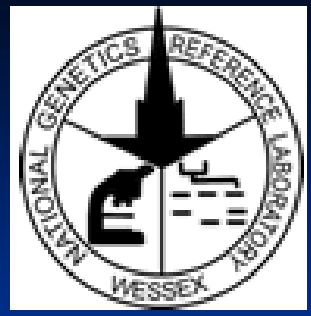

New techniques for DNA methylation analysis

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Salisbury NHS Foundation Trust

Techniques

- MS-MLPA
- Pyrosequencing
- High resolution melt curve analysis
- Mass Spectrometry

Prader Willi and Angelman Syndromes

Two clinically distinct phenotypes that map to 15q11-q13

PWS

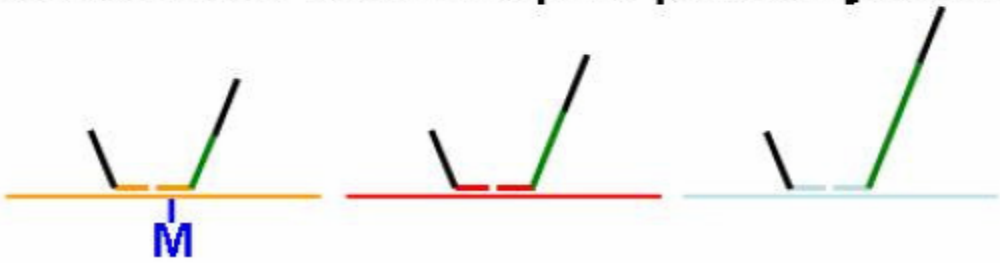
- Caused by loss of the paternal (unmethylated) contribution
  - Paternal deletion (~70%)
  - Maternal UPD (~30% cases)
  - Mutation in the imprinting region causing abnormal methylation (<2%)

AS

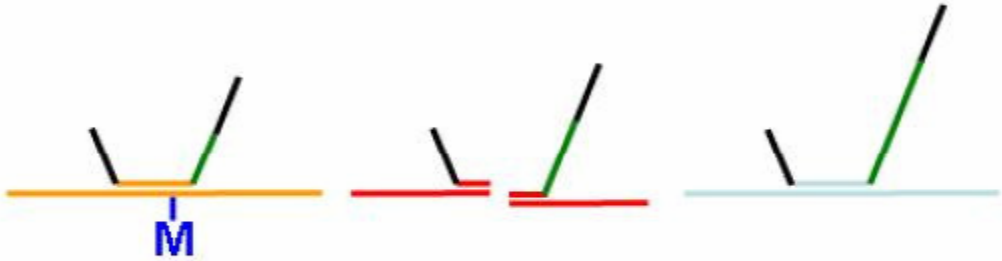
- Caused by loss Maternal (methylated) contribution
  - Maternal deletion (~70%)
  - Paternal UPD (~5% cases)
  - Mutation in the imprinting region causing abnormal methylation (~5%)
- A single gene, UBE3A has been implicated as the AS gene

MS-MLPA

1. Denaturation and Multiplex probe hybridization

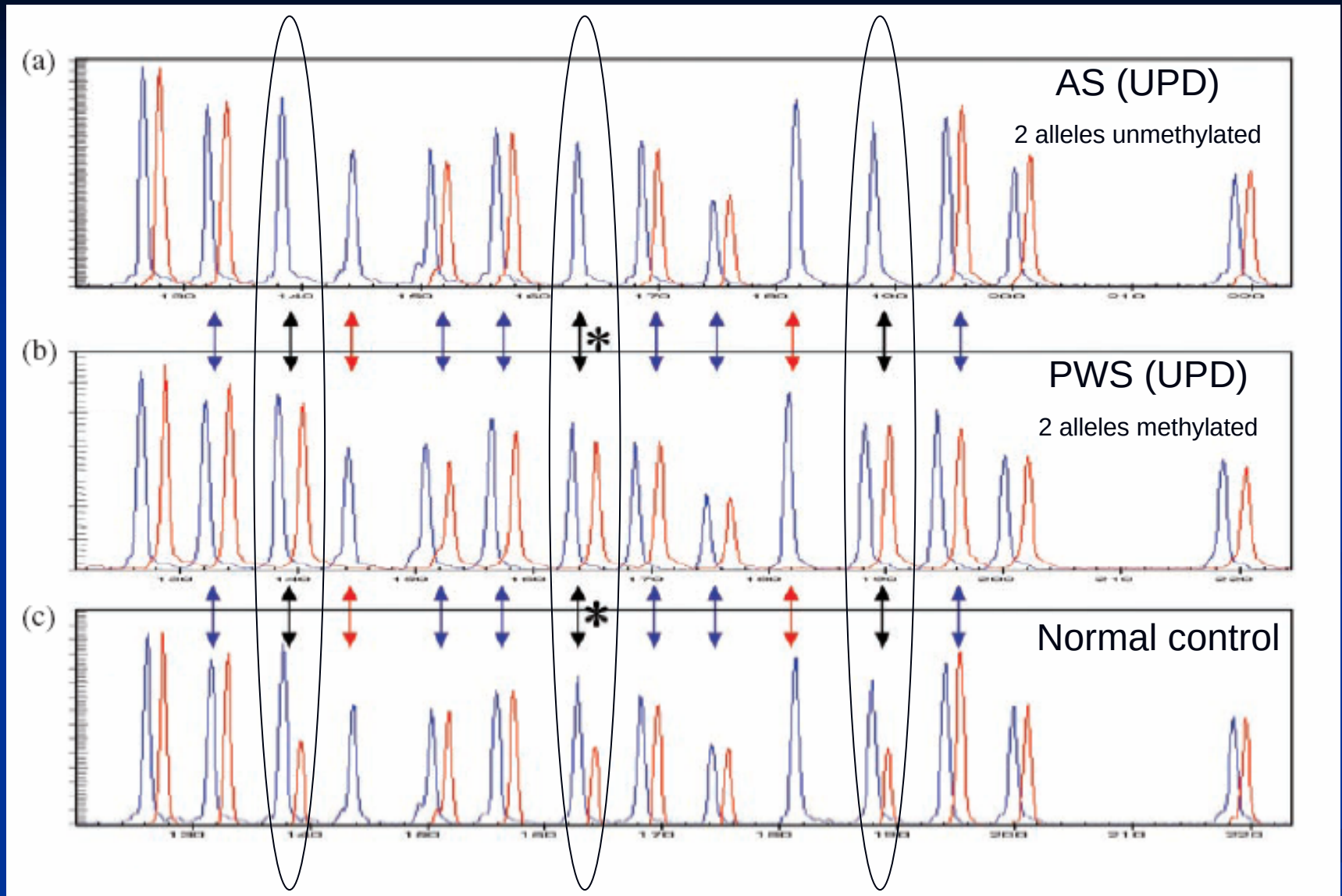


2. Simultaneously Ligation and Digestion with methylation sensitive *endonucleases*



3. PCR using one universal primer pair

Only undigested (methylated) and ligated probes are exponentially amplified


(a)
AS (UPD)
2 alleles unmethylated
(b)
PWS (UPD)
2 alleles methylated
(c)
Normal control
*
*
Undigested
Digested

Advantages

- No bisulphite treatment required
- Multiple targets tested simultaneously
- Genomic and epigenomic information
- Relative quantitation

Disadvantages

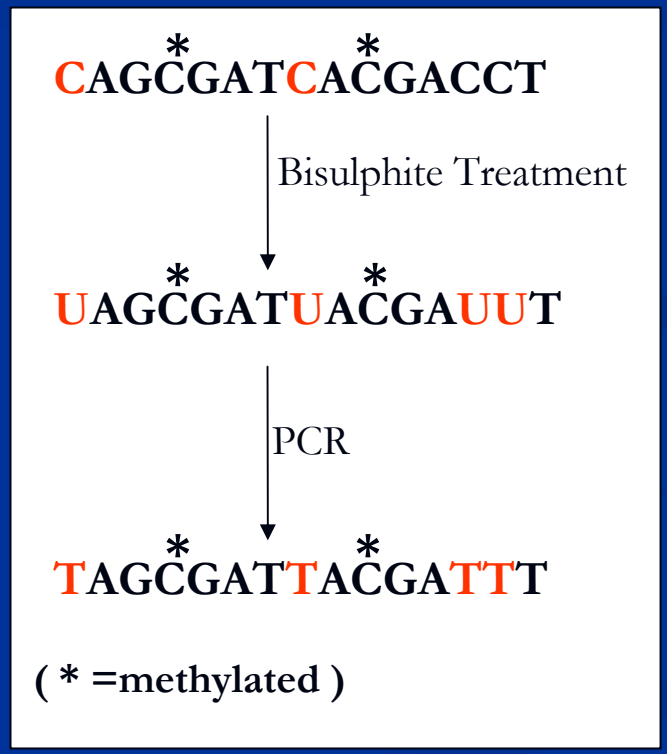
- Possibly not as sensitive as bisulphite based techniques
- Single base variations at HhaI site may results in false positive/negatives

Techniques

- MS-MLPA
- Pyrosequencing
- High resolution melt curve analysis
- Mass Spectrometry

Bisulphite Treatment

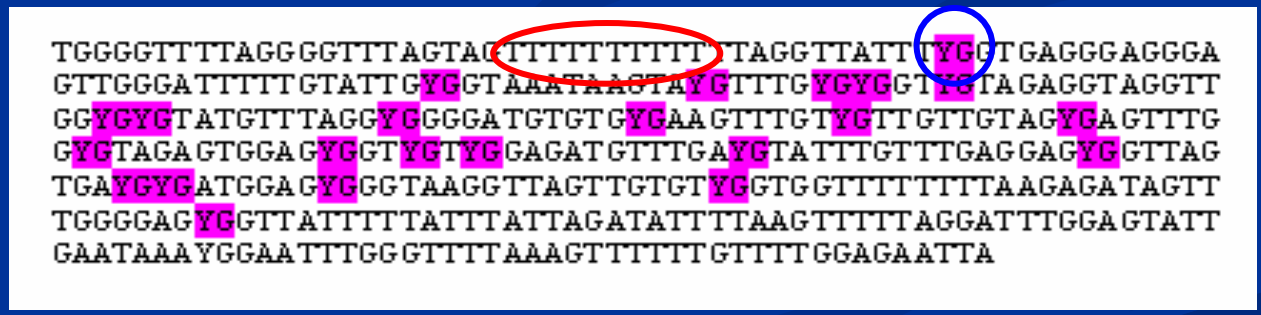
- Bisulphite treatment causes ummethylated Cytosines to convert to Uracil while methylated cytosines remain unchanged.



SNRPN 1
(Genomic)

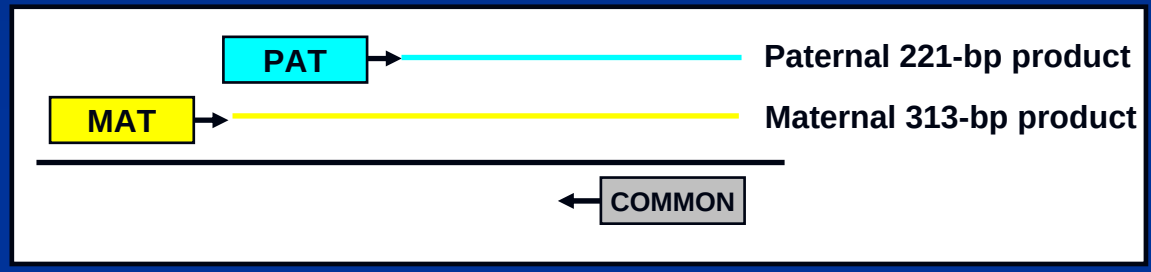
SNRPN 1

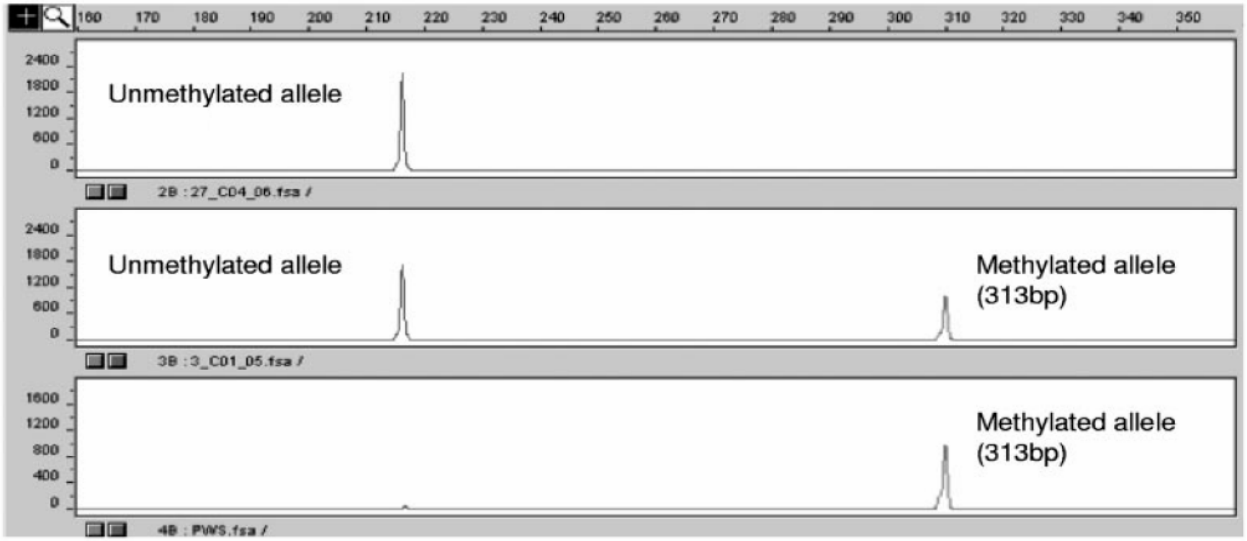
(After bisulphite)

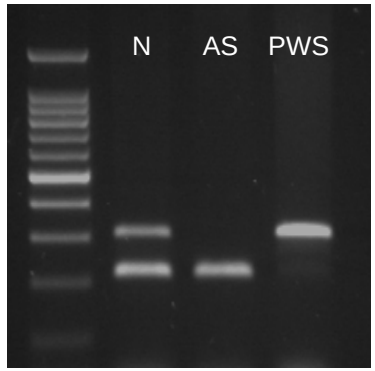


MS PCR

GATTTTTGTATTGYGGTAAATAAGTAYGTTTGYGYGGTYGTAGAGGTAGGTTGGYGYGTATG
TTTAGGYGGGGATGTGTGYGAAGTTTGTYGTTGTTGTAGYGAGTTTGGYGTAGAGTGGAGYG
GTYGTYGGAGATGTTTGAYGTATTTGTTTGAGGAGYGGTTAGTGAYGYGATGGAGYGGGTAA
GGTTAGTTGTGTYGGTGGTTTTTTTTAAGAGATAGTTTGGGGAGYGGTTATTTTTATTTATT
AGATATTTTAAGTTTTTTAGGATTTGGAGTATTGAATAAAYGGAATTTGGGTTTTAAAGTTTT
TTGTTTTGGAGAATTA







Pyrosequencing

Pyrosequencing assay design

TGGGGTTTTAGGGGTTTAGTAGTTTTTTTTTTTTAGGTTATTTYGGTGAGGGAGGGAGTTGG
GATTTTTGTATTGYGGTAAATAAGTAYGTTTGYGYGGTYGTAGAGGTAGGTTGGYGYGTATG
TTTAGGYGGGGATGTGTGYGAAGTTTGTYGTTGTTGTAGYGAGTTTGGYGTAGAGTGGAGYG
GTYGTYGGAGATGTTTGAYGTATTTGTTTGAGGAGYGGTTAGTGAYGYGATGGAGYGGGTAA
GGTTAGTTGTGTYGGTGGTTTTTTTAAGAGATAGTTTGGGGAGYGGTTATTTTTATTTATT
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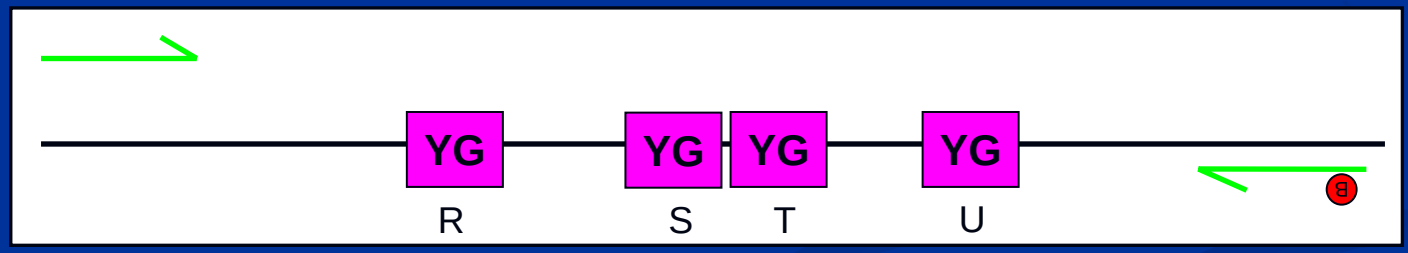
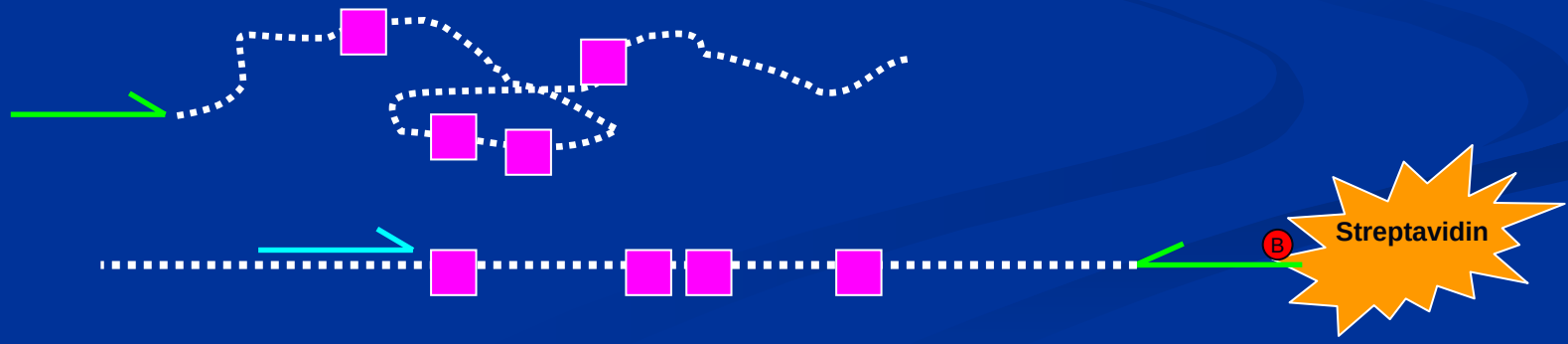
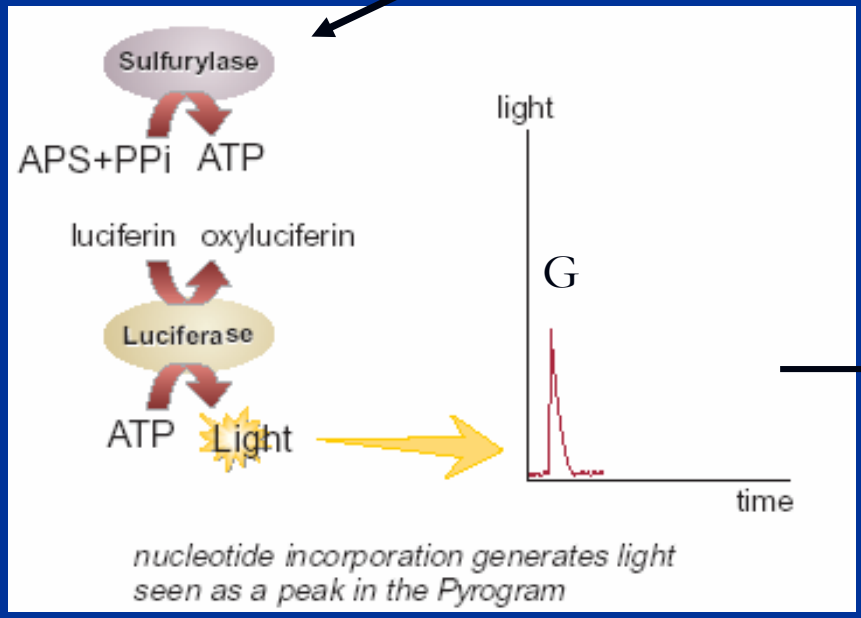
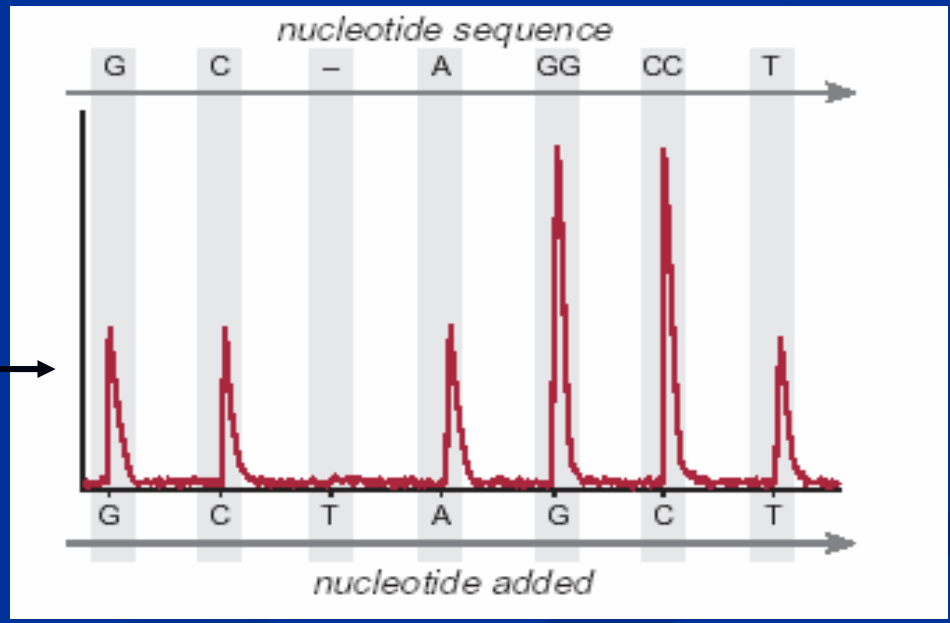


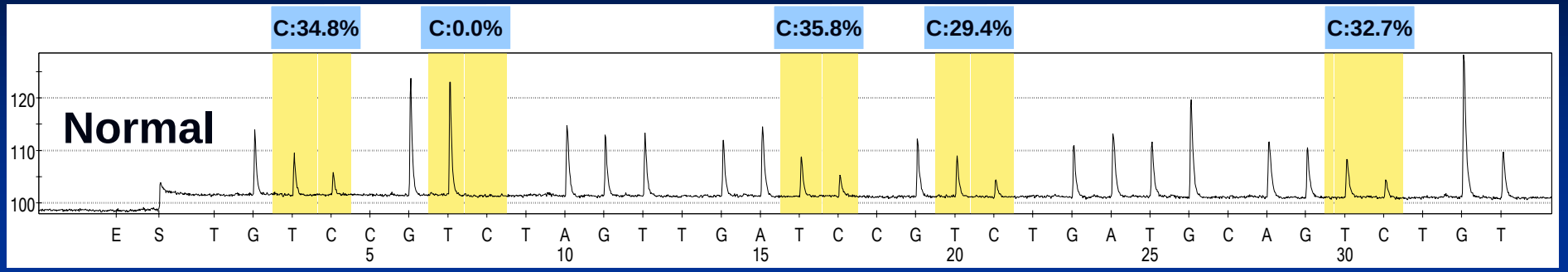


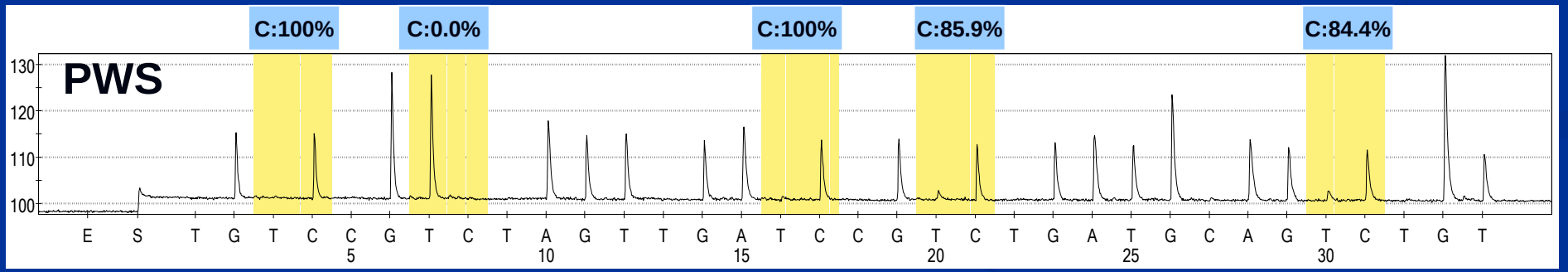
Principle of Pyrosequencing

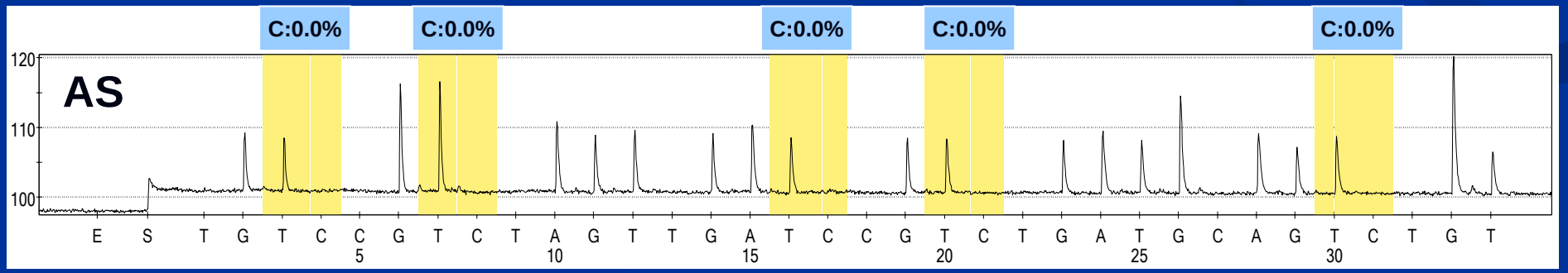




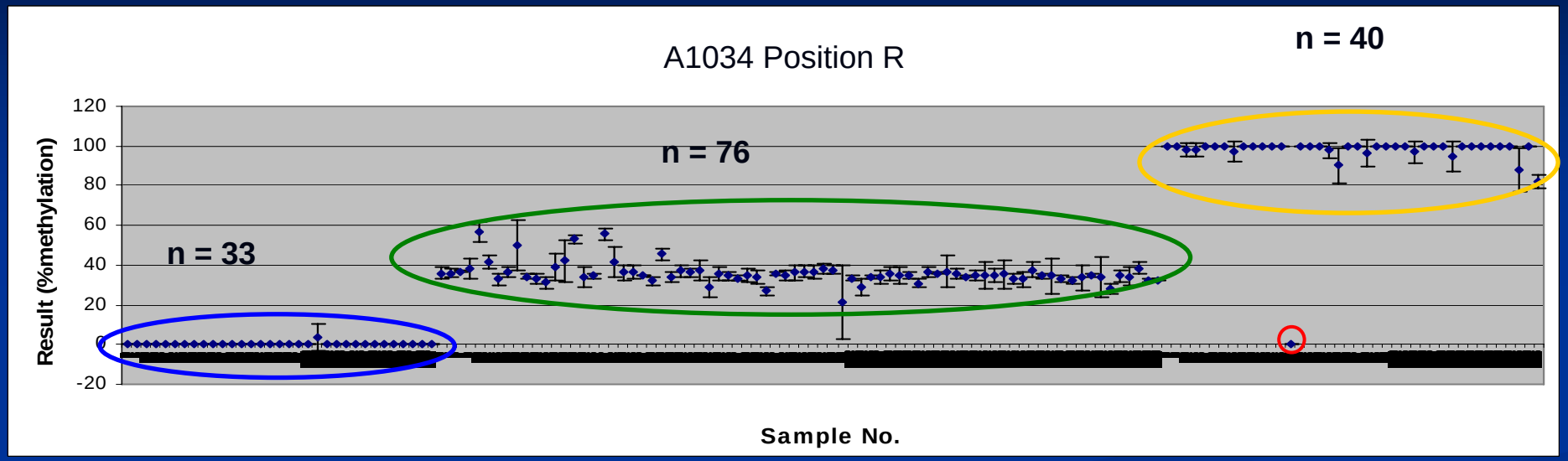
Pyrograms

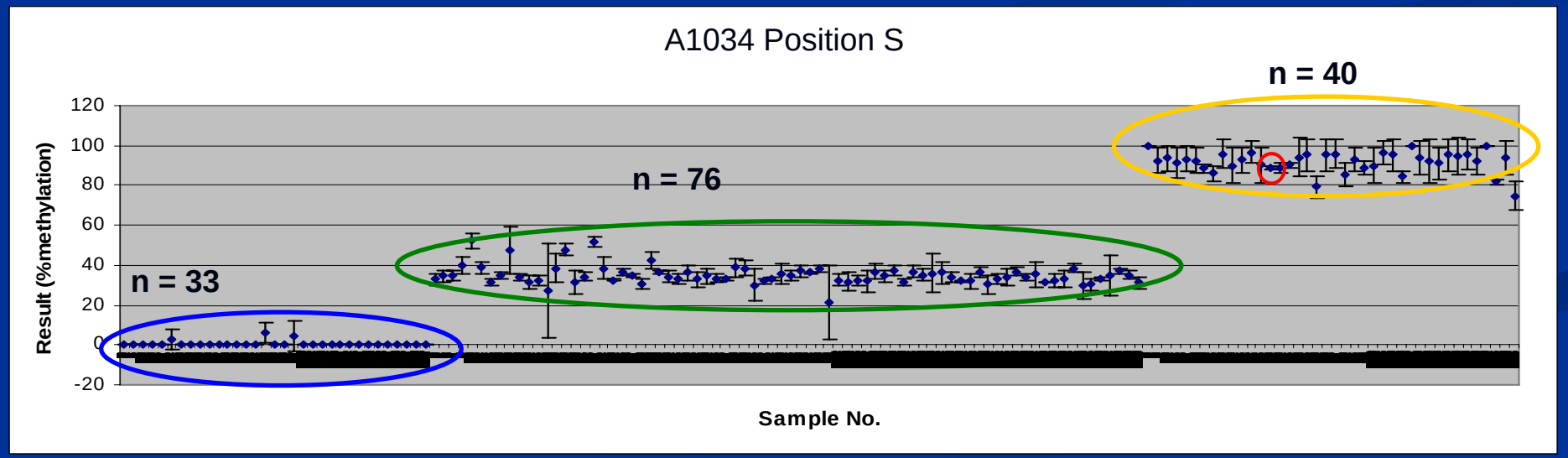

C:34.8%
C:0.0%
C:35.8%
C:29.4%
C:32.7%
Normal
120
110
100
E S T G T C C G T C T A G T T G A T C C G T C T G A T G C A G T C T G T
5
10
15
20
25
30


C:100%
C:0.0%
C:100%
C:85.9%
C:84.4%
PWS
130
120
110
100
E S T G T C C G T C T A G T T G A T C C G T C T G A T G C A G T C T G T
5
10
15
20
25
30

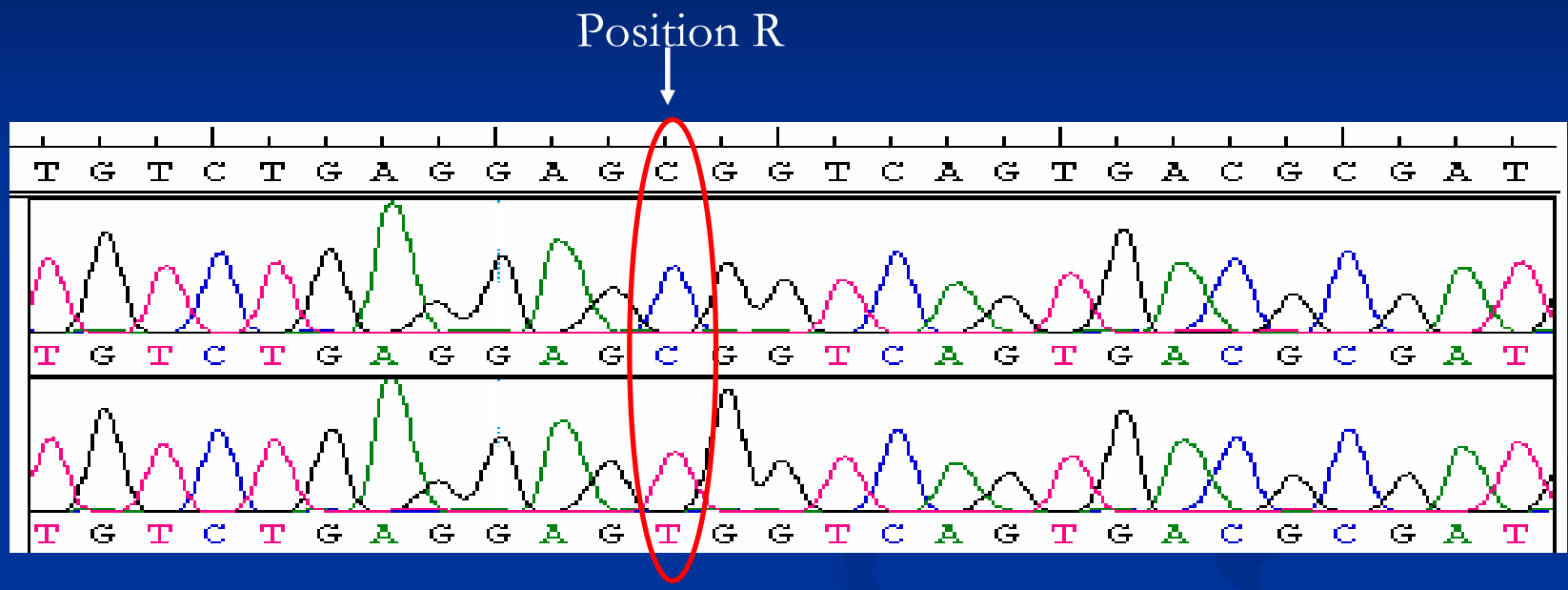

C:0.0%
C:0.0%
C:0.0%
C:0.0%
C:0.0%
AS
120
110
100
E S T G T C C G T C T A G T T G A T C C G T C T G A T G C A G T C T G T
5
10
15
20
25
30

Pyrosequencing Results





Sample 58



PWS Bisulphited sequence

T G T T T G A G G A G C G G T T A G T G A C G C G A T

Sample 58 Bisulphited sequence

T G T T T G A G G A G T G G T T A G T G A C G C G A T

Advantages

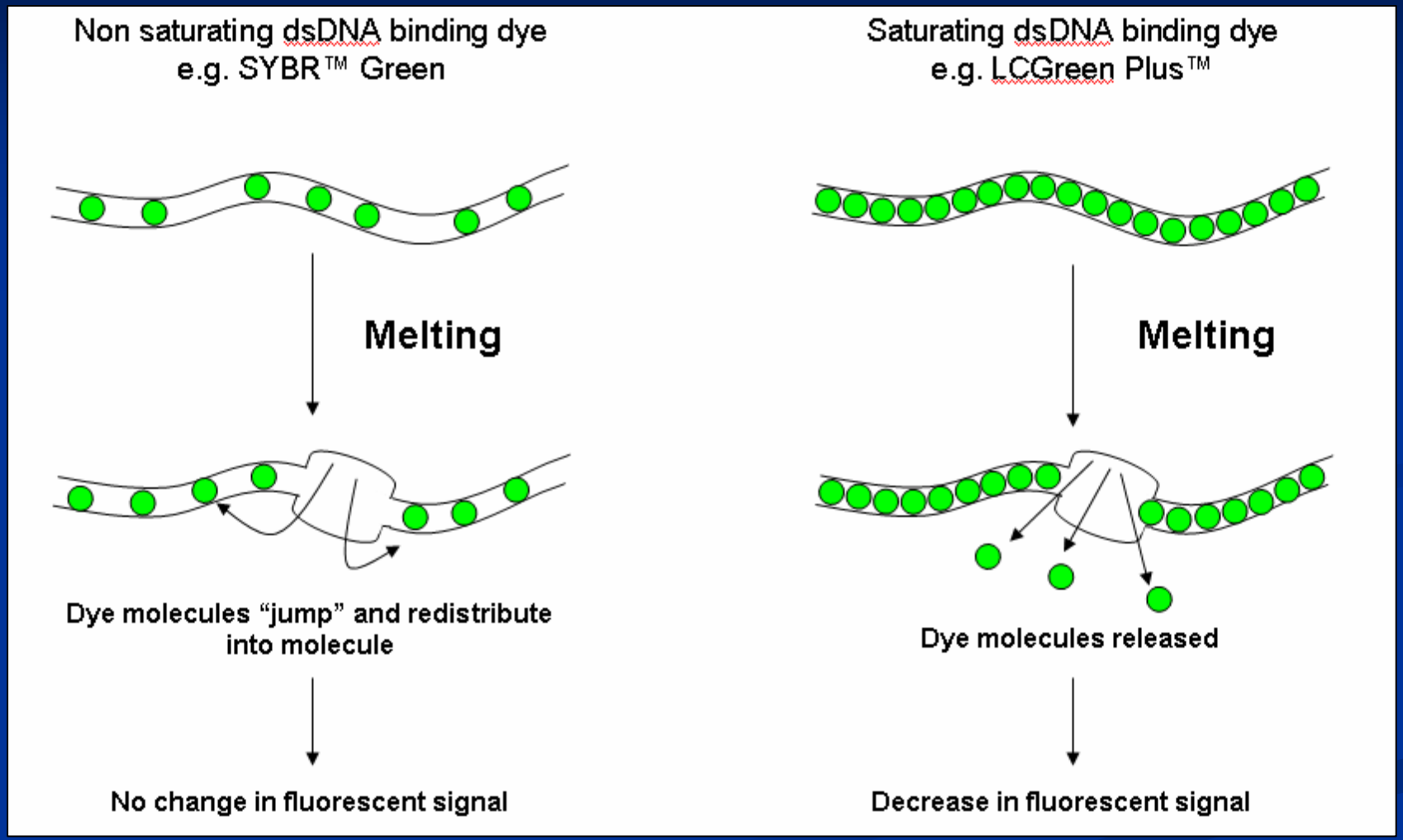
- AQ software allows accurate quantification of methylation at multiple CpG sites within amplicon
- PCR primers independent of methylation state
- Confidence scores (passed, checked or failed) alert user to the quality of assay data
- Reference peaks incorporated into the analysis add confidence to data collection – also bisulphite treatment controls are included
- Results are presented in sequence context so sequence variants will be identified
- Assays relatively inexpensive & rapid
- Has potential to detect mosaicism

Disadvantages

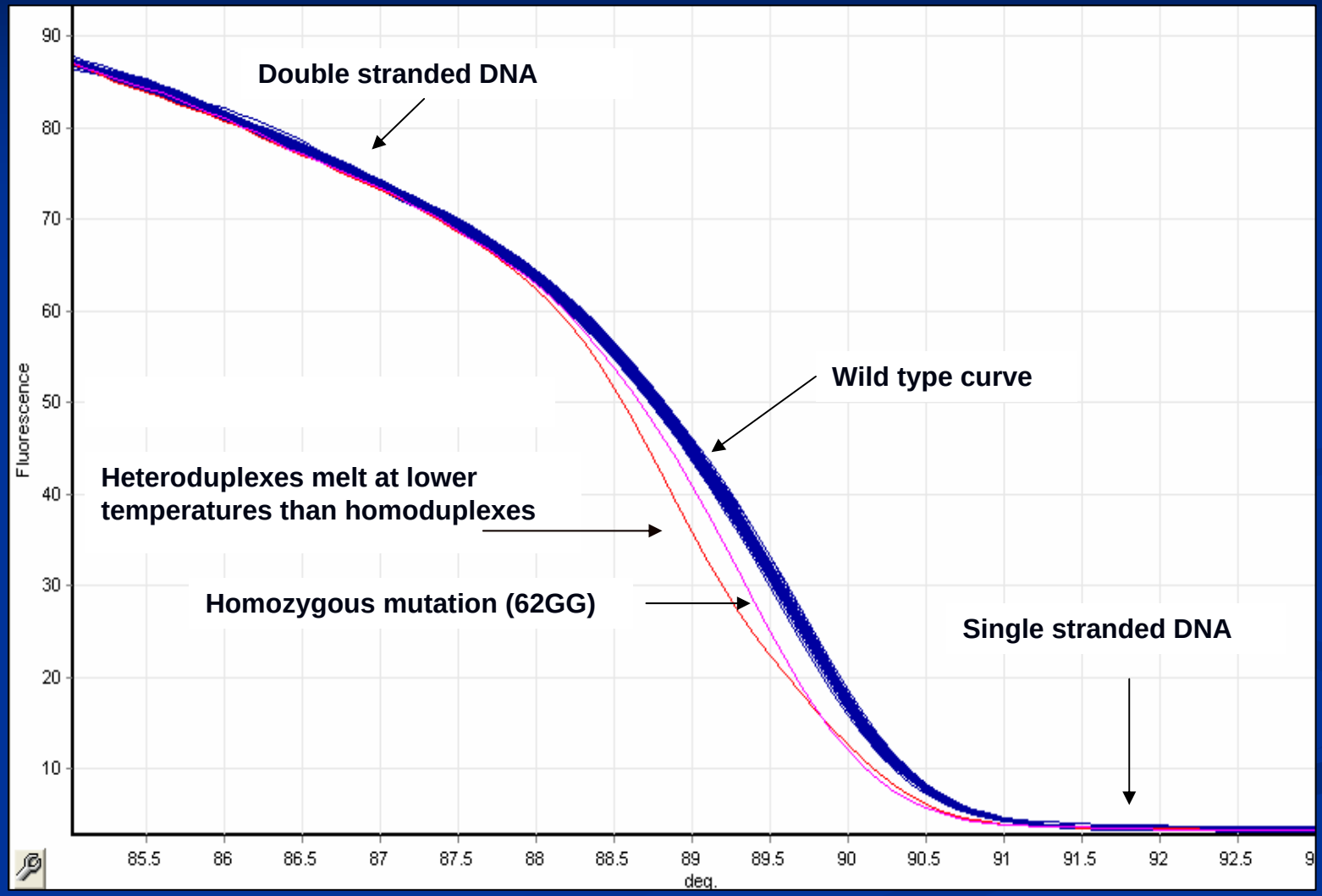
- Further work is still necessary to establish the cause behind the abnormal methylation e.g. UPD, deletion or an imprinting mutation

High resolution melt curve analysis

High resolution melt curve analysis



High resolution melt curve analysis



NORMAL

AGGGAGTTGGGATTTTTGTATTGYGGTAAATAAGTAYGTTTGYGYGGTYGTAGAGGTAGGTTGGYGYGTATG
TTTAGGYGGGGATGTGTGYGAAGTTTGTYGTTGTTGTAGYGAGTTTGGYGTAGAGTGGAGYGGTYGTYGGAG
ATGTTTGAYGTATTTGTTTGAGGAGYGGTTAGTGAYGYGATGGAGYGGGTAAGGTTAGTTGTGTYGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

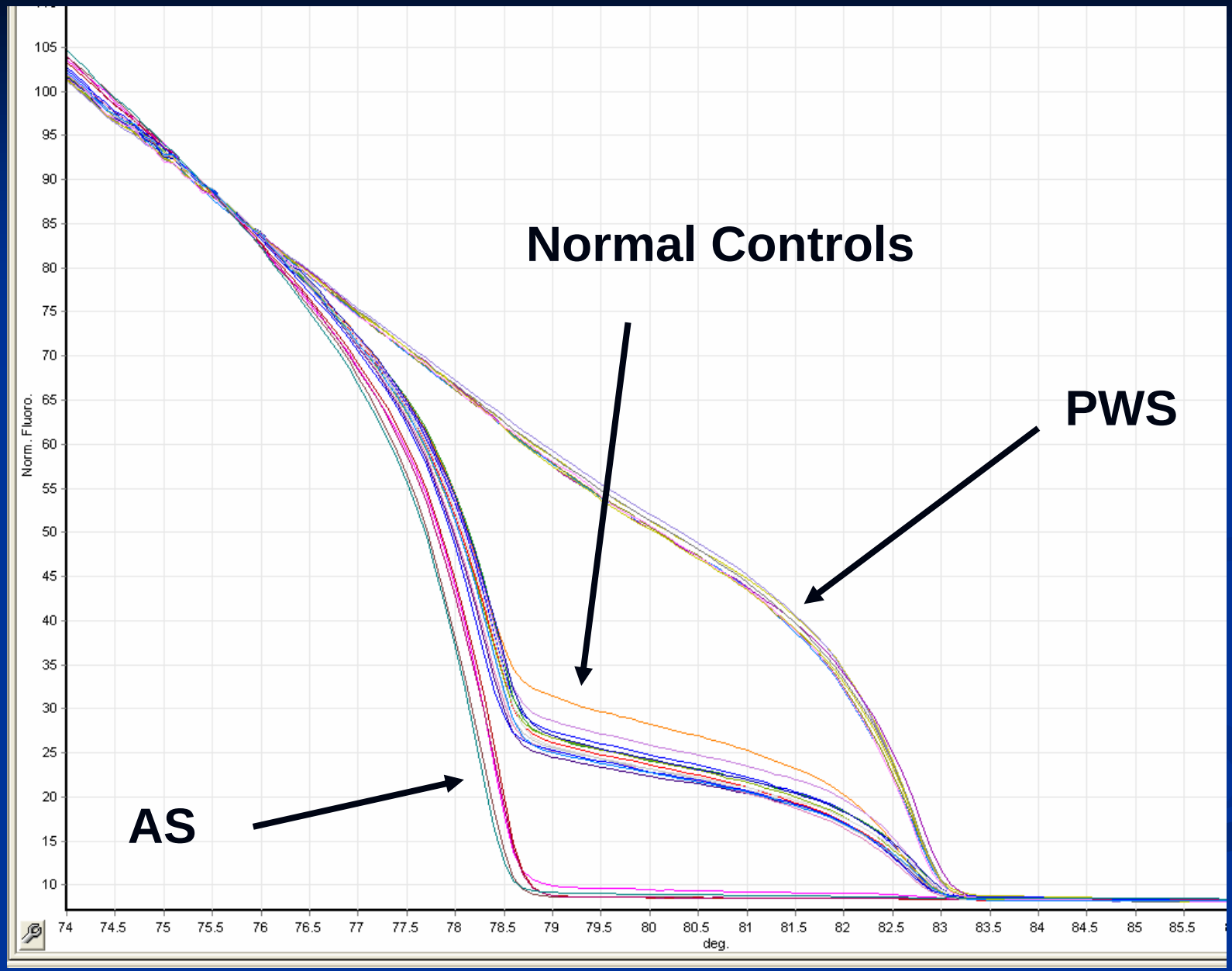
PWS

AGGGAGTTGGGATTTTTGTATTGCGGTAAATAAGTACGTTTGCGCGGTCGTAGAGGTAGGTTGGCGCGTATG
TTTAGGCGGGGATGTGTGCGAAGTTTGTCGTTGTTGTAGCGAGTTTGGCGTAGAGTGGAGCGGTCGTCGGAG
ATGTTTGACGTATTTGTTTGAGGAGCGGTTAGTGACGCGATGGAGCGGGTAAGGTTAGTTGTGTCGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

AS

AGGGAGTTGGGATTTTTGTATTGTGGTAAATAAGTATGTTTGTGTGGTTGTAGAGGTAGGTTGGTGTGTATG
TTTAGGTGGGGATGTGTGTGAAGTTTGTTGTTGTTGTAGTGAGTTTGGTGTAGAGTGGAGTGGTTGTTGGAG
ATGTTTGATGTATTTGTTTGAGGAGTGGTTAGTGATGTGATGGAGTGGGTAAGGTTAGTTGTGTTGGTGGTT
TTTTTTAAGAGATAGTTTGGGG

21 sites can vary


Normal Controls
PWS
AS
Norm. Fluoro.
deg.

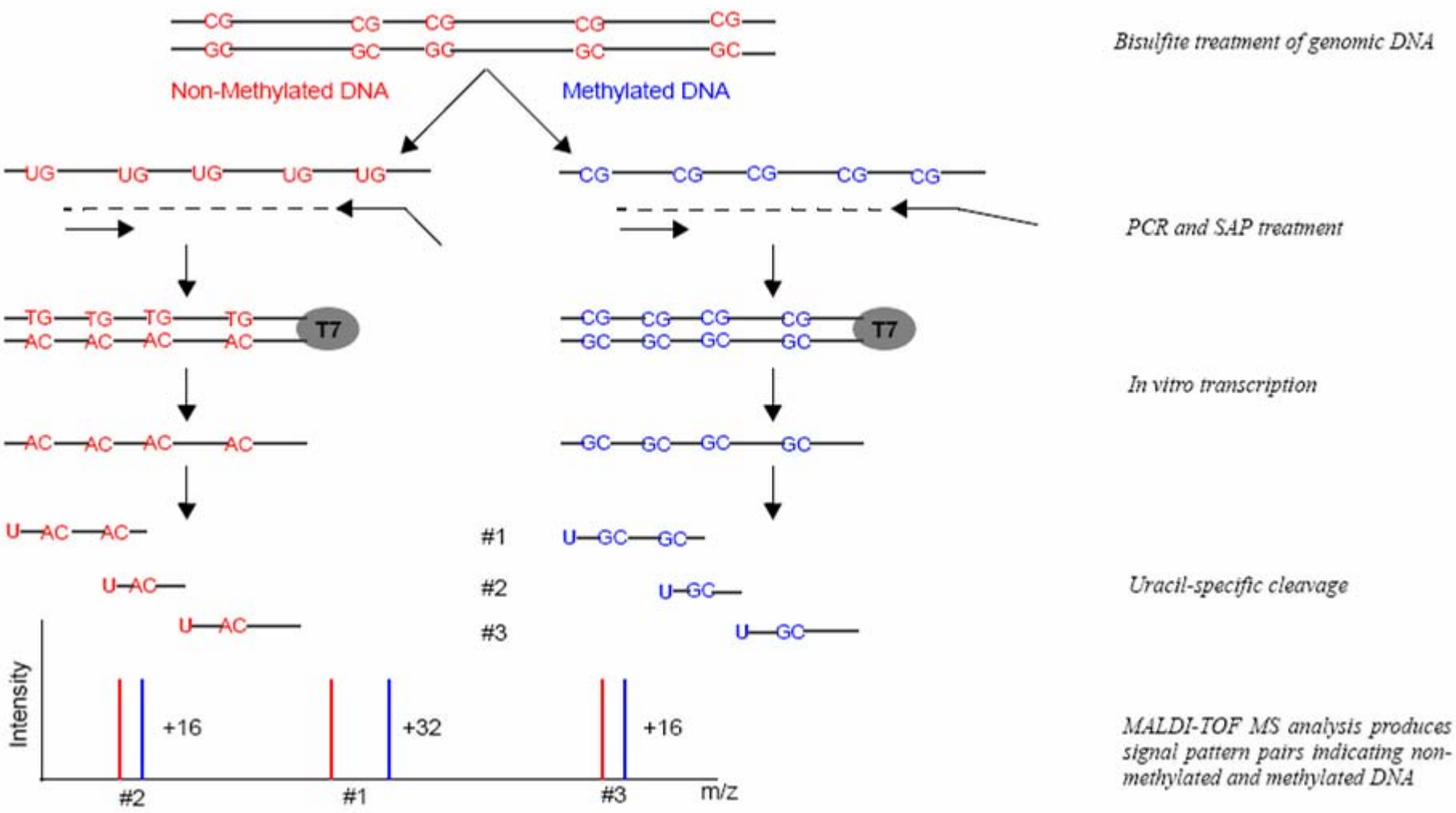
Advantages

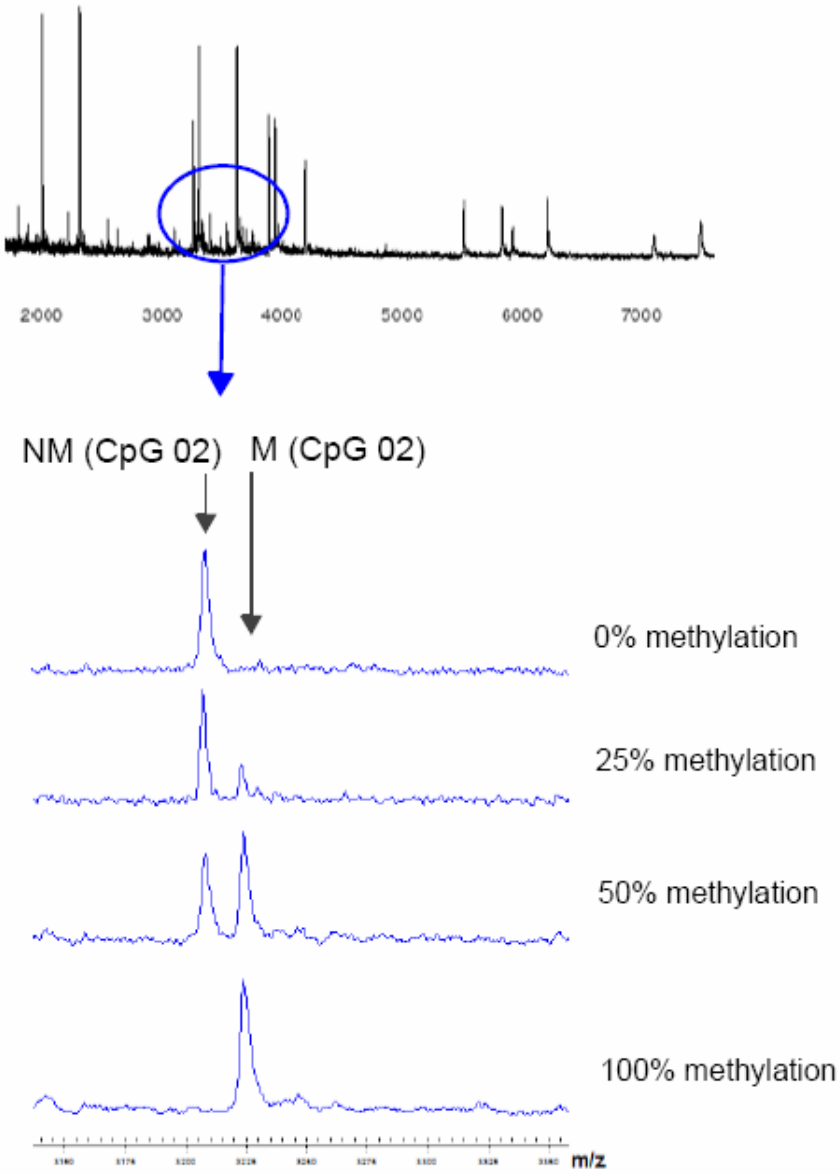
- Closed tube method
- No post PCR processing and no separation step
  - improves analysis time
  - reduces contamination risk
- Rapid
- Inexpensive - requires the use of only PCR reagents and dsDNA binding dye
- PCR primers independent of methylation state
- Global information about methylation/sequence composition of amplicon

Disadvantages

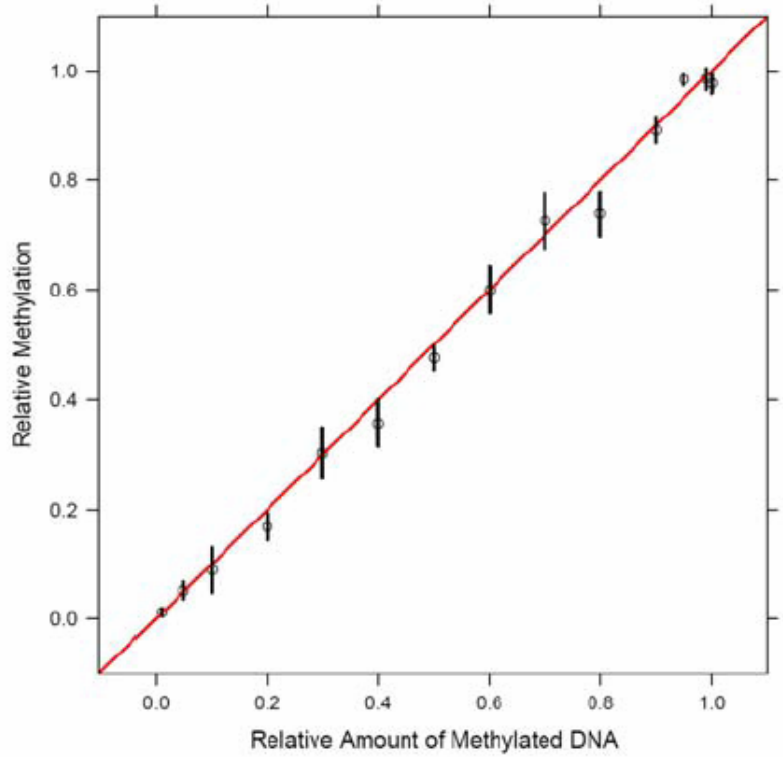
- Further work is still necessary to establish the cause behind the abnormal methylation e.g. UPD, deletion or an imprinting mutation

Mass Spectrometry


Non-Methylated DNA
Methylated DNA
T7
T7
#1
#2
#3
Intensity
+16
+32
+16
#2
#1
#3
m/z
Bisulfite treatment of genomic DNA
PCR and SAP treatment
In vitro transcription
Uracil-specific cleavage
MALDI-TOF MS analysis produces signal pattern pairs indicating non-methylated and methylated DNA



The non-methylated (NM) DNA fragment (5'-AACAAA**CA**AT-3') and the methylated (M) DNA fragment (5'-AACAAA**CG**AT-3') show a mass difference of 16 Da.



Graph showing the relationship between the estimated relative methylation and the relative amount of methylated DNA spiked into the mixture.

Advantages

- PCR primers independent of methylation state
- Quanitfication of methylation (to 5%) at all CpG sites within amplicons up to 600bp
- Useful for large scale projects to identify ‘useful’ CpG sites
- High throughput
- High precision

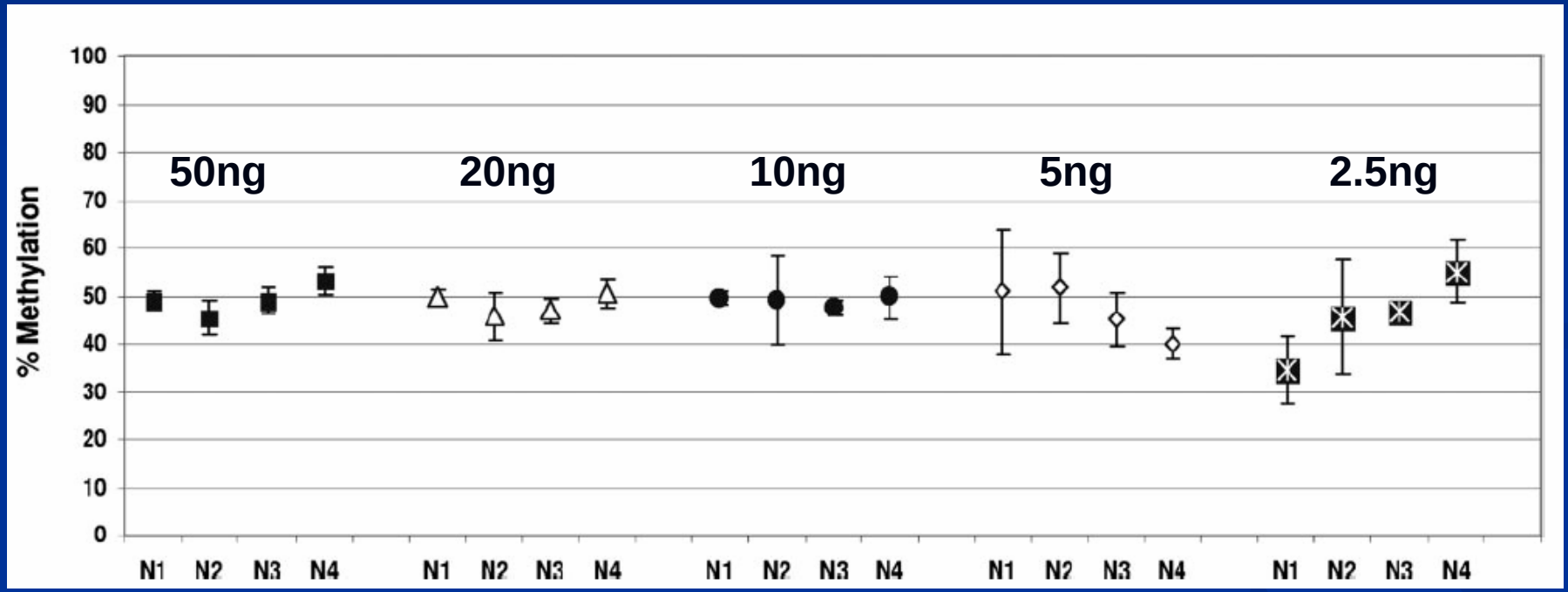
Disadvantages

- Expensive
- Extensive post PCR handling – relies on success of multiple reactions
- Need access to equipment and specialised data analysis software

Quality control issues

- Quality and quantity of DNA
- Bisulphite treatment
  - quality
  - batches
- Preferential amplification
- Use of standard curves

Quality and concentration of DNA

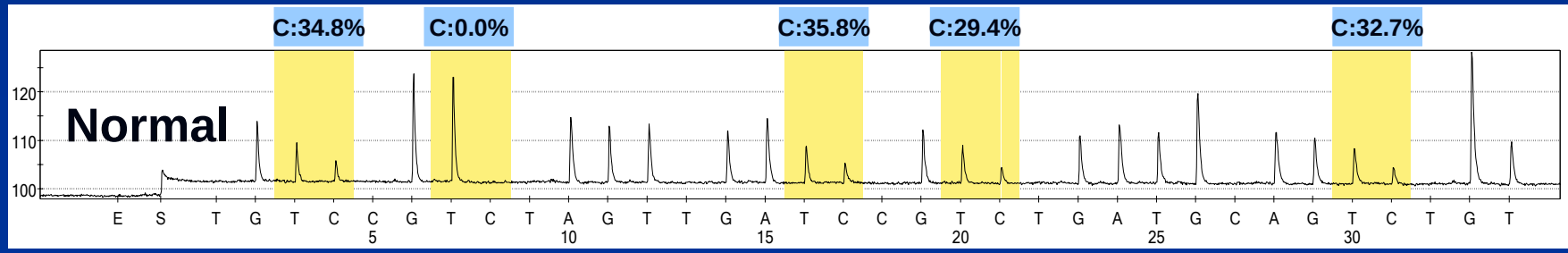


Bisulphite treatment

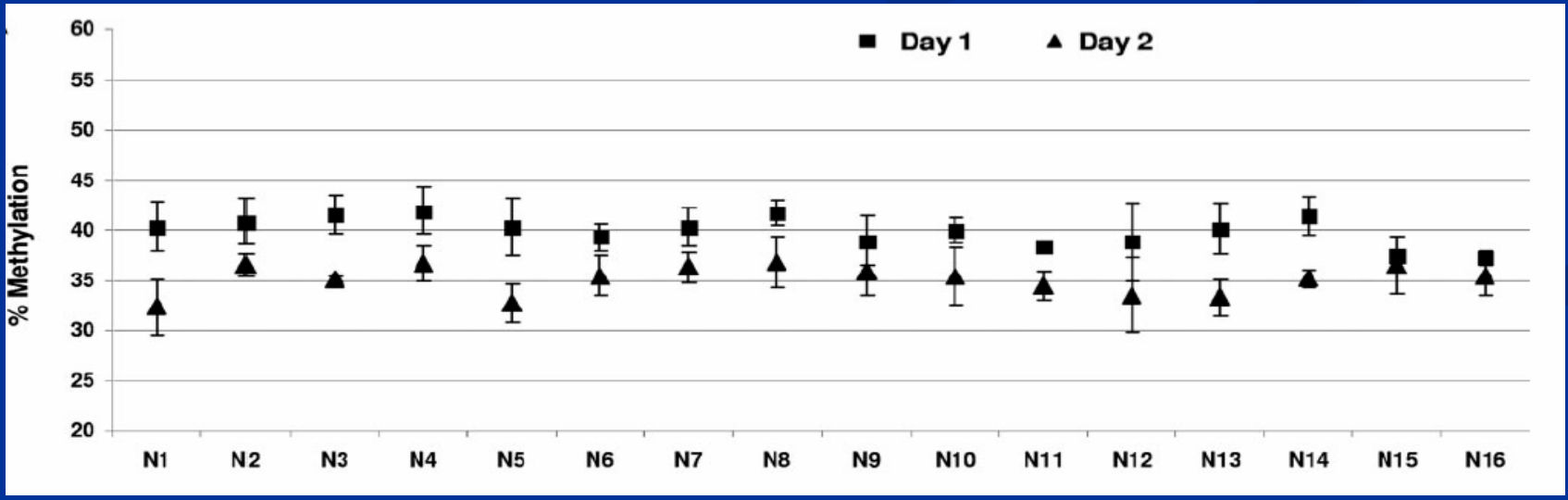
Total conversion

Pyrosequencing has in built control sites to monitor whether bisulphite conversion is complete

Most techniques do not have controls to monitor this



Batches



Preferential amplification and standard curves