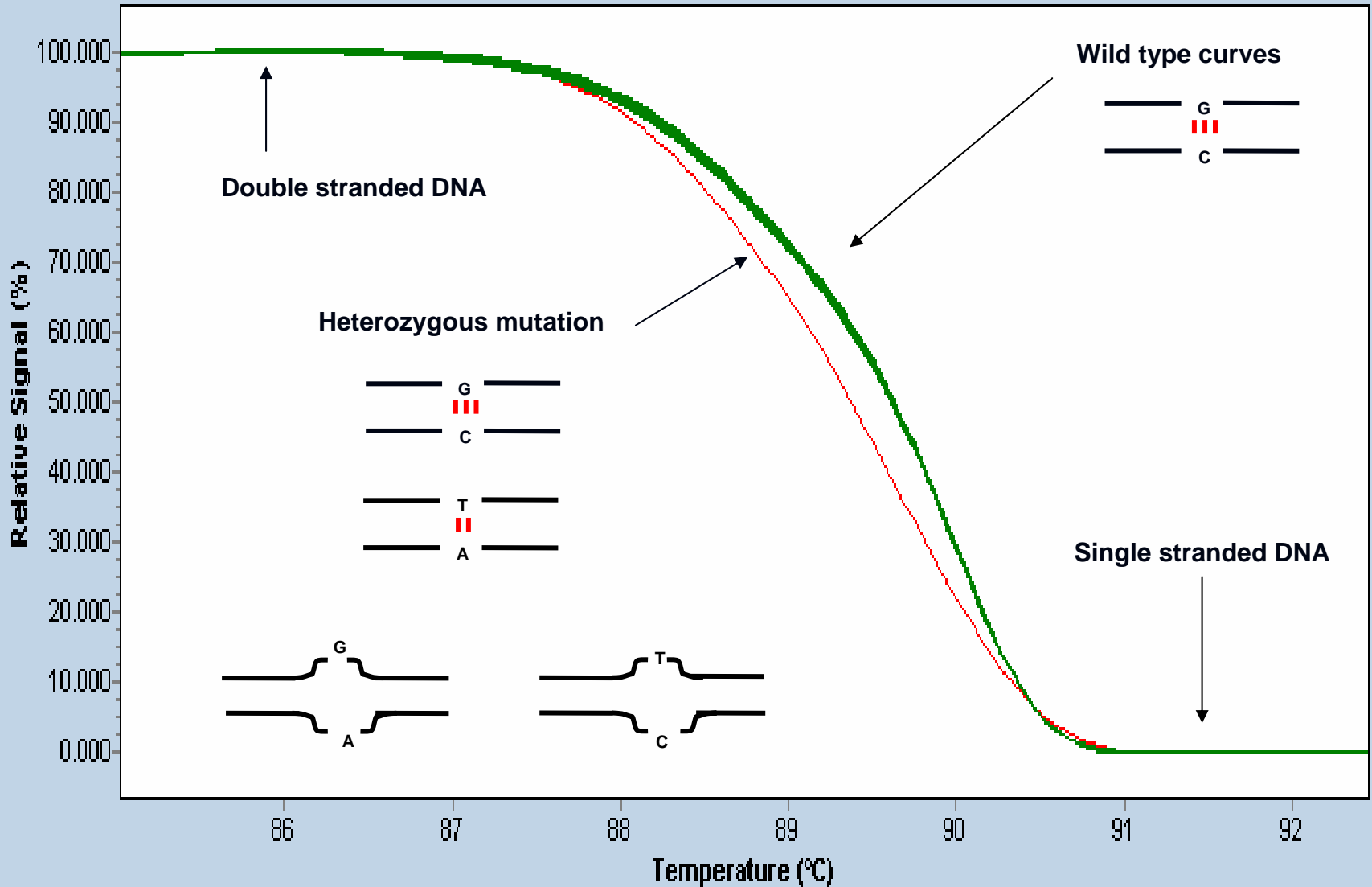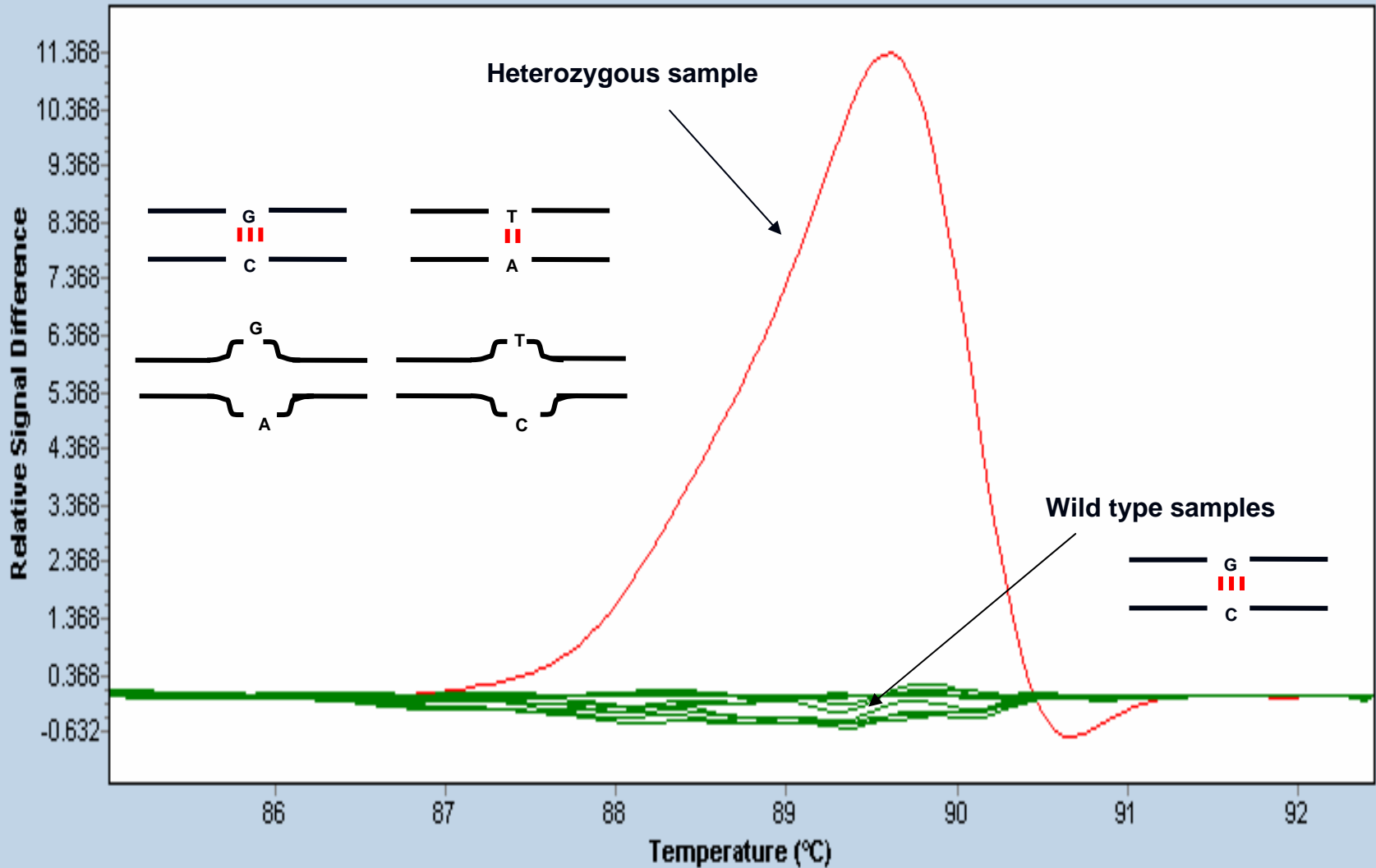# High Resolution Melt Curve Analysis
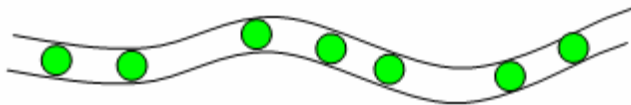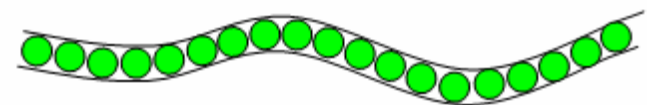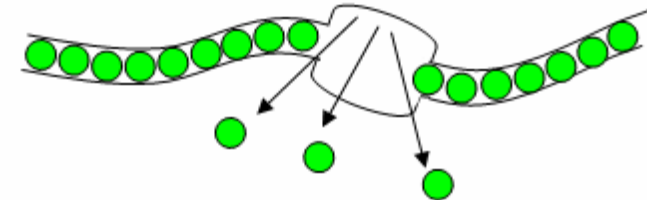
High Resolution Melt Curve Analysis

Normalized and Temp-Shifted Melting Curves

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

■ Over half (51 – 75%) of all genetic test performed in the UK involve mutation scanning for 'private' mutations in large genes

  e.g.   Hereditary breast cancer (BRCA1&2)

          Hereditary colorectal cancer (hMLH1 and MSH2)

          Marfan syndrome (FBN1)

Genetics White Paper June 2003

" Our inheritance, our future – realising the potential of genetics in the NHS"

2.20 High throughput automated testing technology ..will become essential to deliver quality results quickly. Robotics and other automation will mean that testing at vastly increased speeds and the ability to do more tests at once will be possible, and the cost per test reduced considerably

2.26 … by 2006 genetic test results should be available to the following standards:

Within 8 weeks for unknown mutations in a large gene

Use of pre-screening techniques for mutation scanning

Mutation Screening for hereditary Breast Cancer

Only *c.*15% of patients referred are found to have a mutation in BRCA1 or BRCA2

Screen 85 PCR amplicons per patient

Therefore the predicted detection rate for a pathogenic mutation per fragment is 0.18%

- most of the amplicons we screen will be normal

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

but the techniques must have **high sensitivity** (no false negative results)

Mutation Scanning for Hereditary Non Polyposis Colon Cancer using LightCycler 480c

- HNPCC is caused by germline mutation in a mismatch repair gene (MLH1, MSH2, MSH6, PMS2) or associated with tumours exhibiting microsatellite instability

- Characterised by increased risk of colon (and other) cancers

- Diagnosis based on Amsterdam Clinical Criteria

- Germline mutations in MLH1 and MSH2 account for approx 90% of detected mutations in families with HNPCC

- Mutation scanning of MLH1 and MSH2 requires analysis of 35 amplicons

Mutation Scanning for Hereditary Non Polyposis Colon Cancer using LightCycler 480

■ Analysed 4 amplicons from hMLH1 (Exons 1, 7 & 13) and MSH2 (Exon 10) in 36 patients, wild type, heterozygous and homozygous plasmid and 56 normal controls:
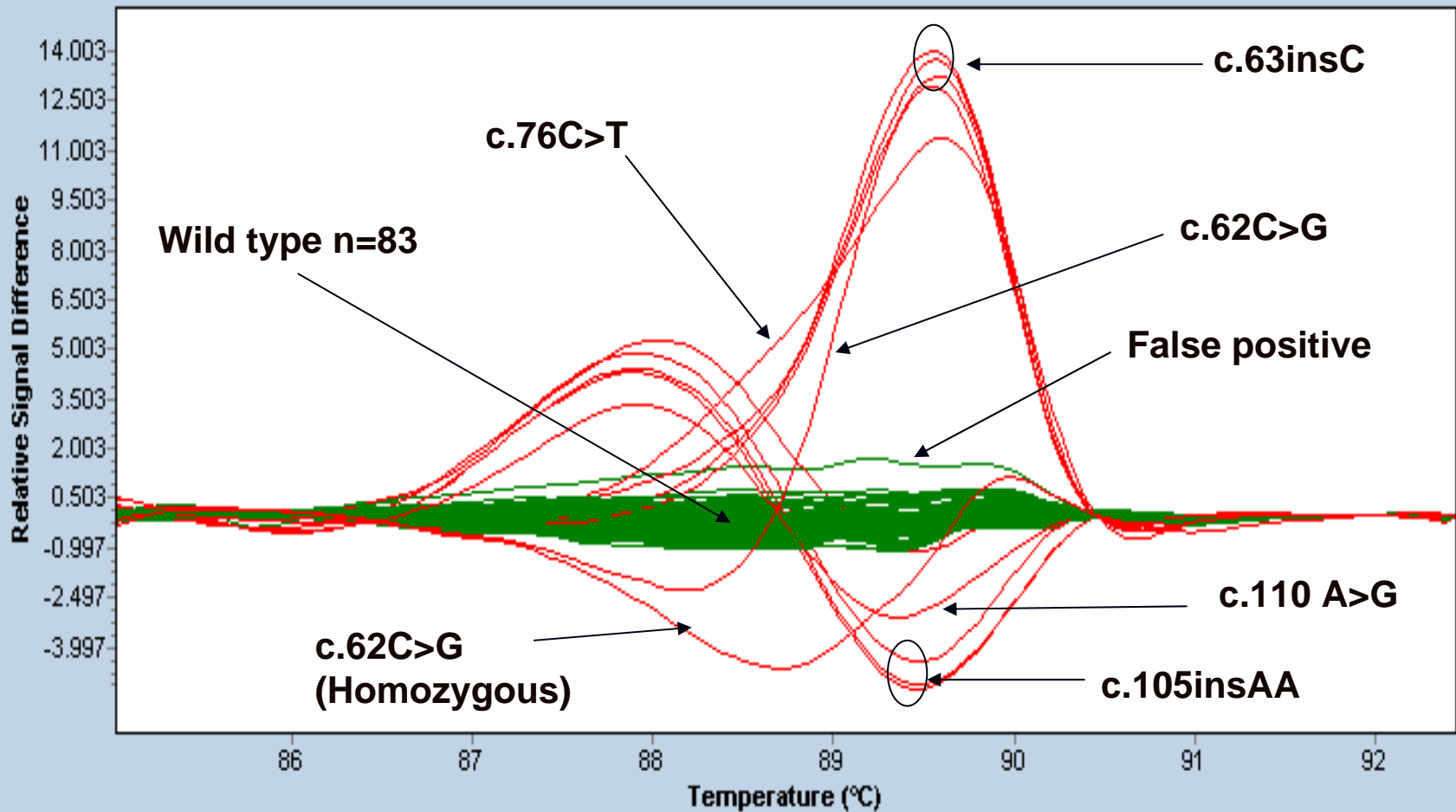
- Size range — 139bp – 277bp
- GC content — 37% - 57%
- Types of mutation — All possible heteroduplex types

  ins C, ins AA

  del A, del C, del CA

  21 unique mutations

■ Amplicons amplified and reaction efficiency monitored using real time PCR

■ High resolution melting profiles analysed using LightCycler 480 software

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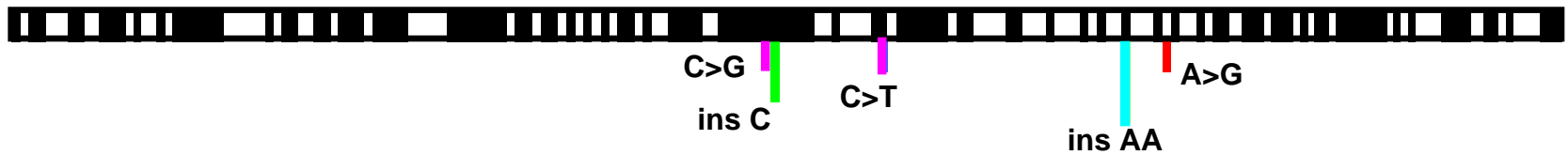
Results

Amplicon	MLH1 Exon 1	MLH1 Exon 7		MLH1 Exon 13	MSH2 Exon10
Specificity setting in analysis	0.9	0.5	0.65	0.8	0.3
True positive	11	7	8	20	13
False positive	1	4	9	2	1
True negative	82	82	77	74	80
False negative	0	1	0	0	0
Sensitivity TP / (TP+FN)	100 %	87.5 %	100 %	100 %	100 %
Specificity TN / (TN+FP)	98.8 %	95.3 %	89.5 %	97.4 %	98.8 %

Data analysis – some considerations

Expected Results
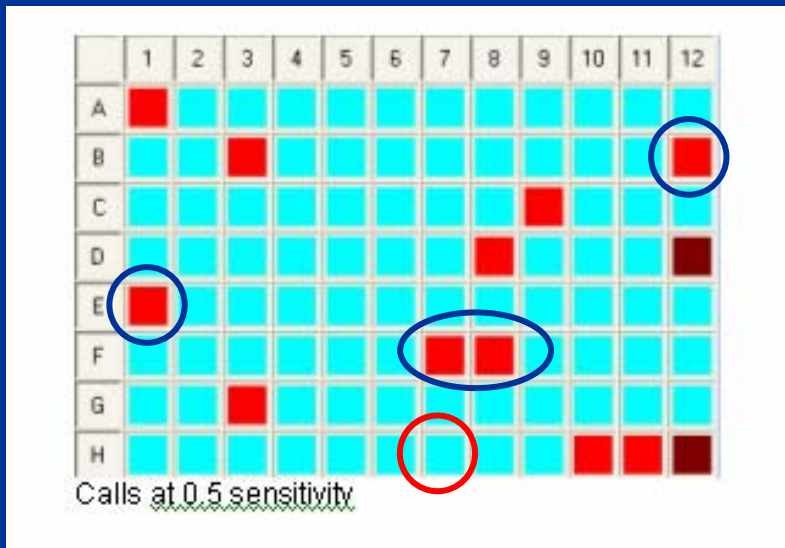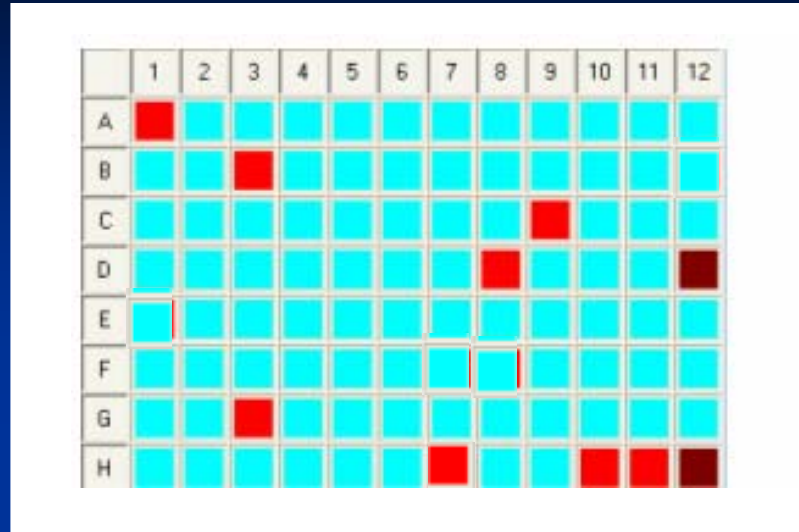
0.5 Sensitivity
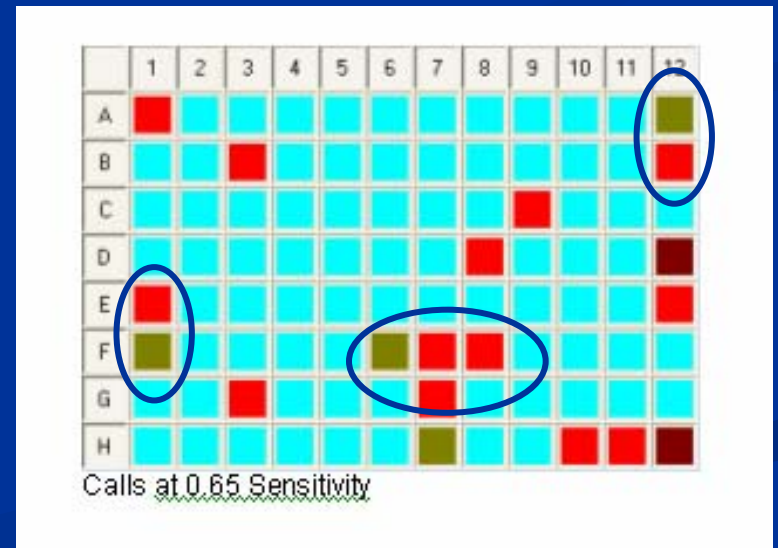
0.65 Sensitivity

Data analysis – some considerations

Analysis setting		0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Dye A	Sensitivity	100	100	91	91	73	55	45	45	0
	Specificity	99	100	100	100	100	100	100	100	100
Dye B	Sensitivity	100	100	100	100	100	82	73	73	27
	Specificity	92	94	96	99	100	100	100	100	100
Dye C	Sensitivity	100	100	100	100	100	100	73	45	0
	Specificity	84	90	93	98	100	100	100	100	100
Dye D	Sensitivity	100	100	100	100	100	100	82	45	0
	Specificity	99	99	99	100	100	100	100	100	100

Factors affecting sensitivity and specificity
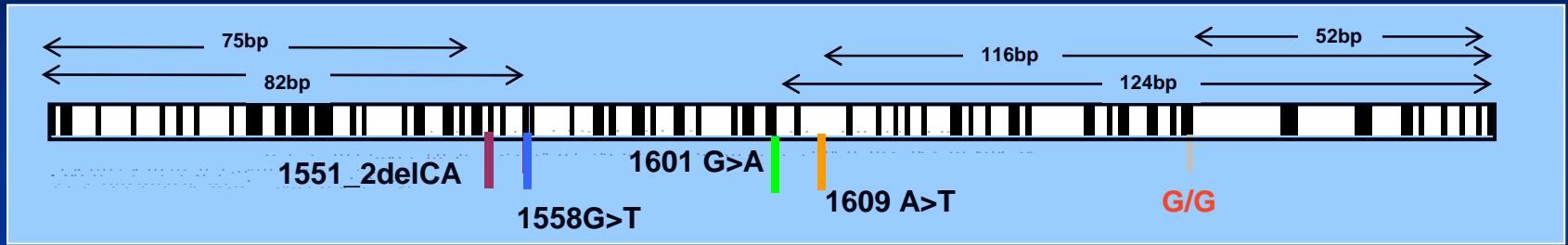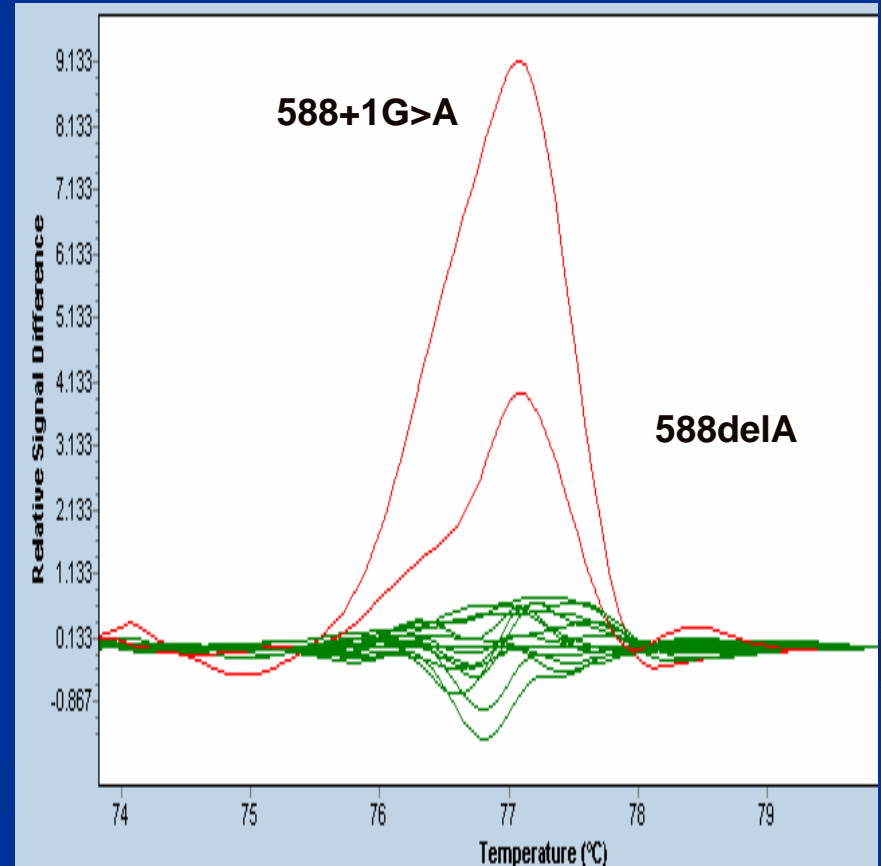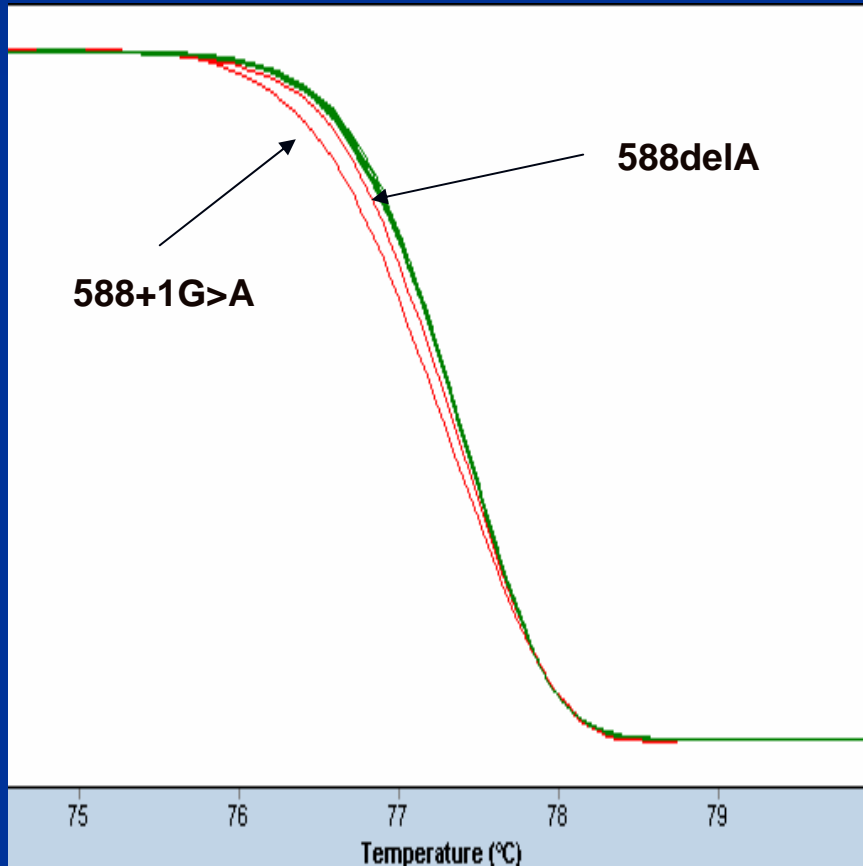
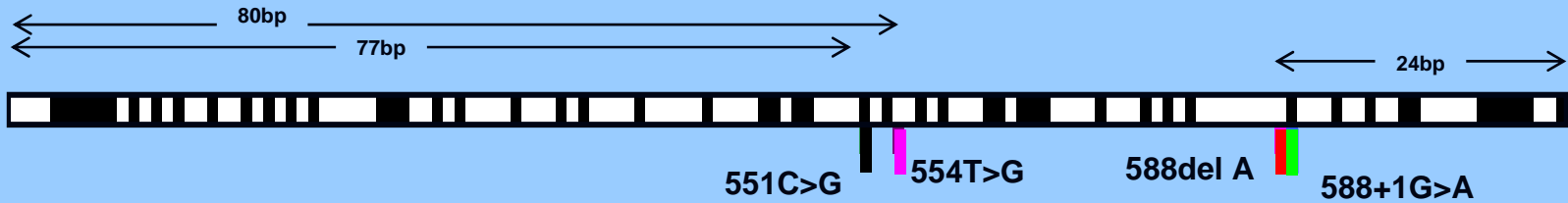# Length of amplicon: Shorter is better
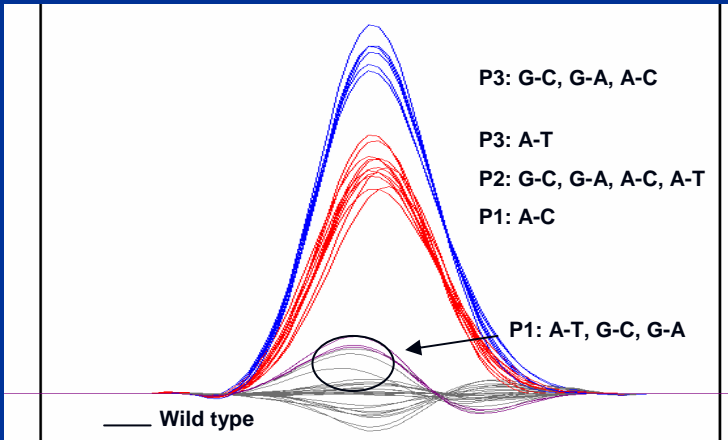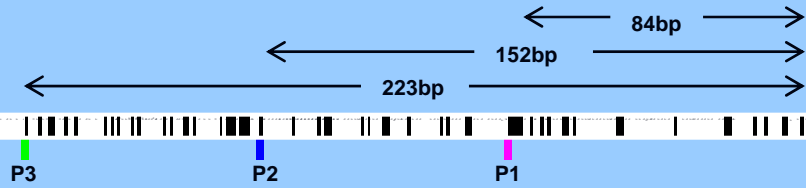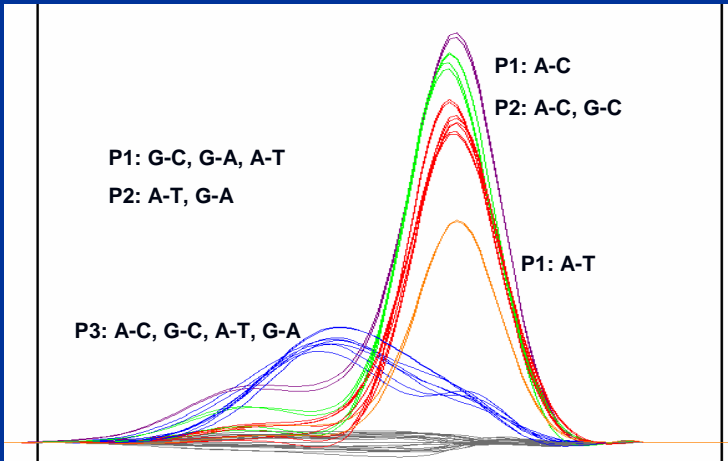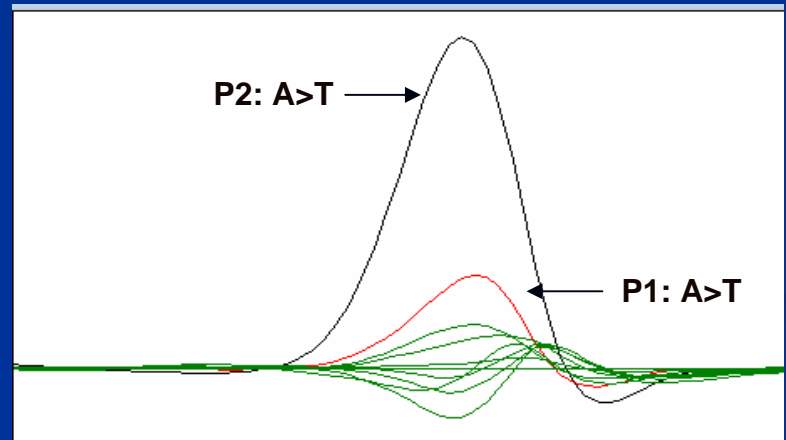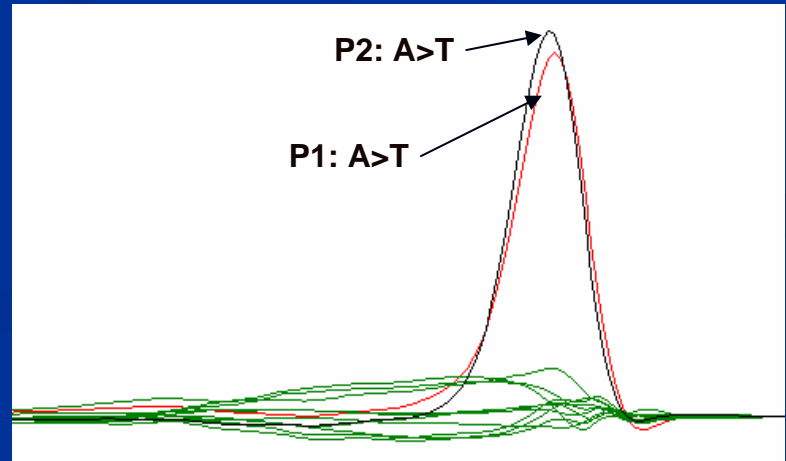
Position in amplicon – no obvious effect

hMSH2 Exon 10 (249bp, 34% GC Rich)
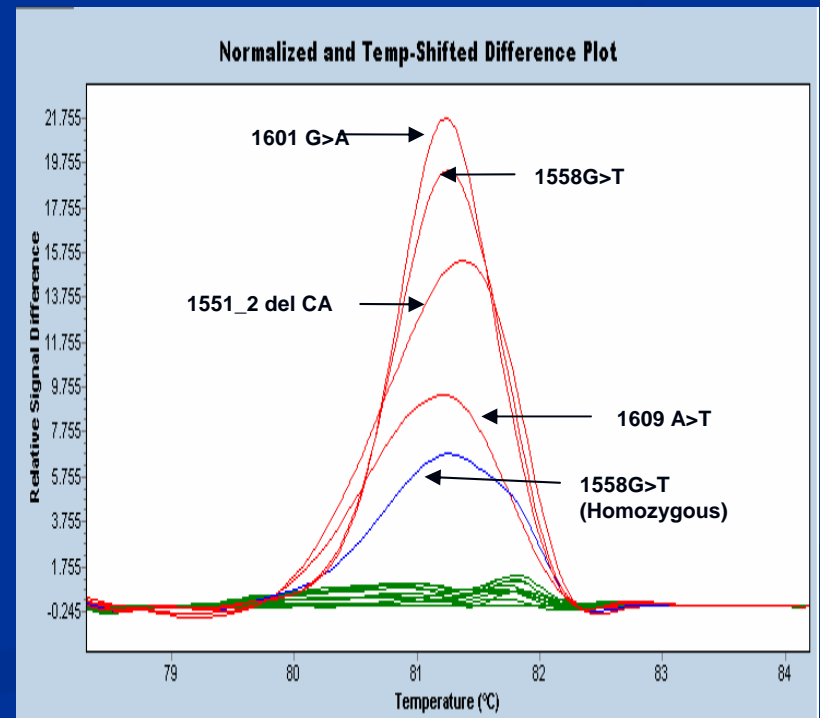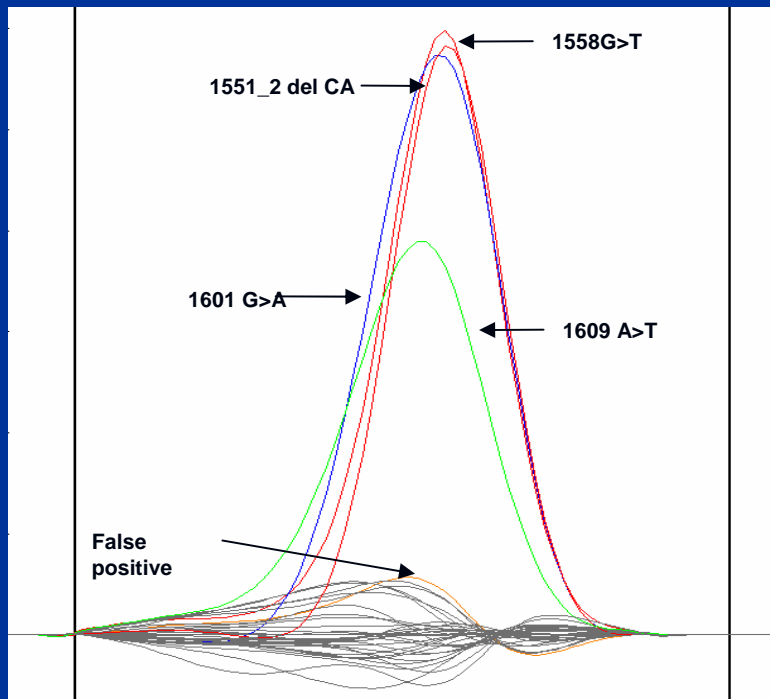
Local sequence context / type of mutation

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

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
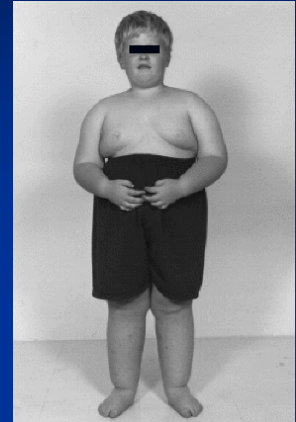
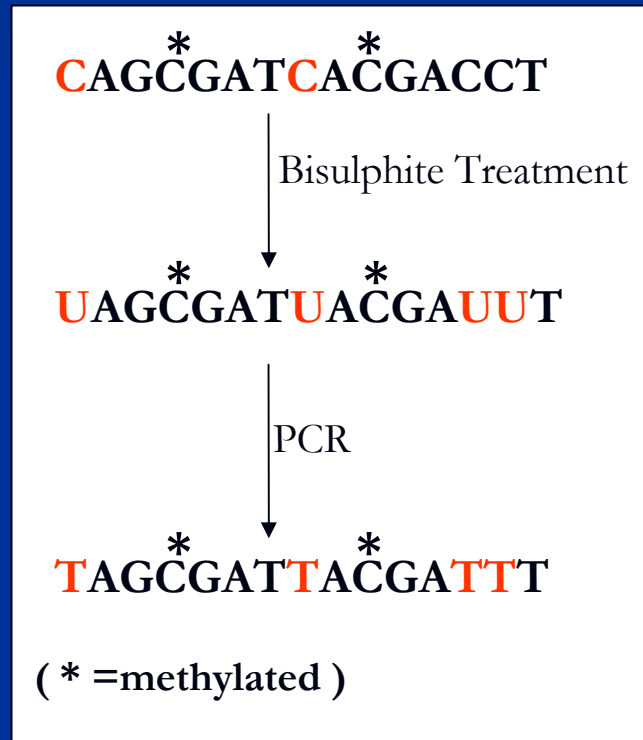**AS** Caused by loss maternal (methylated) contribution

- 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.

Promoter region of SNRPN: 21 CpG sites can vary

HRM for diagnosis of PWS / AS
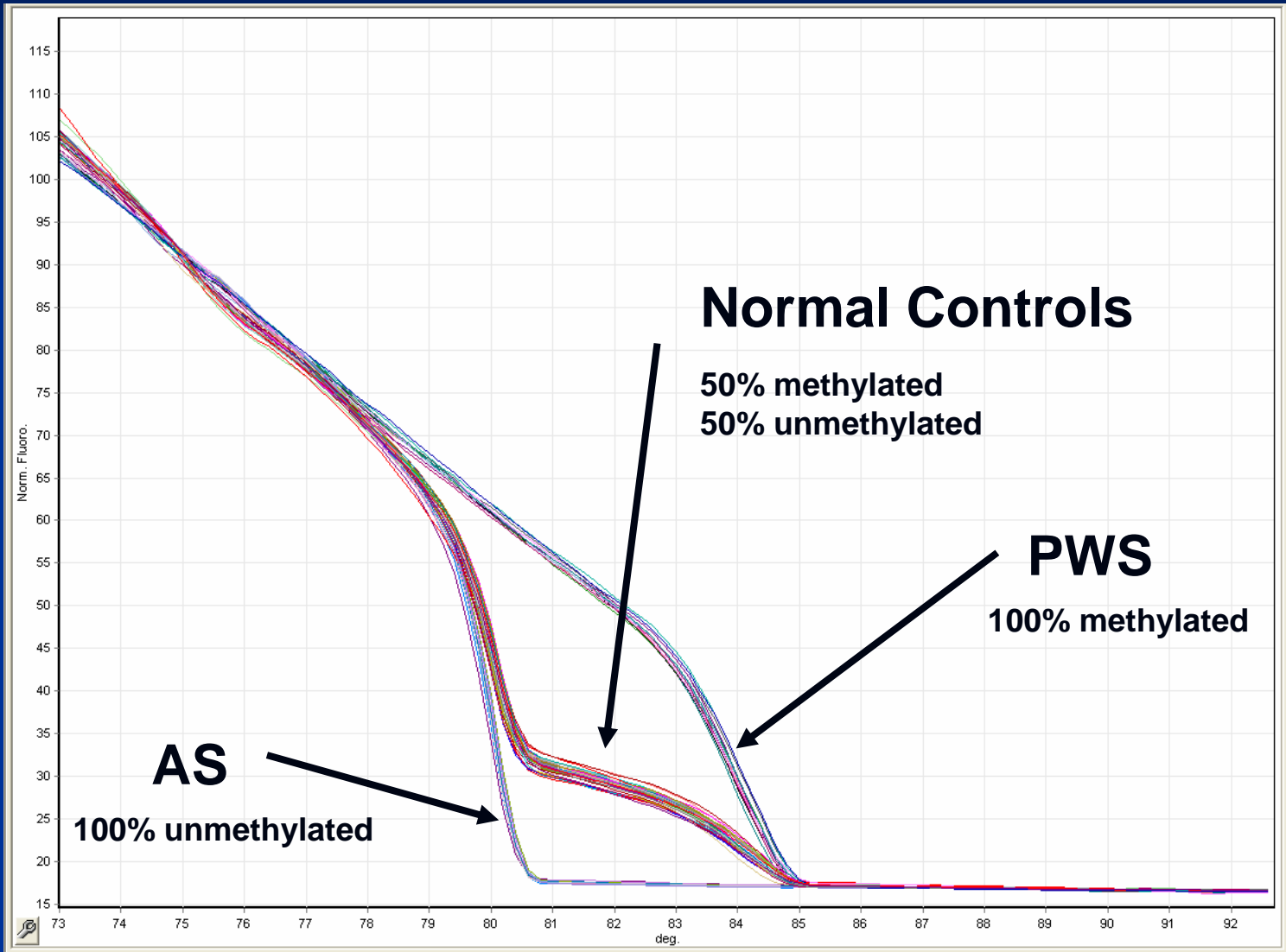
Methylation Sensitive HRM for diagnosis of PWS / AS

- Analysed cohort of PWS (n=39), AS (n=31) and normal controls (n=95) using methylation sensitive high resolution melting assay and compared data with diagnostic MS-PCR assay

- 97.6% samples unambiguously assigned to correct diagnostic category using an 80% confidence percentage threshold

- Correctly identified 2 mosaic AS cases and a PWS patient with putative triplication of SNRPN promoter region on maternal chromosome

- Simple and robust method for screening for alterations in methylation status

- Could be utilised for more complex imprinting disorders e.g. Beckwith Wiedemann syndrome where mosaicism is more commonly observed

Detection of mosaicism
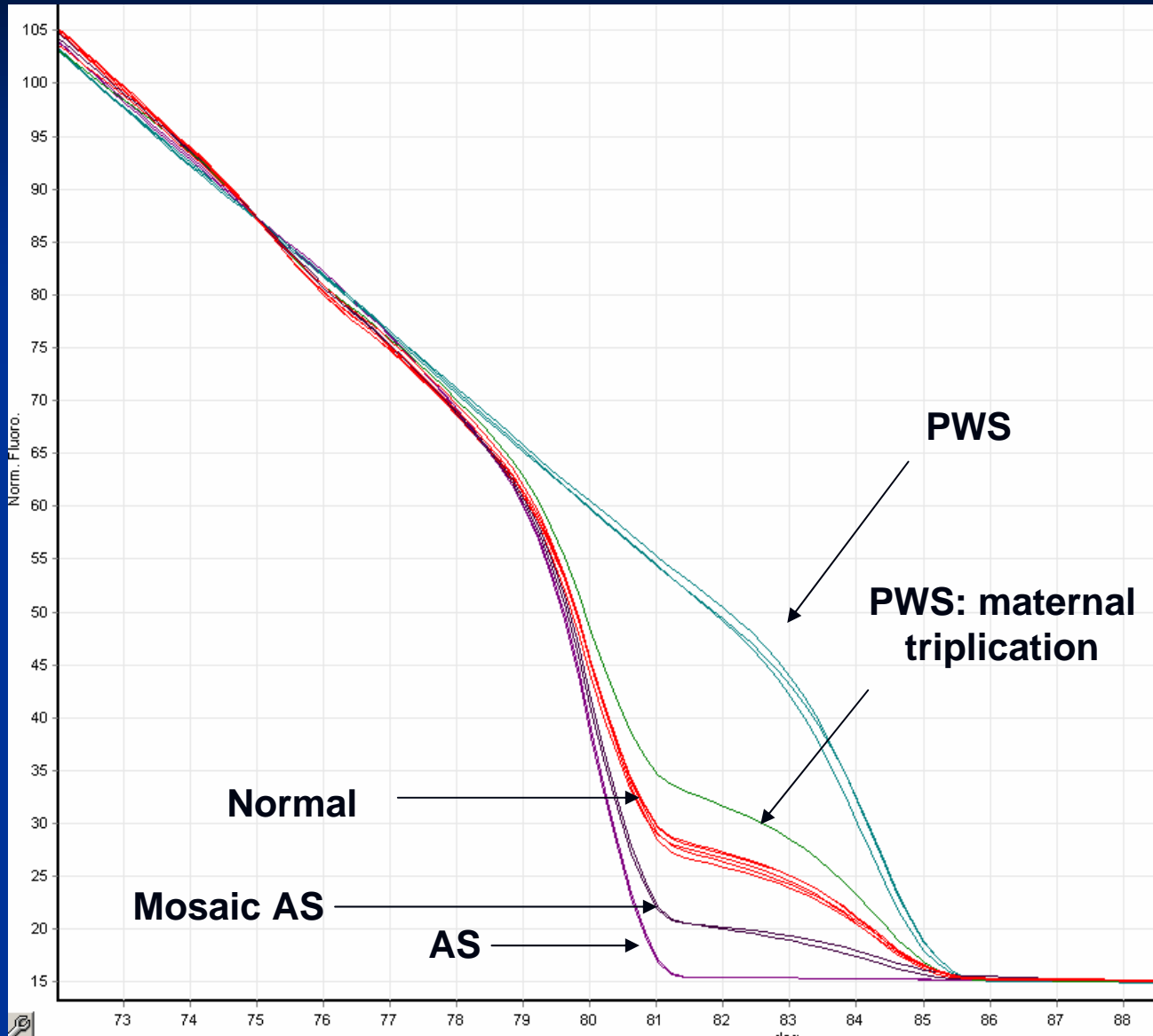
Detection of Acquired Mutations

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
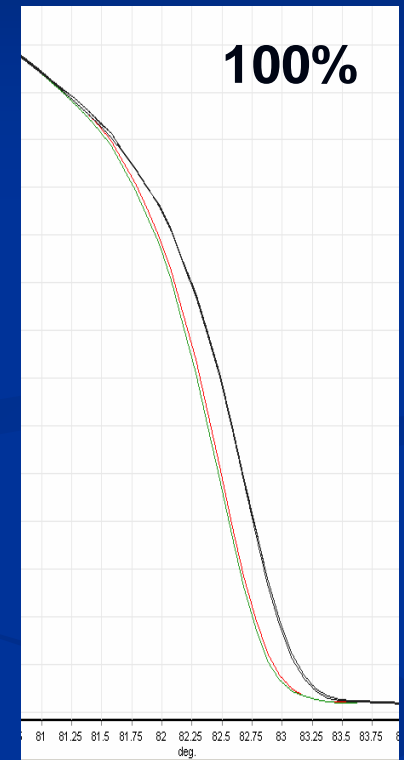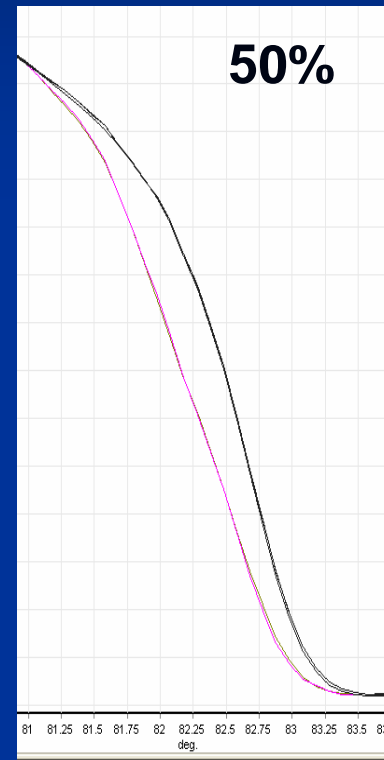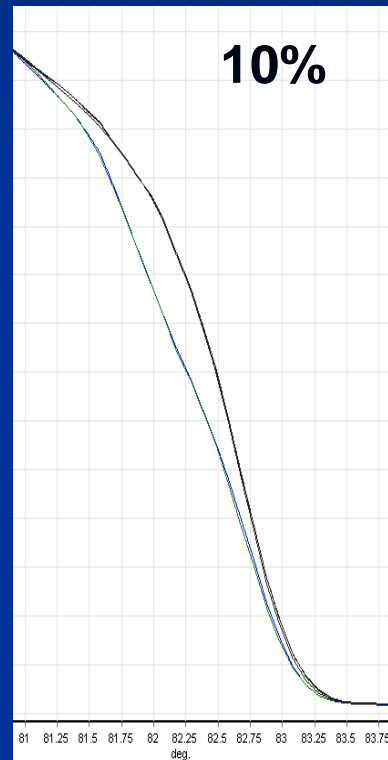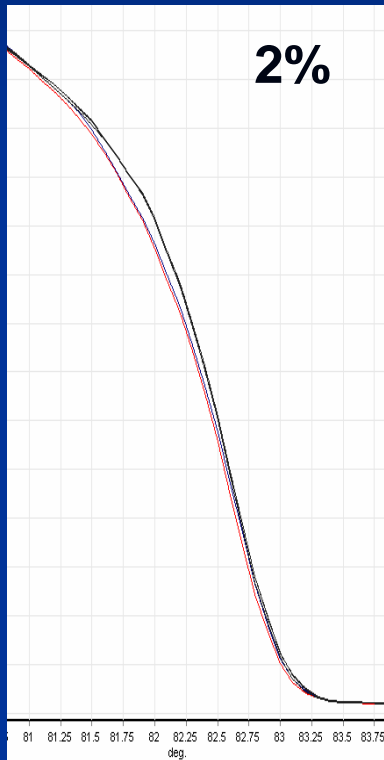
HRM Assays – some considerations

- **PCR optimisation is ESSENTIAL !!**

Very useful to monitor PCR in real time to ensure

- good amplification efficiency (>1.6)
- PCR should take off before 30 cycles
- no primer dimers
- no non-specific products

You will melt whatever you generate in your PCR :

Poor PCR optimisation = messy melt curves!

HRM Assays – some considerations

- **DNA Samples**

Standardise DNA concentration

DNA prepared using different method may show different melt curves

- when using bisulphite treated DNA or historical samples consider 96 well plate DNA clean up methods so samples have the same salt concentration

Inhibitors of PCR decrease amplification efficiency

- can be useful to use a quality control PCR

- **Double stranded DNA binding dyes**

Several dyes are available for high resolution melt curve analysis

- Roche High Resolution Melting Dye (Roche)

- LC Green Plus (Idaho Technology)

- Eva Green (Quantace)

- Syto9 (Invitrogen)

Summary

■ High resolution melting 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

■ Requires no post-PCR handling and no separation step

■ Technique has a mutation detection sensitivity and specificity which is comparable to currently available pre-screening techniques.

■ PCR optimisation and rigorous assay design and validation is essential

■ Capable of detecting some homozygous mutations and 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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