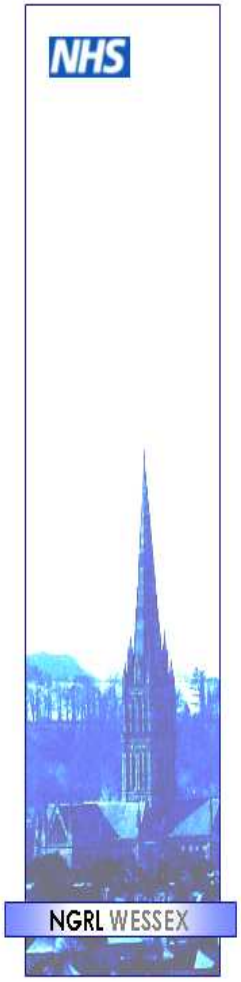
Salisbury NHS
NHS Foundation Trust



High resolution melting for methylation analysis

Helen White, PhD

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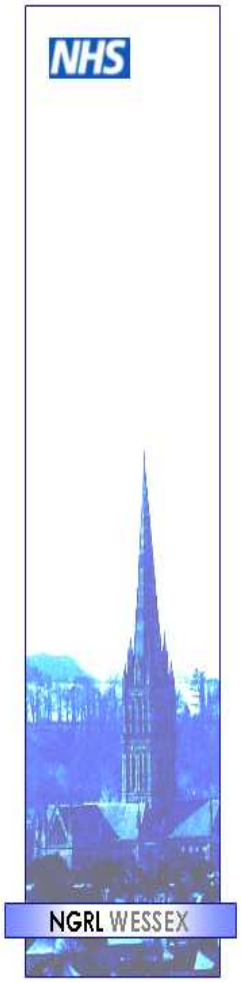
National Genetics Reference Lab (Wessex)

DH Department of Health

Why analyse methylation? – Genomic imprinting



- In diploid organisms somatic cells possess two copies of the genome.

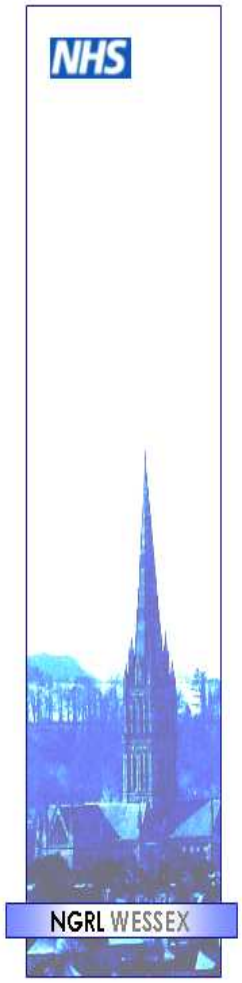
- Each autosomal gene is therefore represented by two copies, or alleles - one copy inherited from each parent at fertilisation.

- For most autosomal genes, expression occurs from both alleles

- However, a small proportion (<1%) of genes are **imprinted**, meaning that gene expression occurs from only one allele

- Genomic imprinting describes the processes involved in introducing functional inequality between two parental alleles of a gene

Why analyse methylation? – Genomic imprinting



- Imprinted genes are expressed in a parent-of-origin-specific manner

  e.g. the allele inherited from the mother e.g. H19
  the allele inherited from the father e.g. IGF2

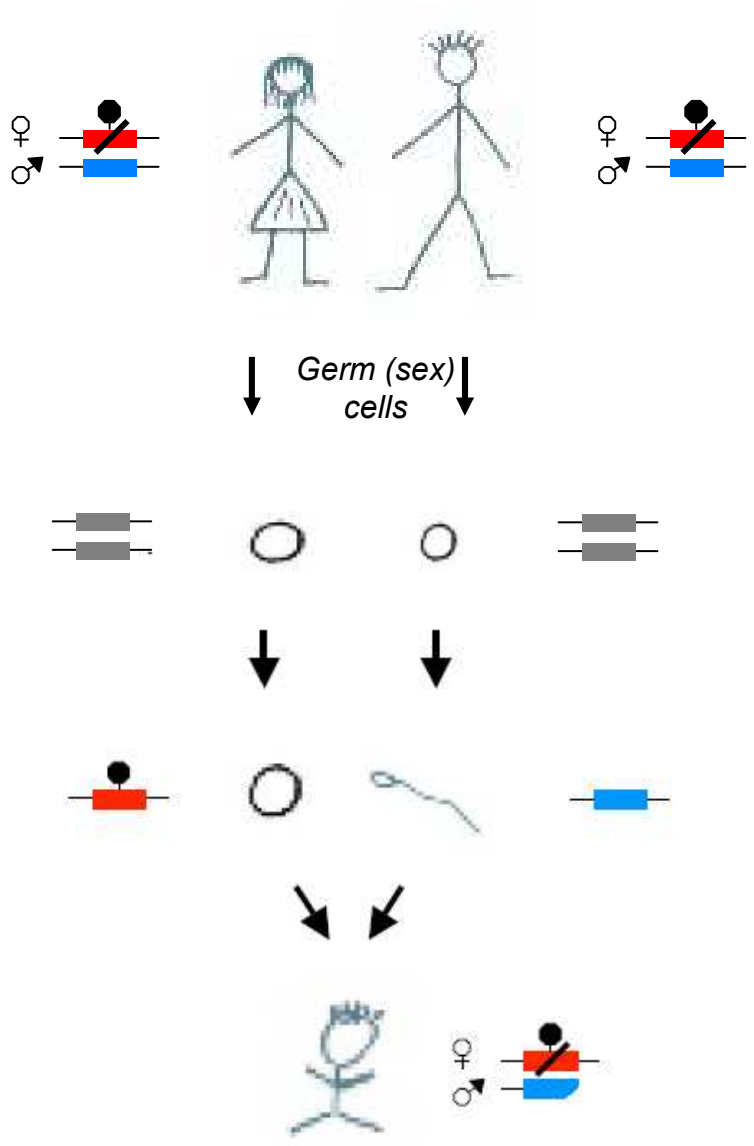
- The nature of the imprint must therefore be **epigenetic** (modifications to the structure of the DNA rather than the sequence).

- Two major mechanisms that are involved in establishing the imprint; these are DNA methylation and histone modifications.

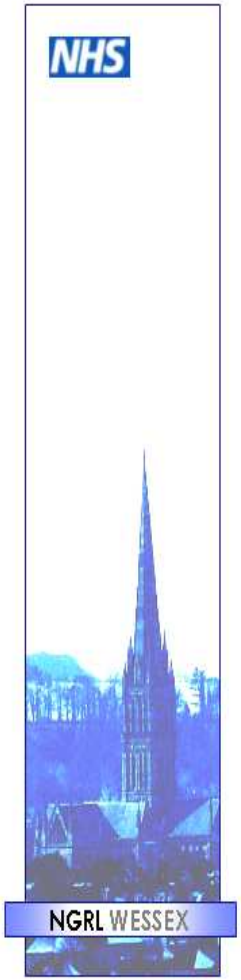
- Majority of imprinted genes have roles in the control of embryonic growth and development, including development of the placenta

- Other imprinted genes are involved in post-natal development, with roles affecting suckling and metabolism

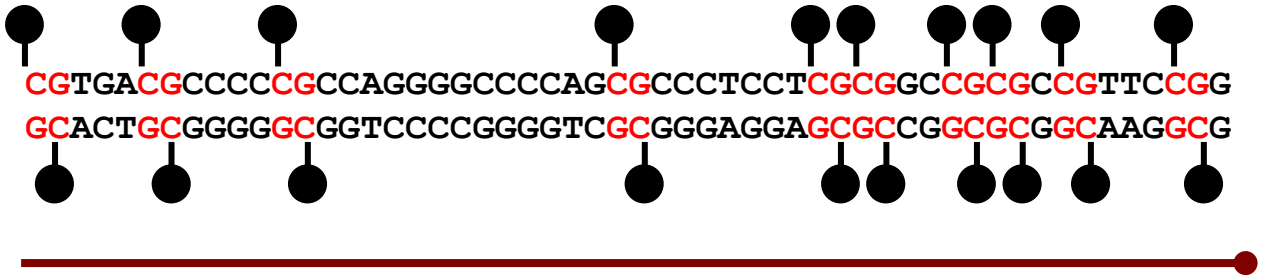
What is the life-cycle of imprinting?



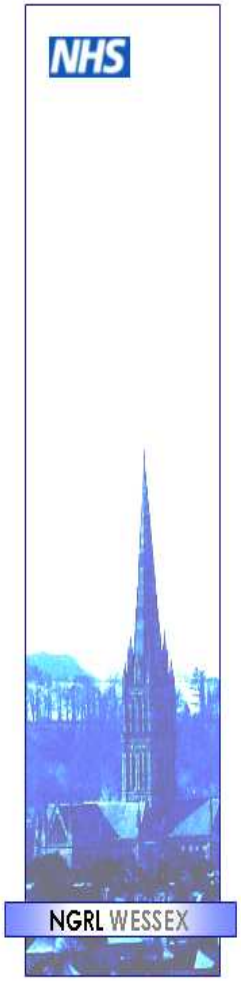
How can HRM be used for methylation analysis?



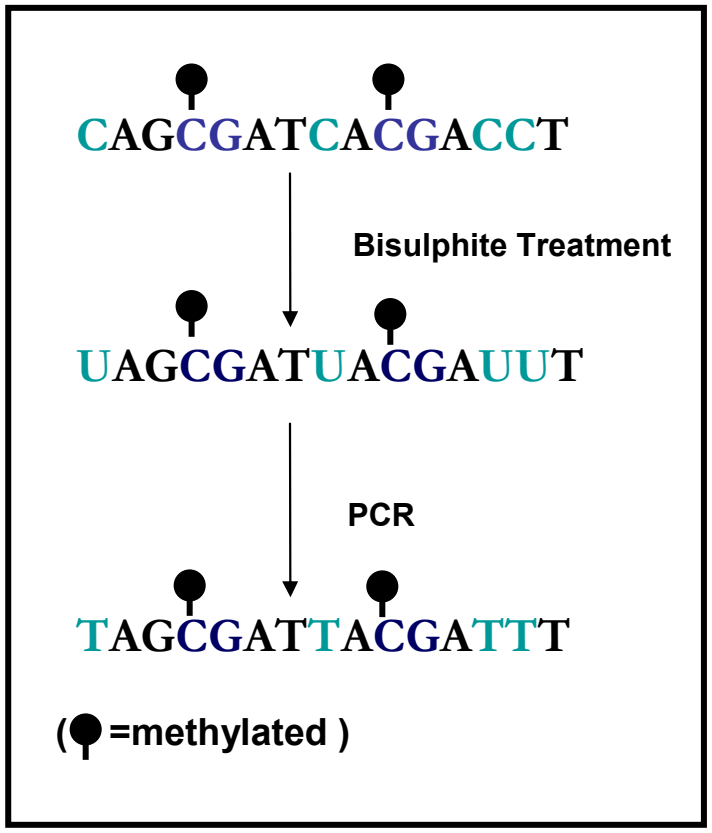
CGTGACGCCCCCGCCAGGGGCCCCAGCGCCCTCCTCGCGGCCGCGCCGTTCCGG
GCACTGCGGGGGCGGTCCCCGGGGTCGCGGGAGGAGCGCCGGCGCGGCAAGGCG



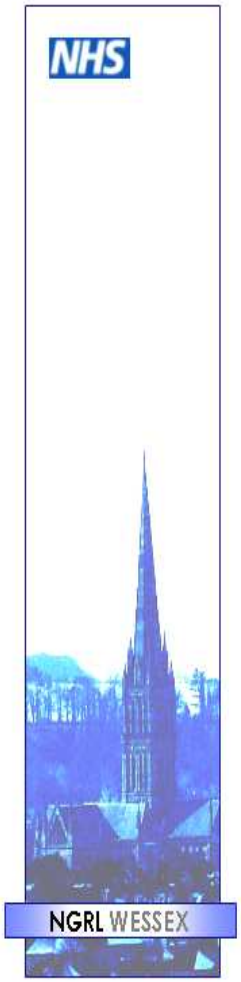
Bisulphite Treatment

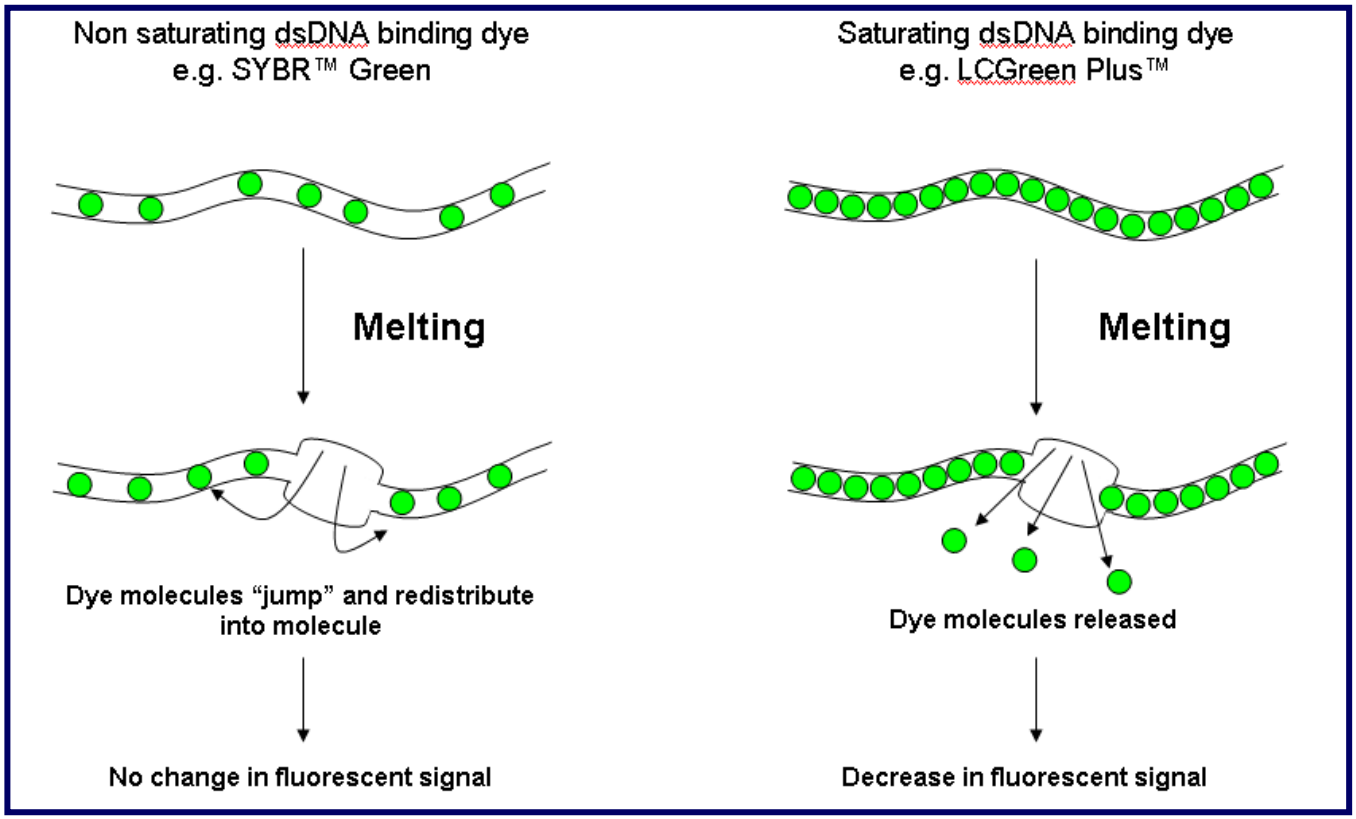


Bisulphite treatment causes ummethylated Cytosines to convert to **Uracil** while methylated cytosines remain unchanged

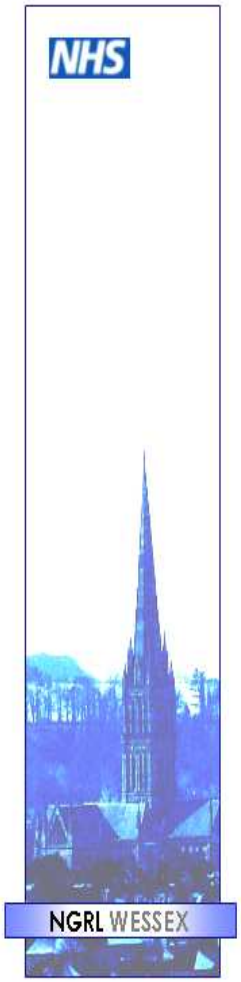


High Resolution Melt Curve Analysis





Populations of DNA produced after bisulphite conversion and PCR amplification



Normal sample: one methylated allele and one unmethylated allele

(MeC remain as C and Unmethylated C converted to T, Y=C or T)

GTGYGAAGTTTGTYGTTGTTGTAGYGAGT

Hypermethylated sample: No unmethylated allele present

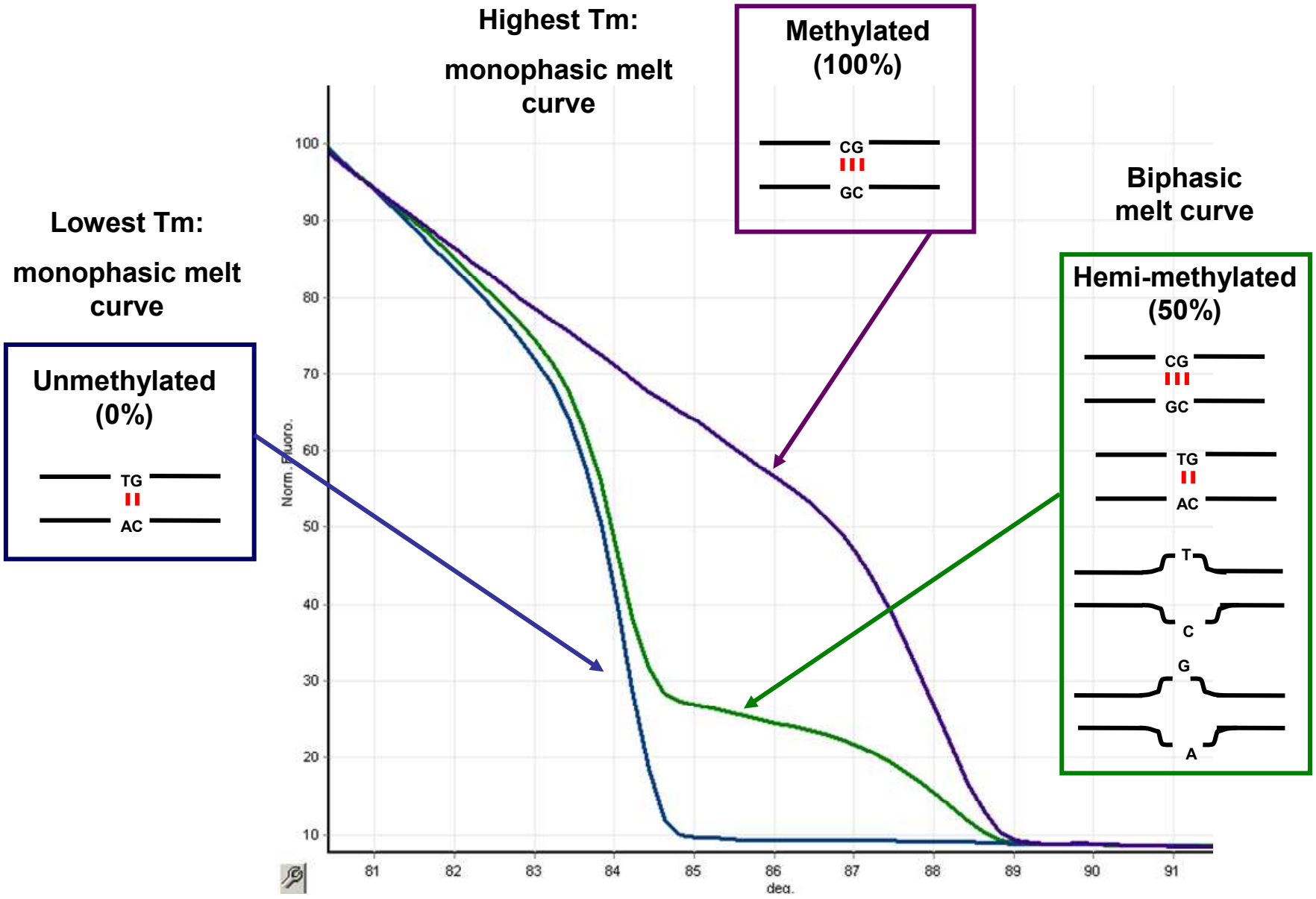
(MeC remain as C)

GTGCGAAGTTTGTCGTTGTTGTAGCGAGT

Hypomethylated sample: No methylated allele present

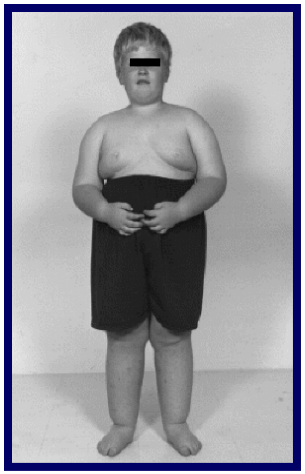
(Unmethylated C converted to T)

GTGTGAAGTTTGTTGTTGTTGTAGTGAGT


Highest Tm:
monophasic melt curve
Methylated (100%)
CG
GC
Lowest Tm:
monophasic melt curve
Unmethylated (0%)
TG
AC
Biphasic melt curve
Hemi-methylated (50%)
CG
GC
TG
AC
T
C
G
A
Norm. Fluoro.
100
90
80
70
60
50
40
30
20
10
81
82
83
84
85
86
87
88
89
90
91
deg.

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

Promoter region of SNRPN

Paternal chromosome 15

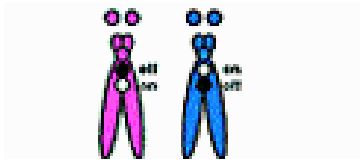
Unmethylated

Maternal chromosome 15

Methylated

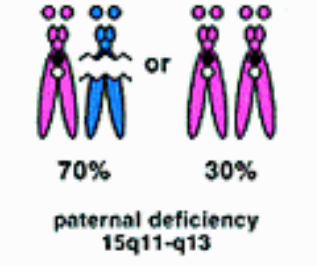
NORMAL

AGGGAGTTGGGATTTTTGTATTGYGGTAAATAAGTAYGTTTGYGYGGTYGTAGAGGTAGGTTGGYGYGTATG
TTTAGGYGGGGATGTGTGYGAAGTTTGTYGTTGTTGTAGYGAGTTTGGYGTAGAGTGGAGYGGTYGTYGGAG
ATGTTTGAYGTATTTGTTTGAGGAGYGGTTAGTGAYGYGATGGAGYGGGTAAGGTTAGTTGTGTYGGTGGTT
TTTTTTAAGAGATAGTTTGGGG



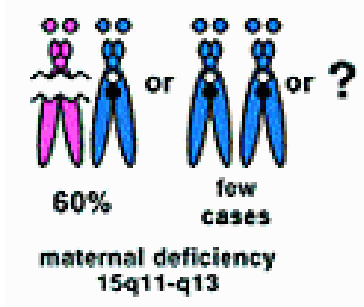
PWS

AGGGAGTTGGGATTTTTGTATTGCGGTAAATAAGTACGTTTGCGCGGTCGTAGAGGTAGGTTGGCGCGTATG
TTTAGGCGGGGATGTGTGCGAAGTTTGTCGTTGTTGTAGCGAGTTTGGCGTAGAGTGGAGCGGTCGTCGGAG
ATGTTTGACGTATTTGTTTGAGGAGCGGTTAGTGACGCGATGGAGCGGGTAAGGTTAGTTGTGTCGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

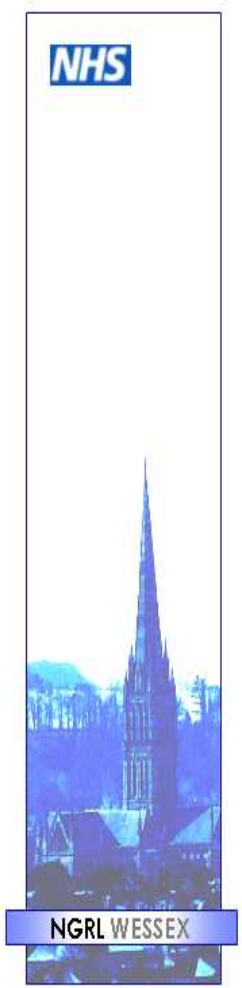


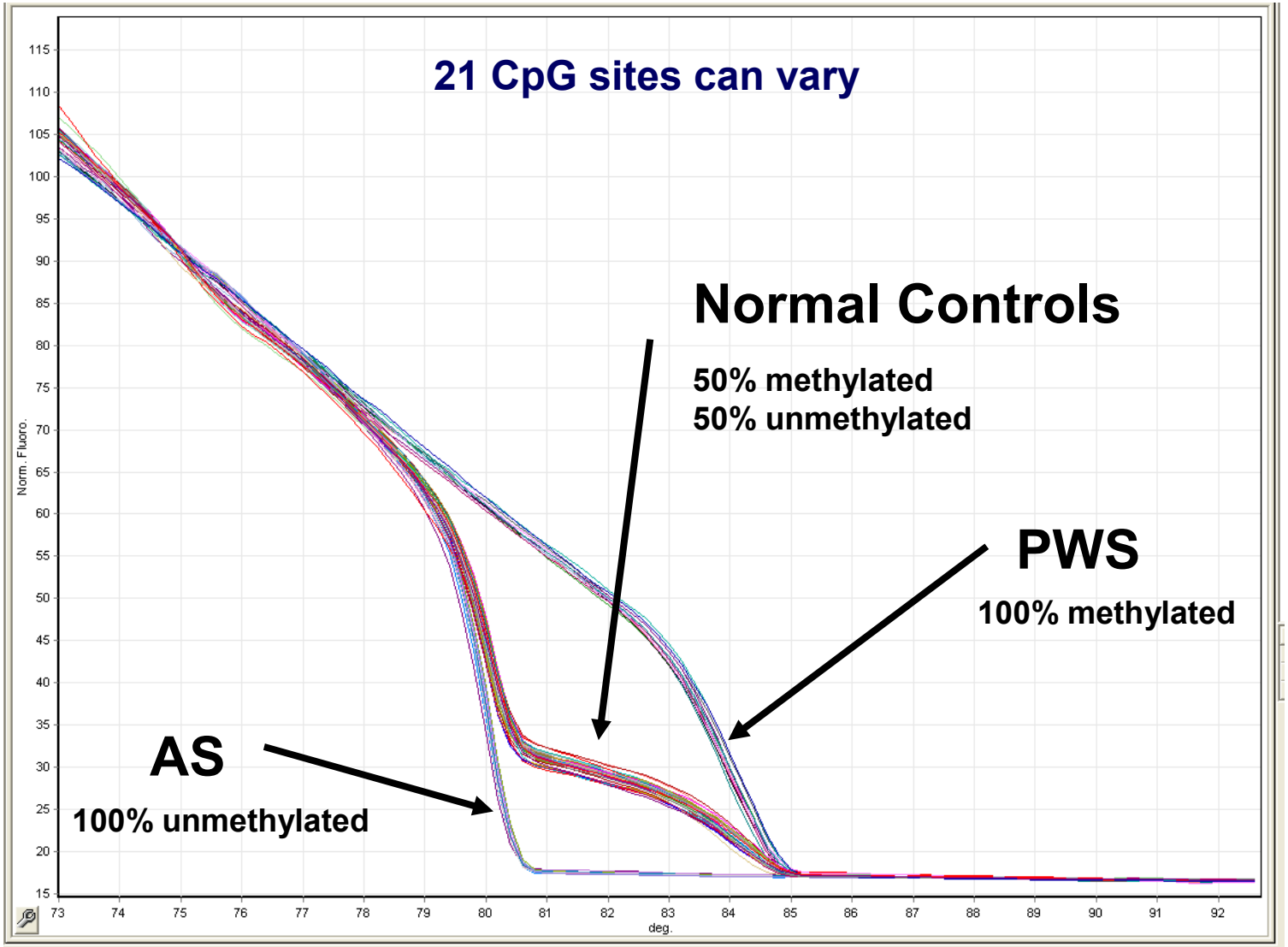
AS

AGGGAGTTGGGATTTTTGTATTGTGGTAAATAAGTATGTTTGTGTGGTTGTAGAGGTAGGTTGGTGTGTATG
TTTAGGTGGGGATGTGTGTGAAGTTTGTTGTTGTTGTAGTGAGTTTGGTGTAGAGTGGAGTGGTTGTTGGAG
ATGTTTGATGTATTTGTTTGAGGAGTGGTTAGTGATGTGATGGAGTGGGTAAGGTTAGTTGTGTTGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

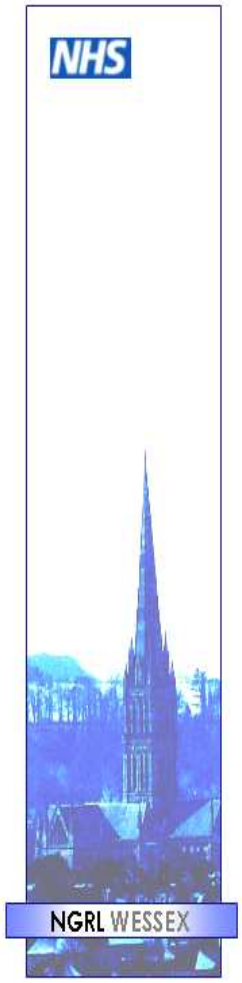


HRM for diagnosis of PWS / AS: Analysis of SNRPN promoter





Methylation Sensitive HRM for diagnosis of PWS / AS

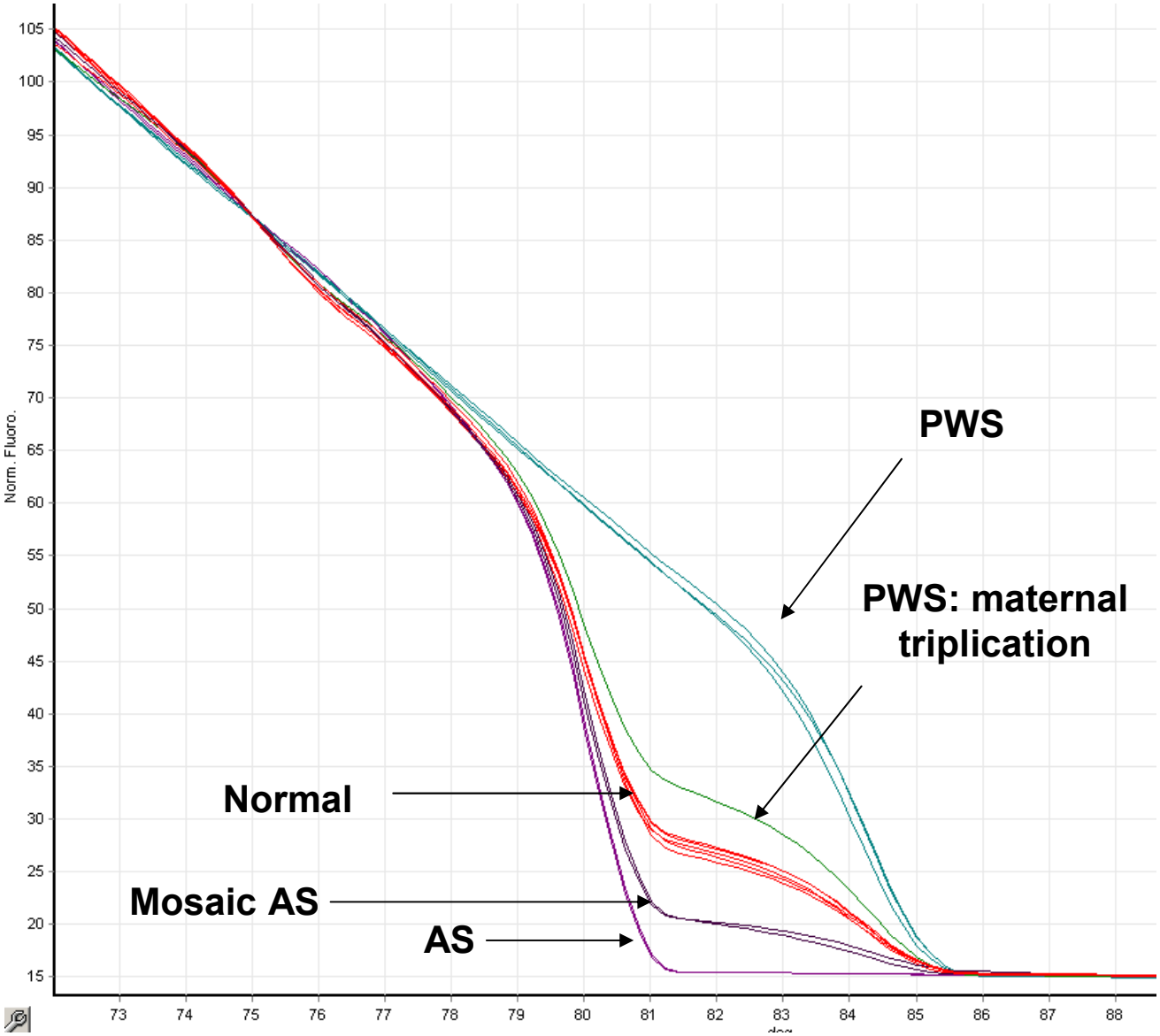


- Analysed cohort of PWS (n=39), AS (n=31) and normal controls (n=95) using methylation sensitive HRM and compared data with diagnostic MS-PCR assay

- 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

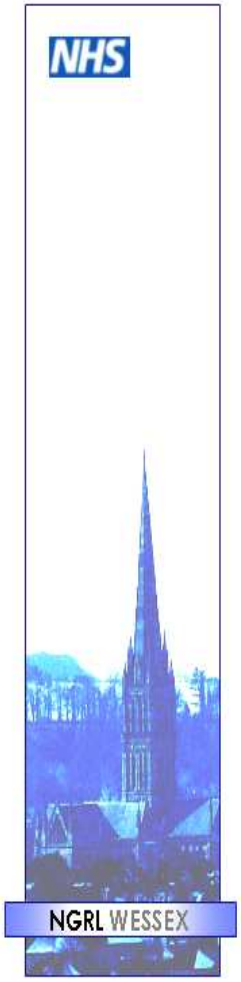
Detection of mosaicism



White HE, Hall VJ & Cross NCP (2007) Clin Chem

HRM methylation analysis for more complex imprinting disorders

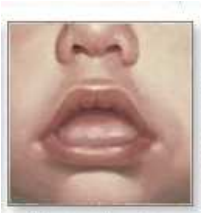
e.g. Beckwith Wiedemann Syndrome



Clinical features:

Overgrowth syndrome affecting 1:13,700


Microcephaly


Macroglossia

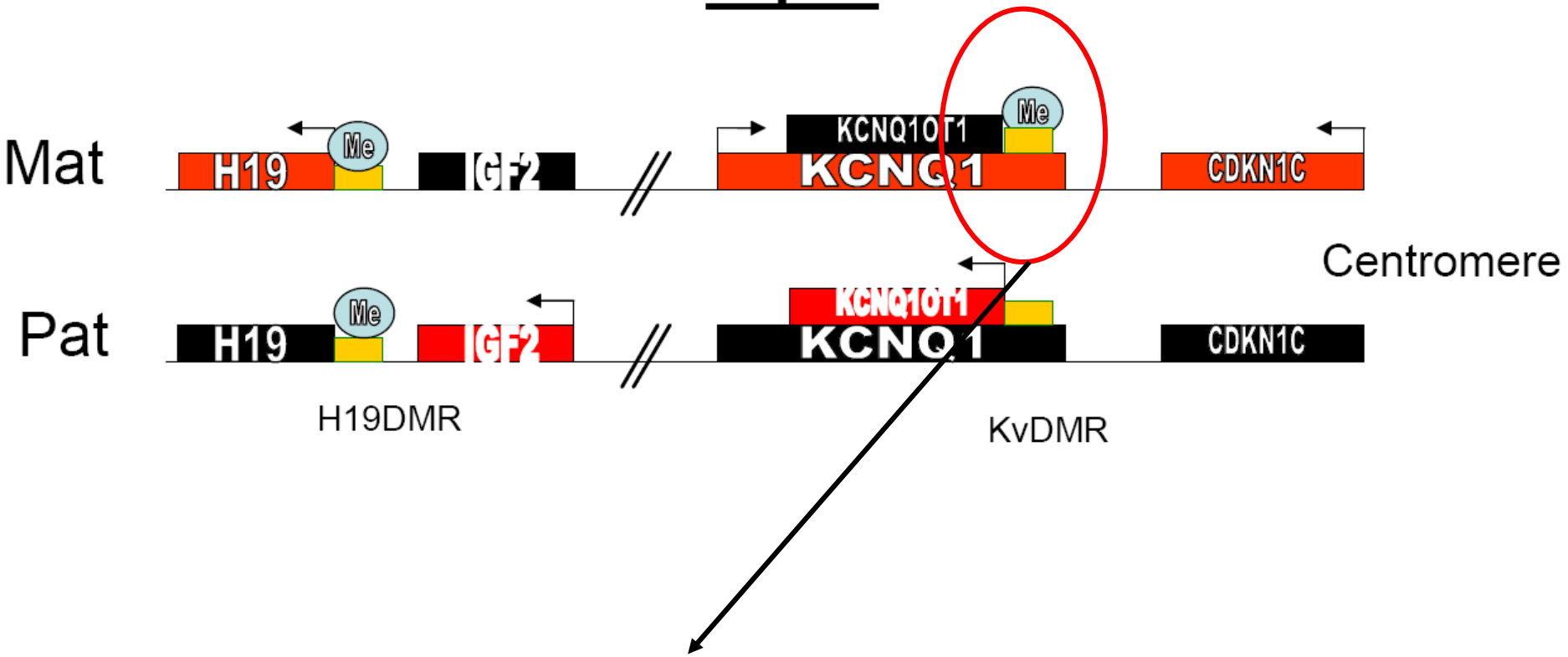
- Macrosomia (Large body size)
- Macroglossia (Large Tongue)
- Visceromegaly (Abnormal enlargement of the large internal organs)
- Embryonal tumours (e.g. Wilms tumour)
- Hemihyperplasia (asymmetric overgrowth of region(s) of the body)
- Neonatal hypoglycaemia
- Ear creases/pits


Umbilical hernia

Genetic causes of BWS:

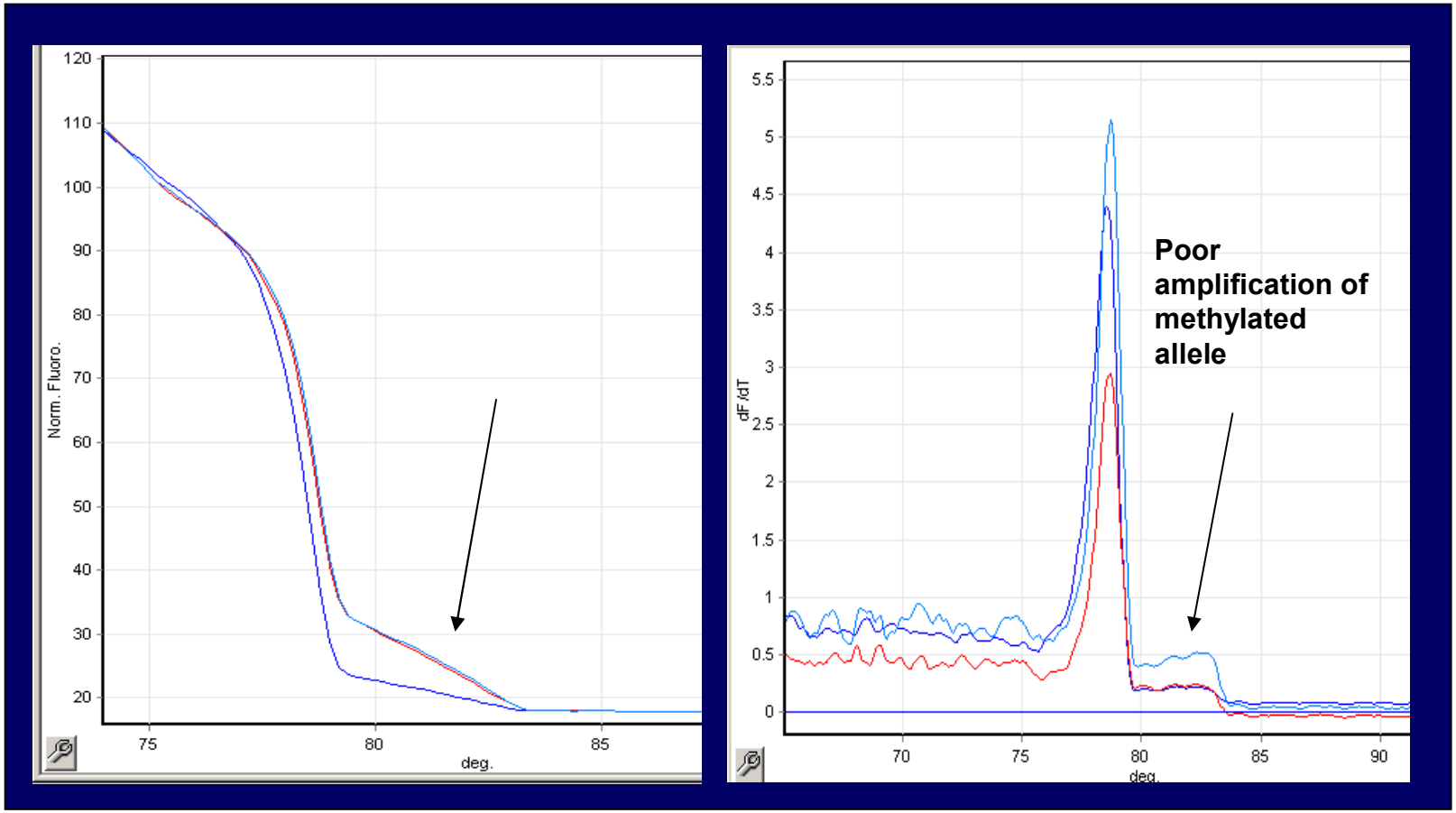
Defective expression of imprinted genes at 11p15.5

11p15

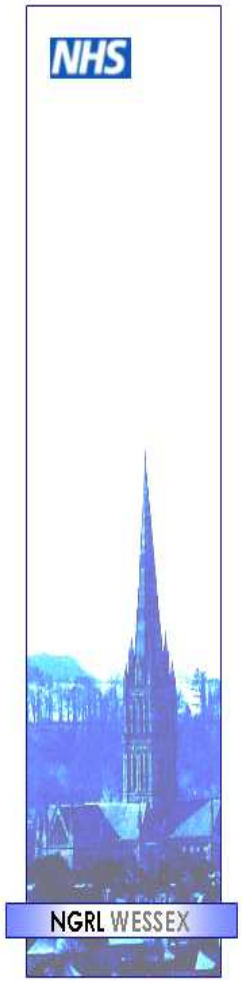


Aim: to design HRM methylation assay to detect **loss of maternal methylation** at KvDMR

Preliminary assay design



Assay redesign



- To increase efficiency of amplification of methylated allele our primers were re-designed

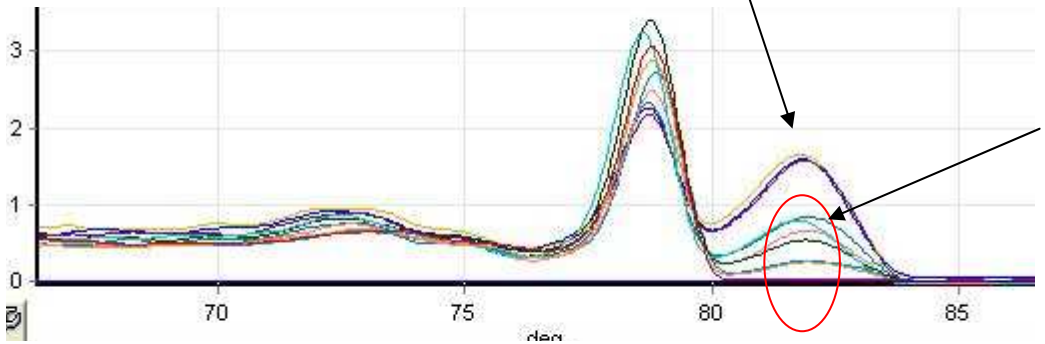
- Instead of primers being non-selective for methylated and unmethylated alleles one primer was designed to incoporate a CpG at the 5' end

- Strategy used by Wojdacz et al to control PCR bias in methylation studies:

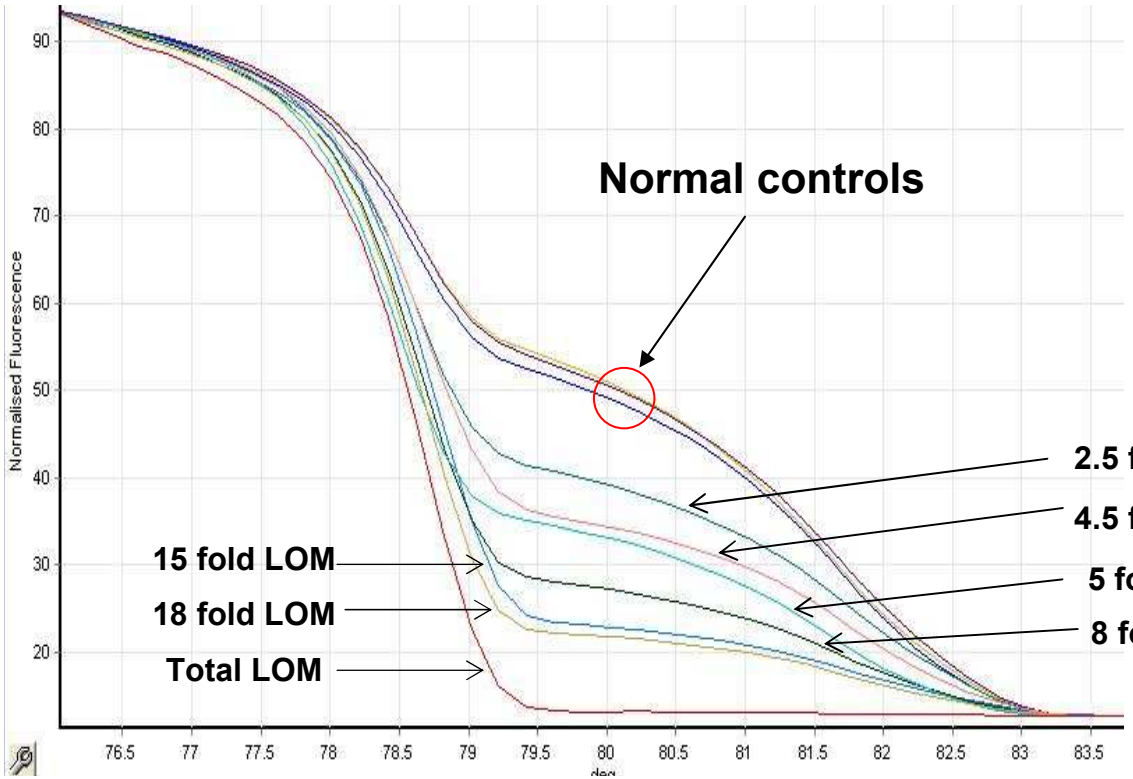
• Wojdacz TK, Hansen LL, Dobrovic A. (2008) A new approach to primer design for the control of PCR bias in methylation studies. BMC Res Notes 28;1:54.

• Wojdacz TK, Hansen LL. (2006) Reversal of PCR bias for improved sensitivity of the DNA methylation melting curve assay. Biotechniques 41(3):274.

Improved amplification of methylated allele in normal individuals



Loss of methylation (LOM) more easily detected in BWS



Normal controls

2.5 fold LOM *

4.5 fold LOM

5 fold LOM

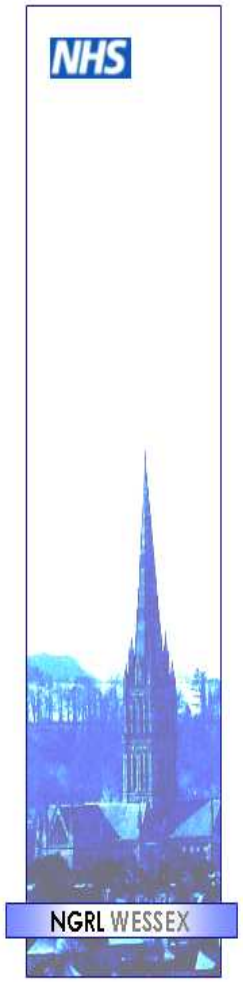
8 fold LOM

15 fold LOM

18 fold LOM

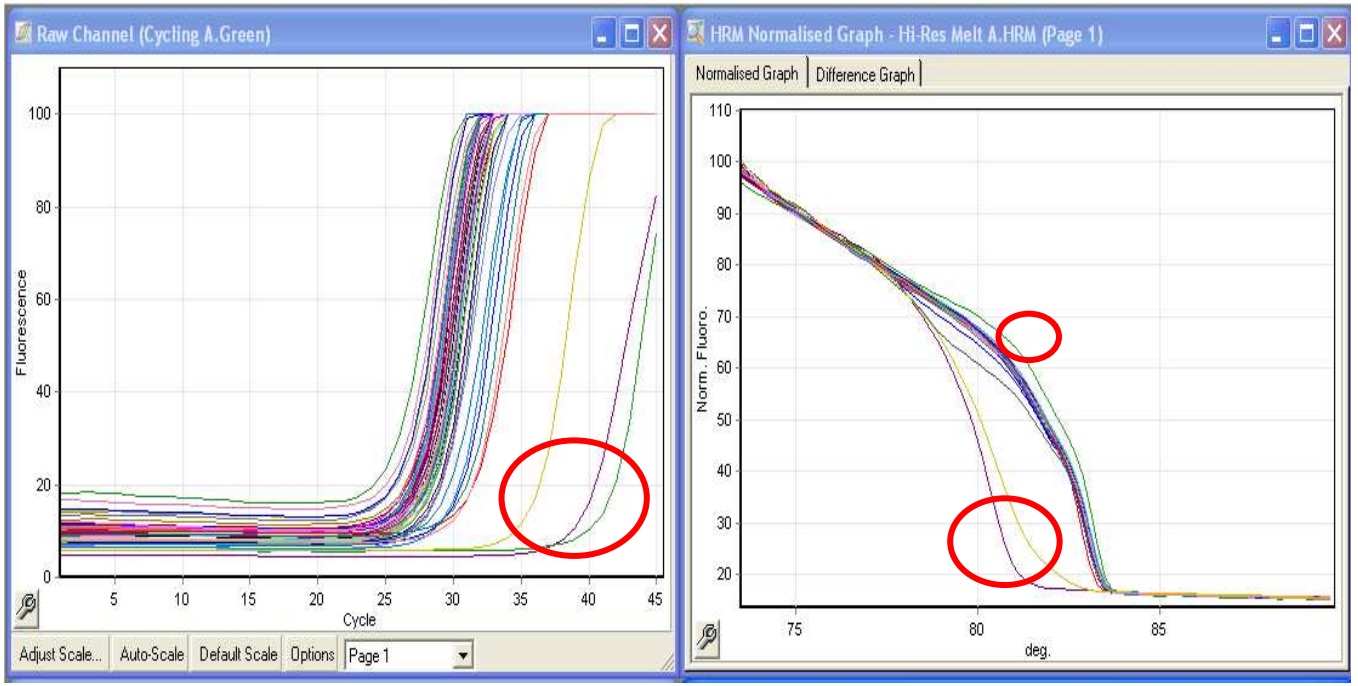
Total LOM

*** By MS-PCR**



Some technical considerations…..

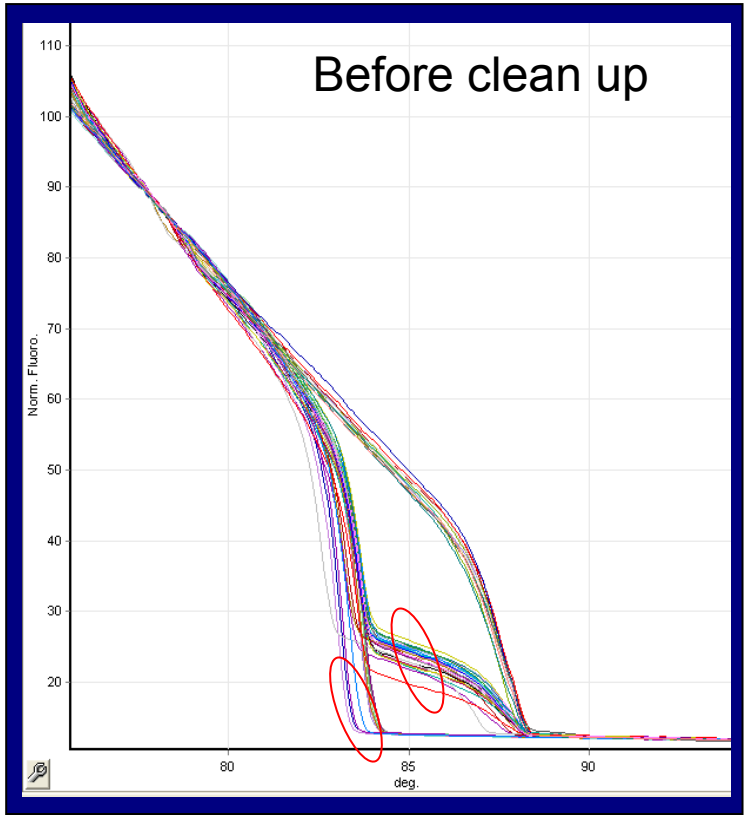
Effect of PCR cycle number, DNA quality and concentration

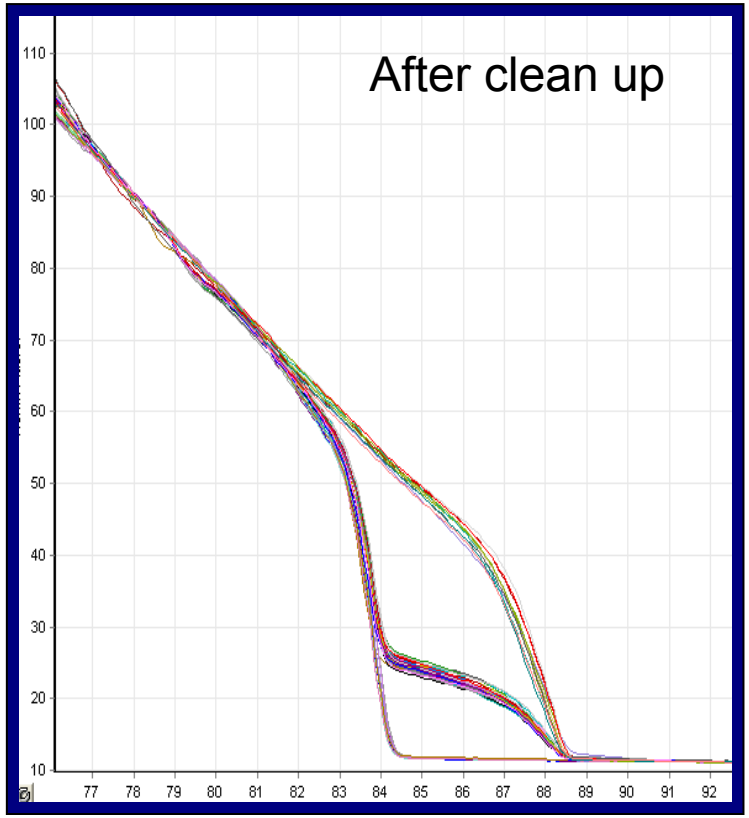


Poor sample quality has the potential to increase false positive results in HRM

Monitoring the PCR in real time allows poor data to be excluded from HRM analysis

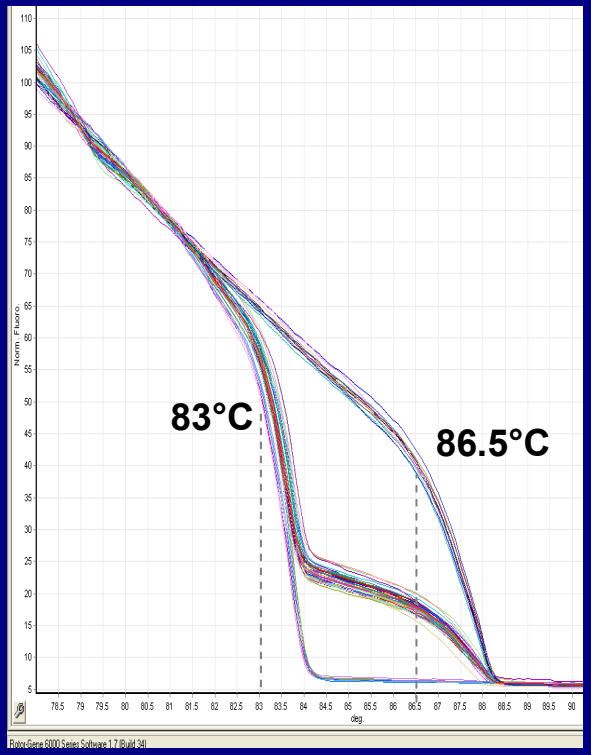
Bisulphite treated DNA



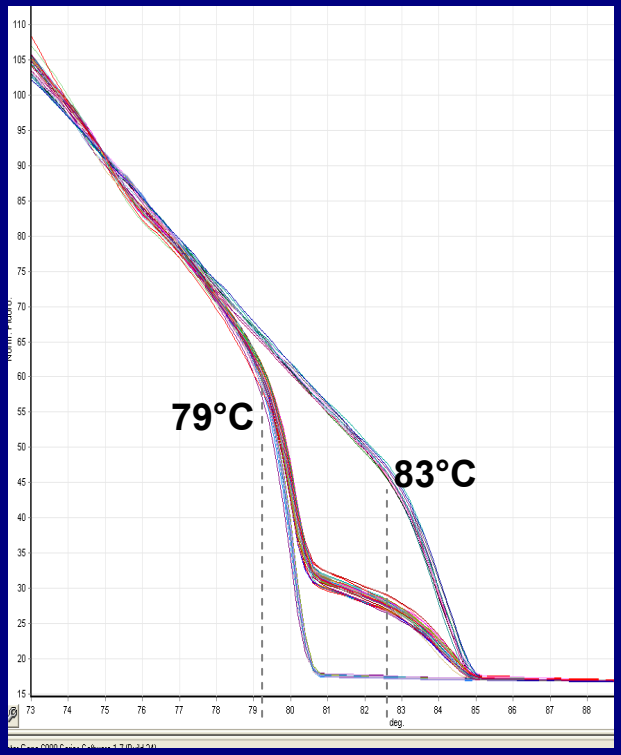


dsDNA binding dyes

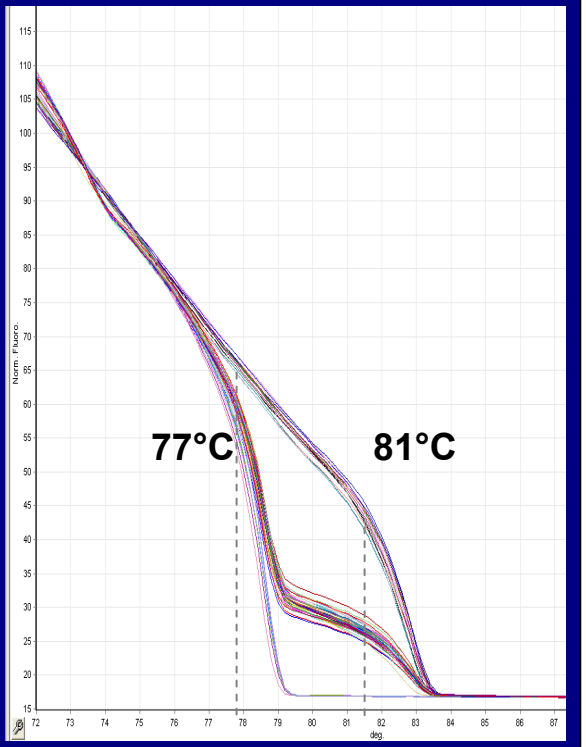
LC Green Plus



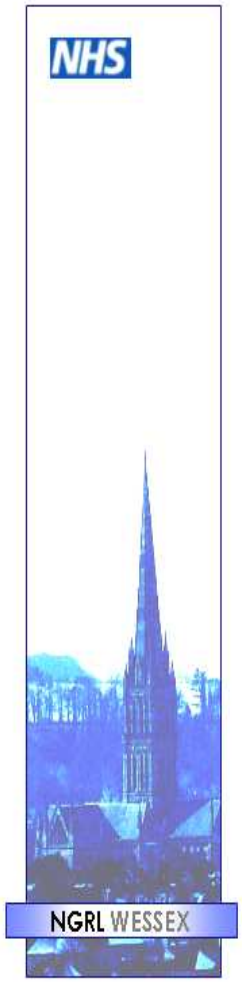
Eva Green



Syto 9



Conclusions



- Following optimisation, MS-HRM is a technically simple and robust method for screening for alterations in methylation status
- MS-HRM has been successfully optimised for PWS / AS screening
- Has potential to be utilised for methylation analysis of more complex imprinting disorders e.g. Beckwith Wiedemann syndrome where mosaicism is more commonly observed

Acknowledgements:

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www.ngrl.org.uk/Wessex