Amplicon Genotyping and Methylation Analysis by HRM

Jonathan Nery
Applications Scientist

illumina®

Introduction

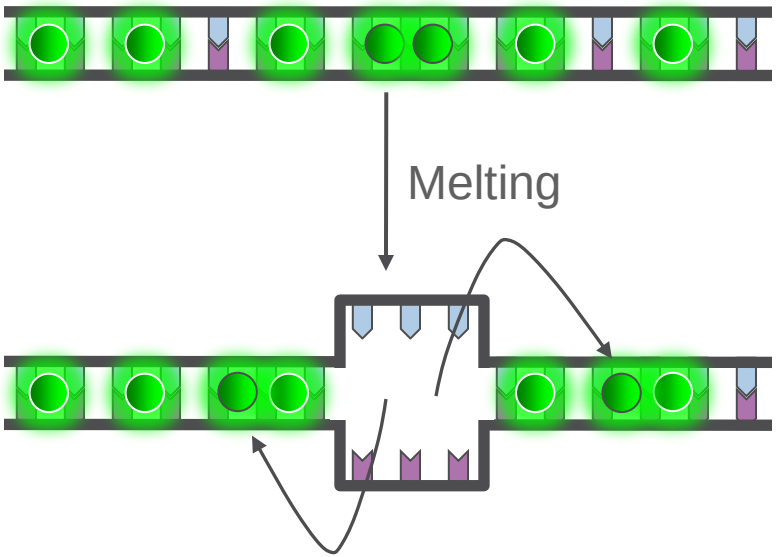
- Requirements for High Resolution Melt
  - Chemistry
  - Instrumentation
  - Software
- Applications
  - Genotyping
    - Class IV SNPs
    - Open platform
  - DNA Methylation Analysis

Requirements for HRM

- **Chemistry**
  - Saturating dsDNA binding dyes
  - Non-inhibitory to PCR
  - SYTO® 9 (Life Tech.), LCGreen™ (Idaho Tech.), EvaGreen™ (Biotium Inc.)
- **Instrumentation**
  - Precise temperature control
  - Minimum of well-to-well thermal or optical non uniformity
  - Fast data acquisition rate for more data points
- **Software**
  - Specialized normalization algorithms
  - Difference plots

Double Stranded DNA Binding Dyes

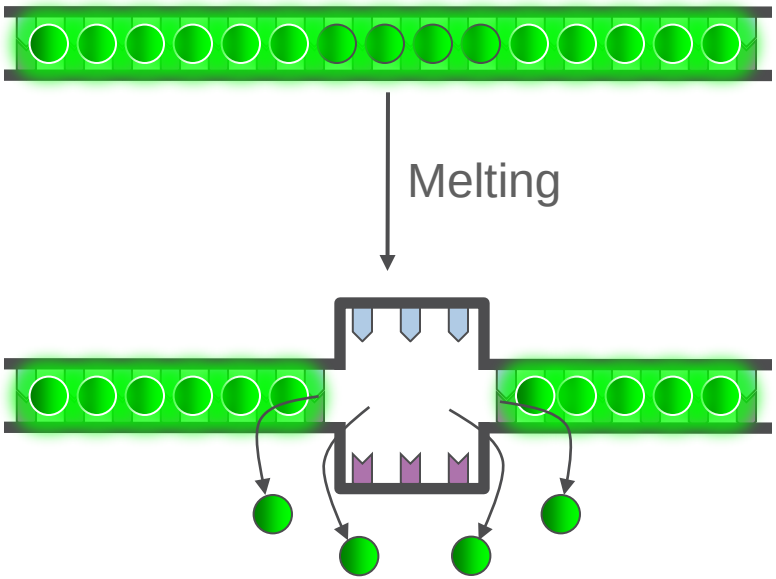
Non-Saturating dsDNA Binding Dye
- SYBR® Green I



Dye molecules redistribute into amplicon

Little to no change in fluorescence

Saturating dsDNA Binding Dye
- SYTO® 9



Dye molecules are released

Decrease in fluorescence

illumina®

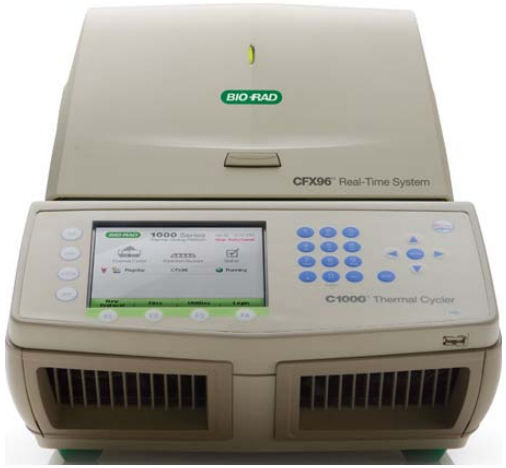
HRM Compatible Real-Time PCR Instruments

Life Technologies
7500Fast/7900Fast/Viia 7

Illumina Eco™

Qiagen Rotor-Gene Q

Idaho Technologies
LightScanner® 32

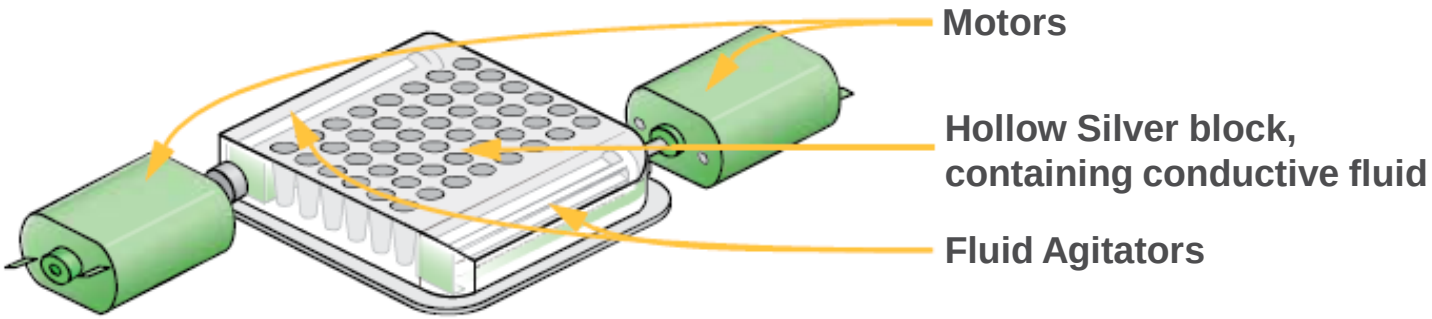


Bio-Rad
CFX96/CFX384

Roche LightCycler® 480

Images not to scale

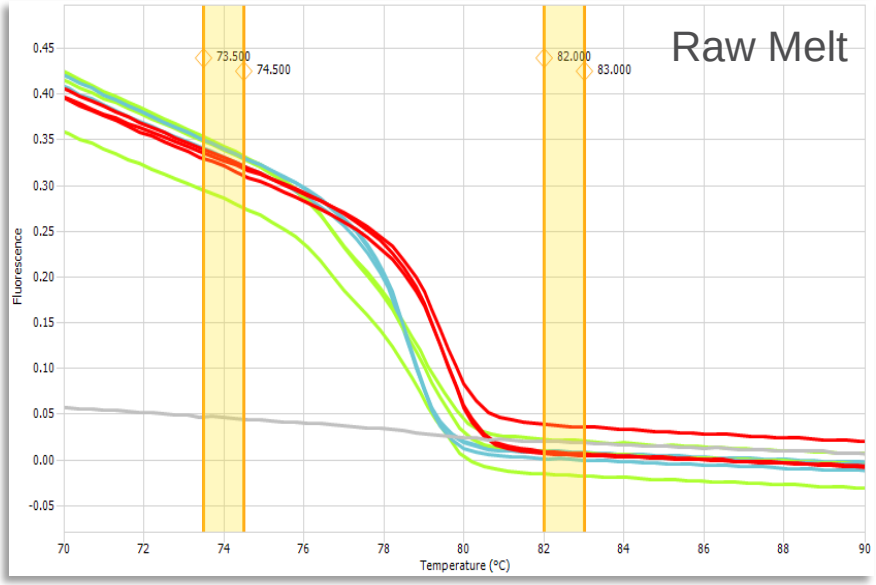
Eco Thermal System

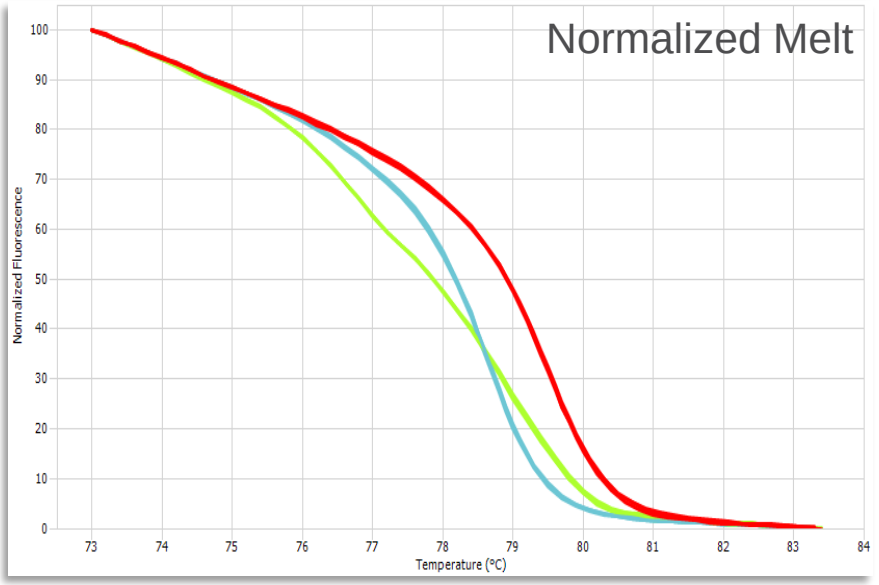


- Temperature uniformity and accuracy of < ±0.1°C
- Ramp rate of > 5°C/sec
- Temperature resolution of 0.1°C
- No temperature shift or calibration required for HRM

Eco Software – Melt Curve Normalization

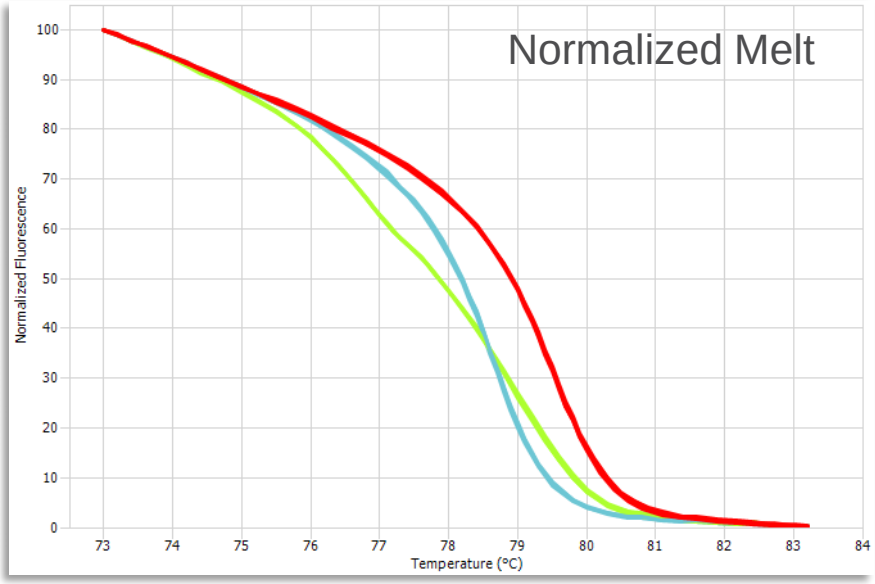
- Raw fluorescence pre- and post- melt normalized to 100% and 0%
- Aligns curves for better differentiation

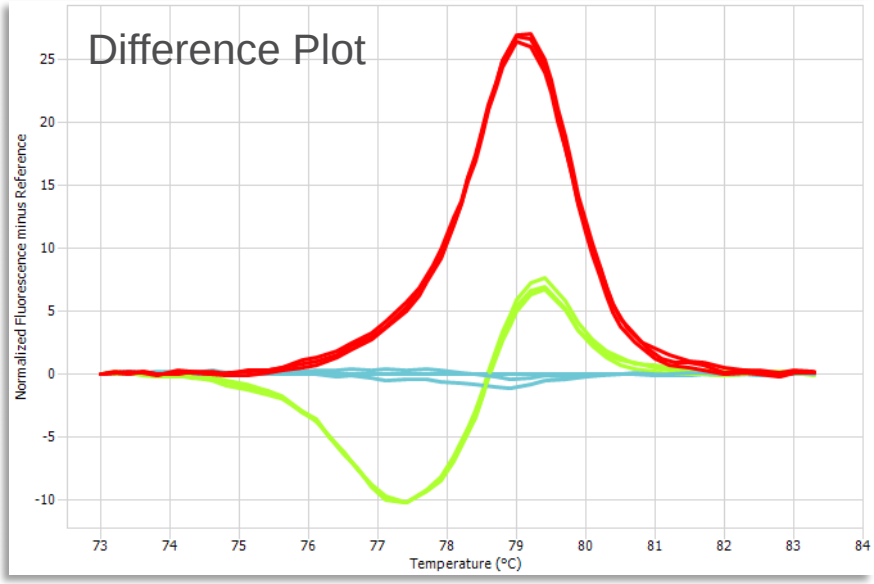




Eco Software – Difference Plot View

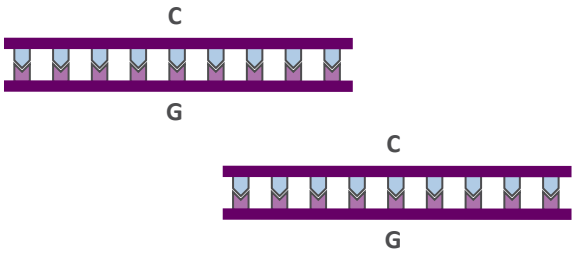
- Alternate view of melt curve to maximize differences
- Subtract a reference curve from each curve



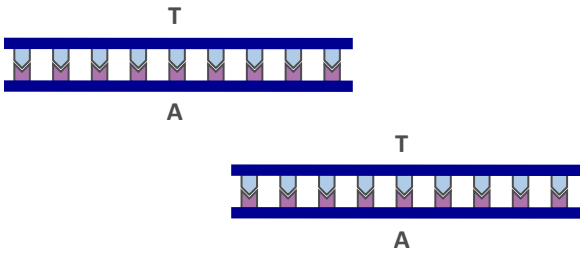


Applications – Amplicon Genotyping

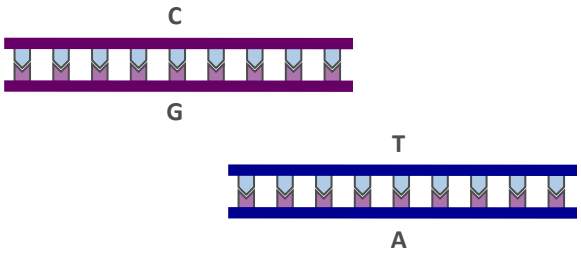
- Three possible genotypes
  - Homozygous Wild-type
  - Homozygous Mutant
  - Heterozygous



Wild-Type



Mutant

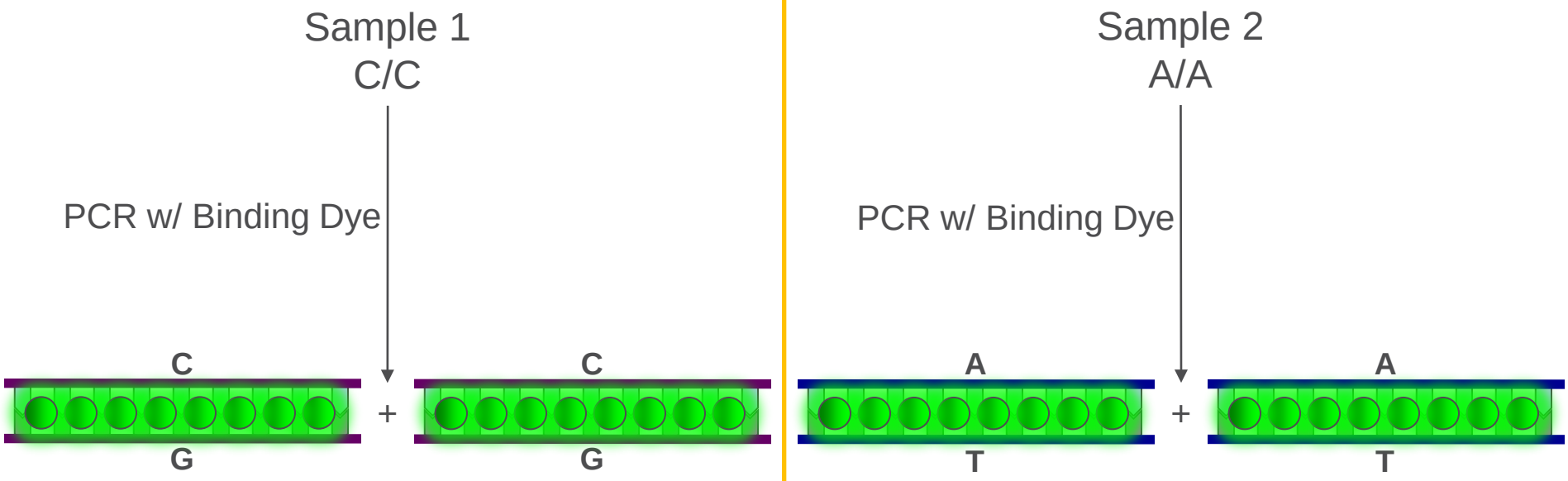


Heterozygote

- Each will produce a unique melting profile that can be differentiated by HRM

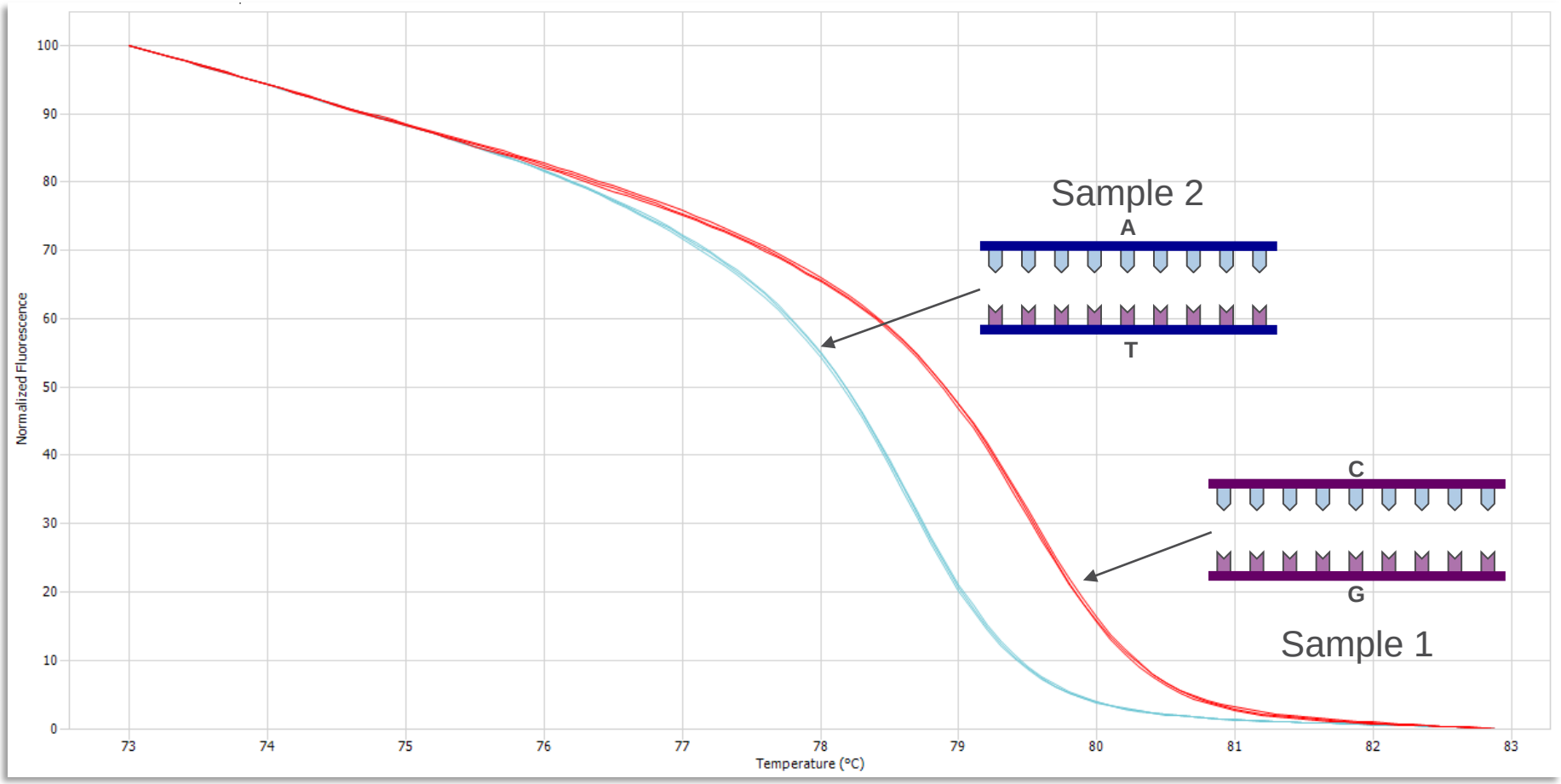
PCR of Homozygous Samples

Class II SNP



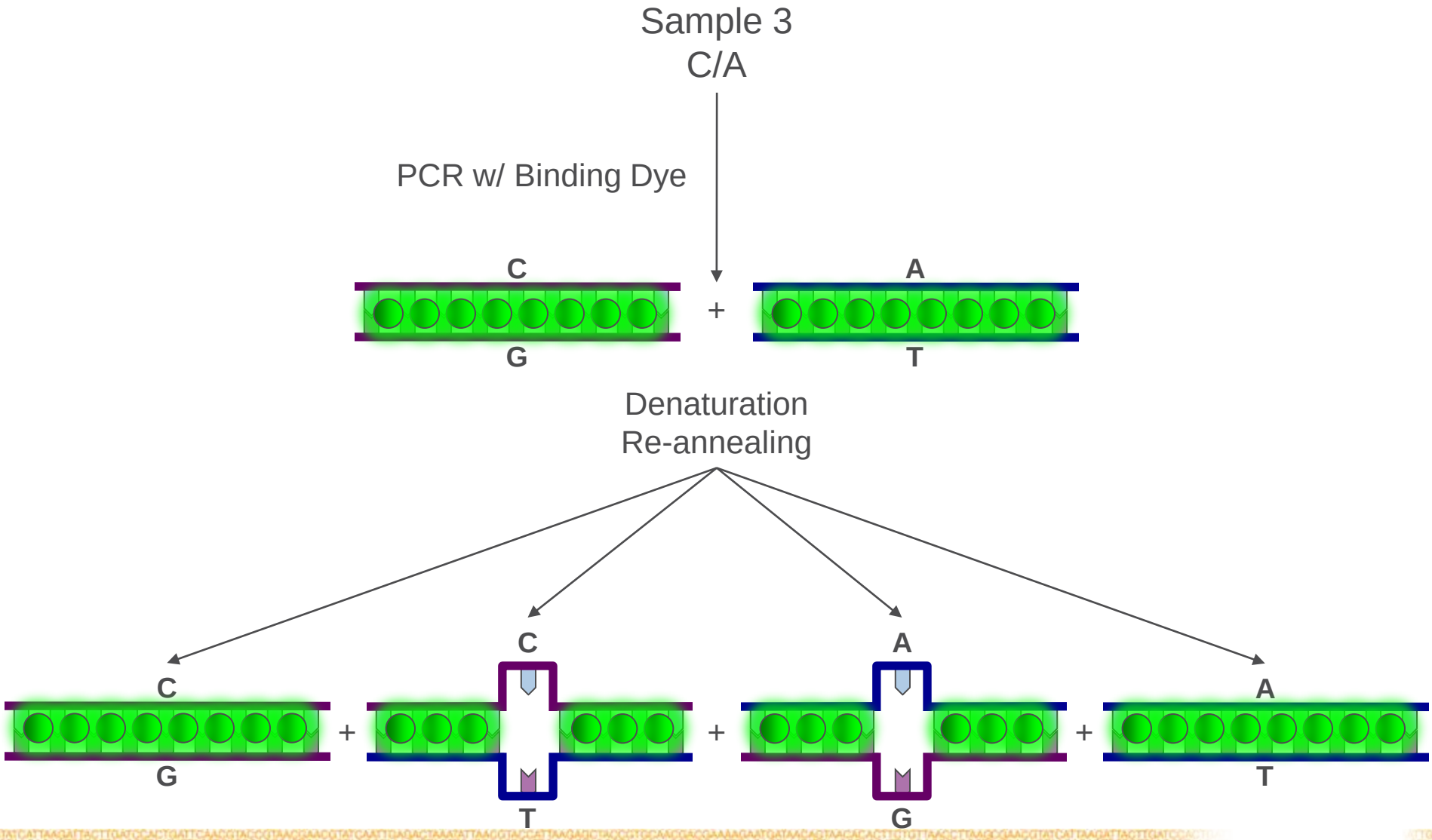
illumina

Melting Profile of Homozygous Samples

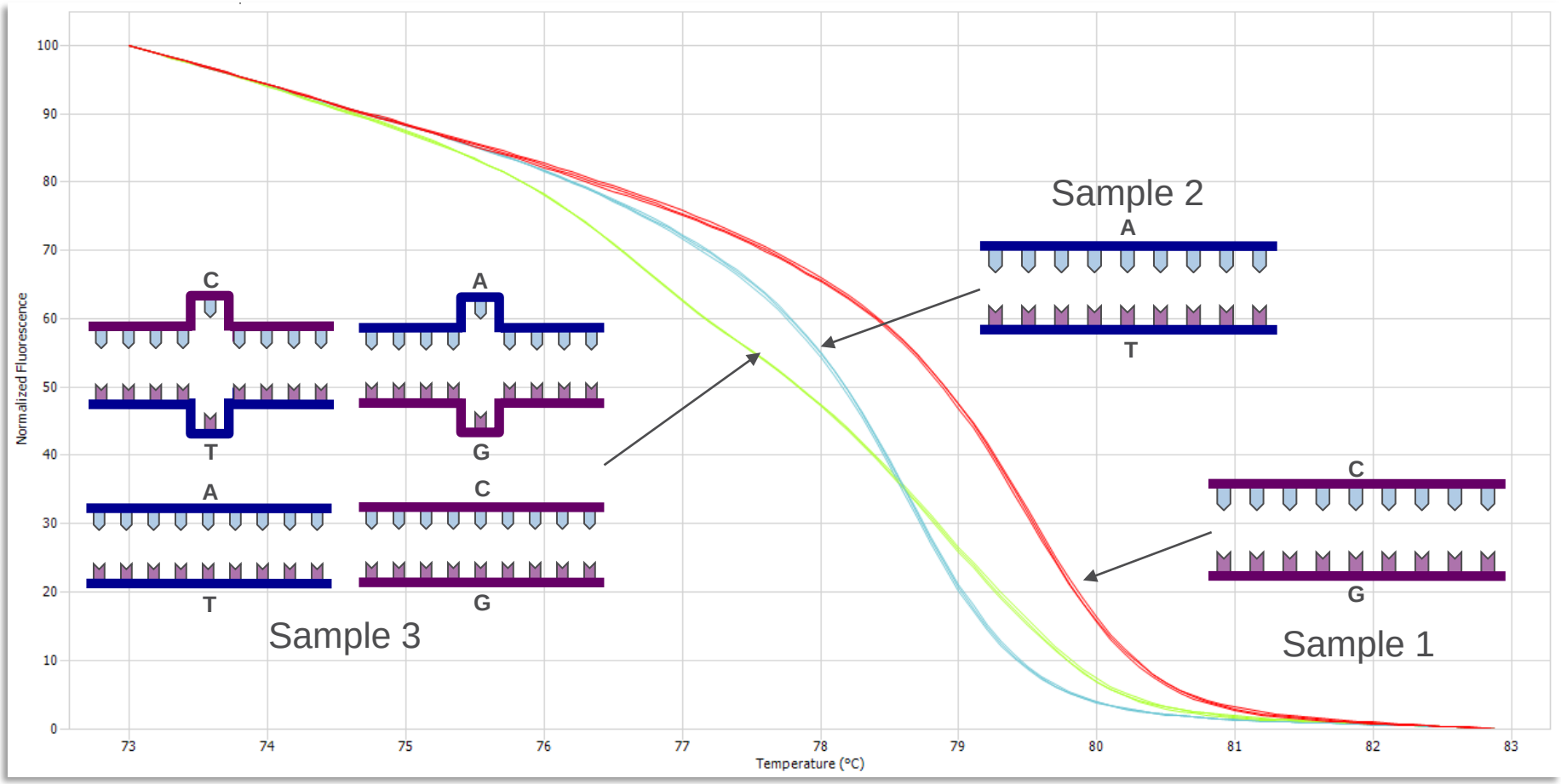


- Homozygous Samples Differentiated by Shift in Melting Temperature

PCR of Heterozygous Sample



Melting Profile of Heterozygous Samples



- Heterozygous Sample Differentiated by Curve Shape

Amplicon T_m Shifts for the 4 SNP Classes

SNP Class	Base Change	Frequency in Human Genome	Approximate T_m Melt Curve Shift
1	C/T and G/A	0.67	> 0.5°C
2	C/A and G/T	0.18	0.4 to 0.5°C / 0.2 to 0.5°C
3	C/G	0.09	0.2 to 0.4°C
4	A/T	0.07	< 0.2°C

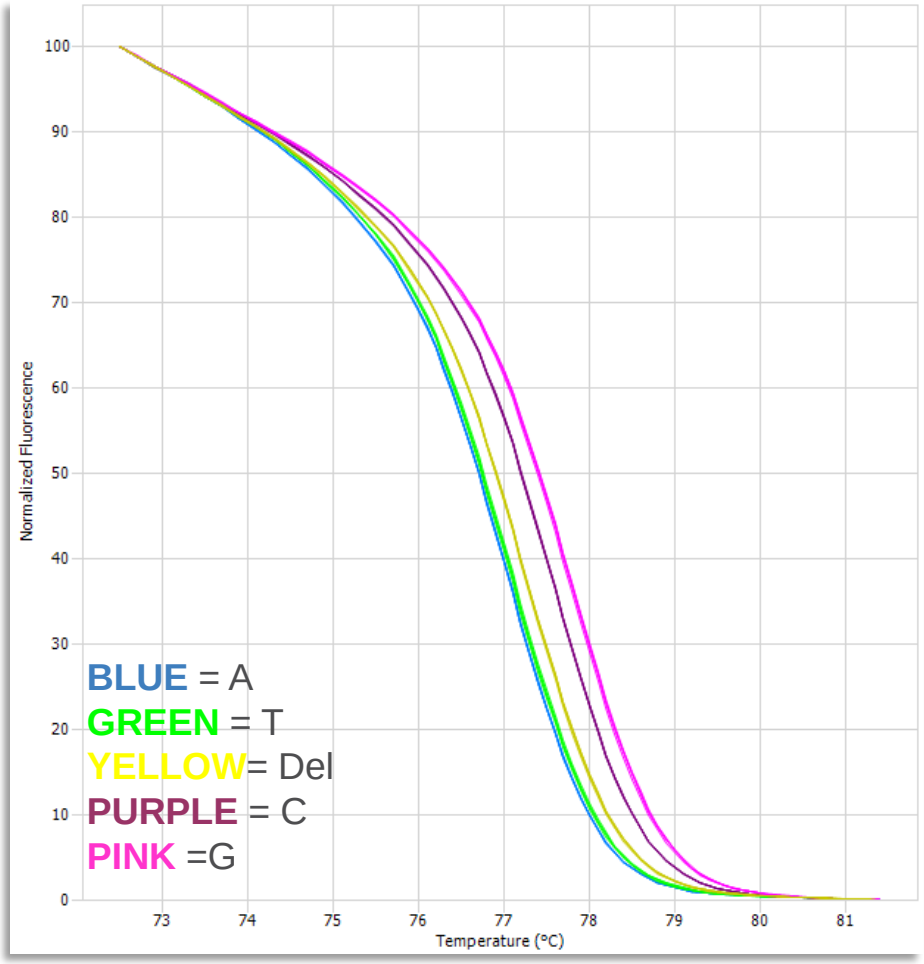
- Amplicon T_m shifts vary from large to very small depending on identity of polymorphism
- Class 4 SNPs are the rarest and most difficult SNP type to discriminate by T_m
- HRM requires highly optimized assays and precise instrumentation to differentiate sequence changes

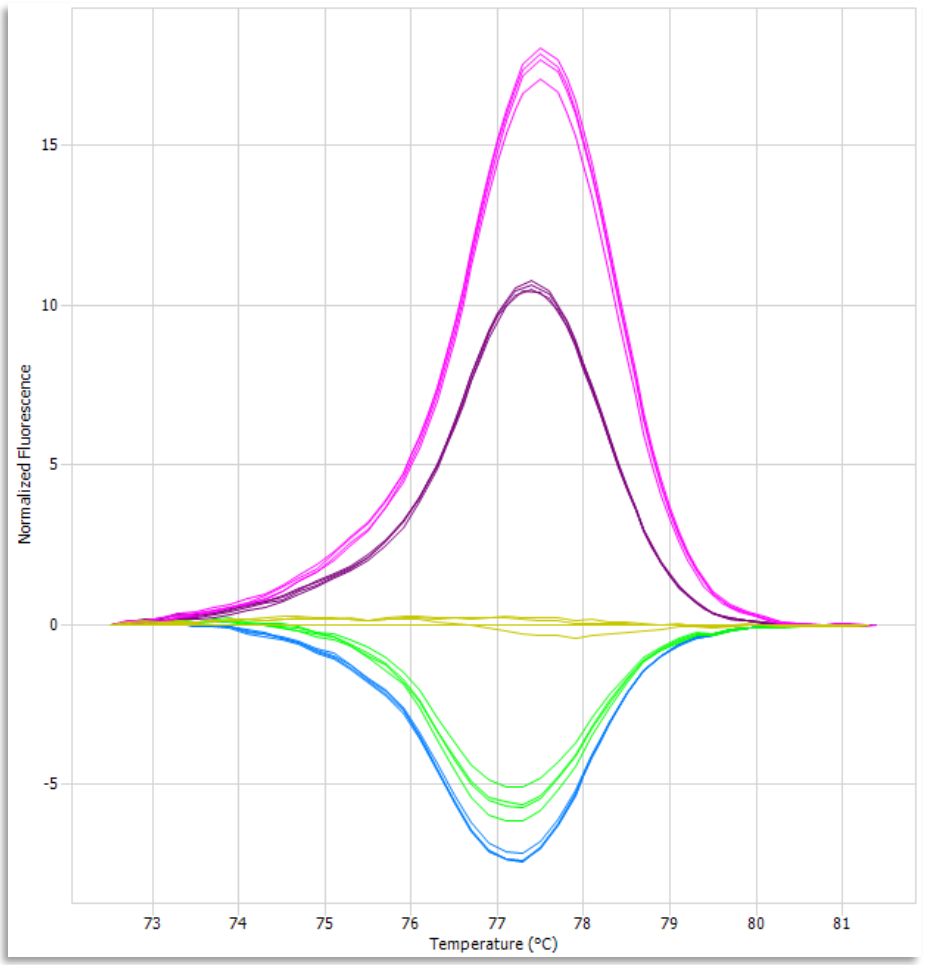
Single Nucleotide Resolution

Primer and Amplicon Sequences (60 and 61 bp):

```
5′- CACCTCACGCAGCACTTACCAA
5′- CACCTCACGCAGCACTTACCAACTACTCATaCAGACTCATTCACCTCACCATGTCACTCGC     76.64°C
5′- CACCTCACGCAGCACTTACCAACTACTCATtCAGACTCATTCACCTCACCATGTCACTCGC     76.82°C
5′- CACCTCACGCAGCACTTACCAACTACTCATcCAGACTCATTCACCTCACCATGTCACTCGC     77.47°C
5′- CACCTCACGCAGCACTTACCAACTACTCATgCAGACTCATTCACCTCACCATGTCACTCGC     77.70°C
5′- CACCTCACGCAGCACTTACCAACTACTCAT:CAGACTCATTCACCTCACCATGTCACTCGC     76.95°C
                                                AAGTGGAGTGGTACAGTGAGCG -5′
```

Normalized Melt and Difference Plot





- Five clear melt profiles with good separation

Class IV SNP Genotyping

- A/T polymorphism within KIAA1683 (rs8110972)

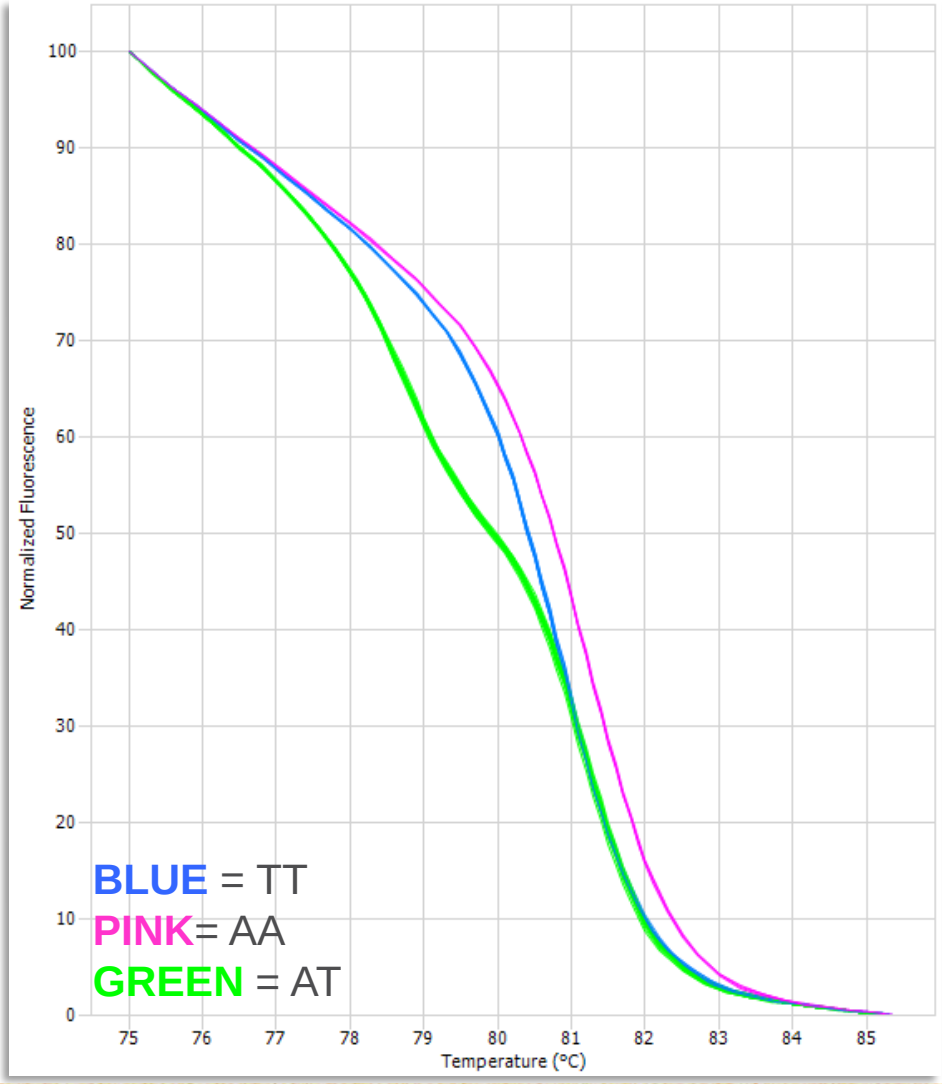
Primer and Amplicon Sequences (48 bp):

```
5′- GGTGACAGCCATGTCTACA
5′- GGTGACAGCCATGTCTACAGGCACAaACACGTGAGGTGGCTTGTCCCC        81.17°C
5′- GGTGACAGCCATGTCTACAGGCACAtACACGTGAGGTGGCTTGTCCCC        80.76°C
                                  ACTCCACCGAACAGGGG –5′
```

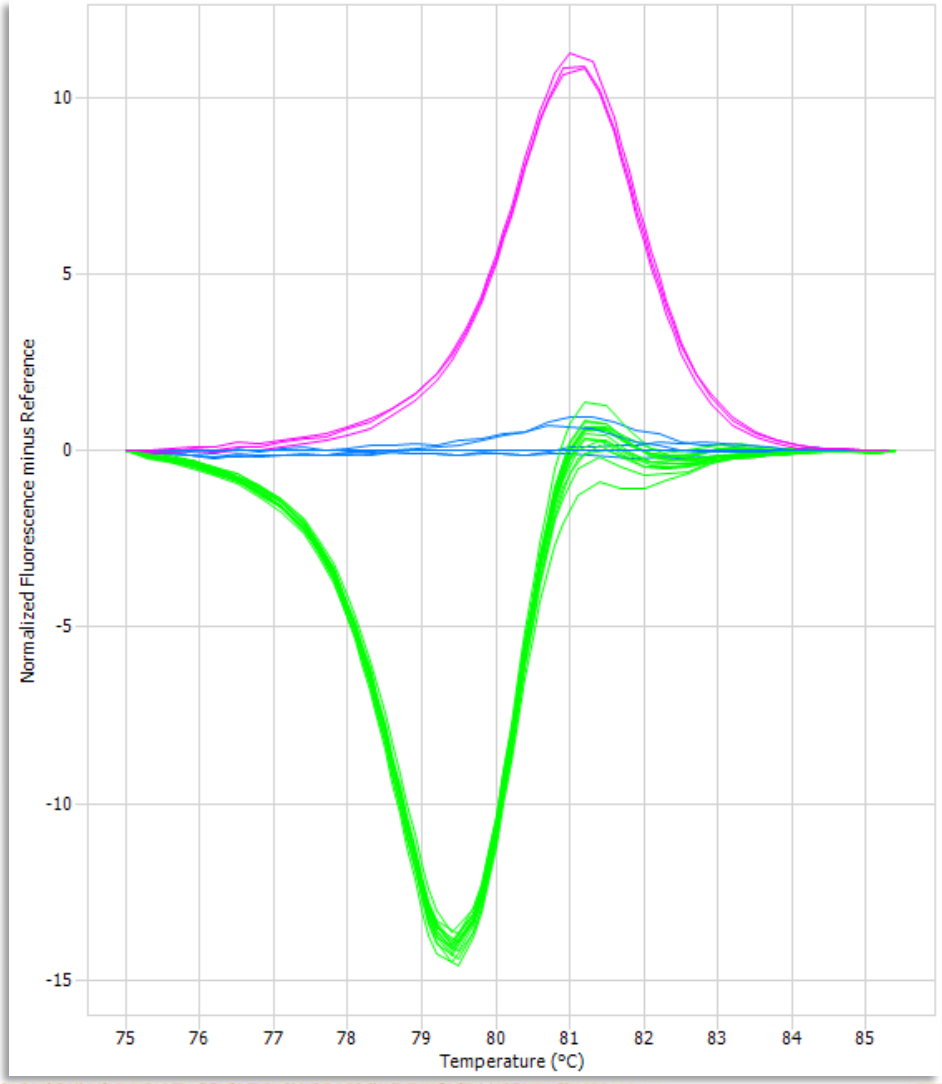
- 8 human genomic DNA samples – 3 technical replicates of each

Class IV SNP Genotyping

Normalized Melt



Difference Plot



illumina

Class IV SNP Genotyping

- A/T polymorphism within ATP-binding cassette, sub-family C (CFTR/MRP), member 12 (ABCC12) gene (rs6500305)

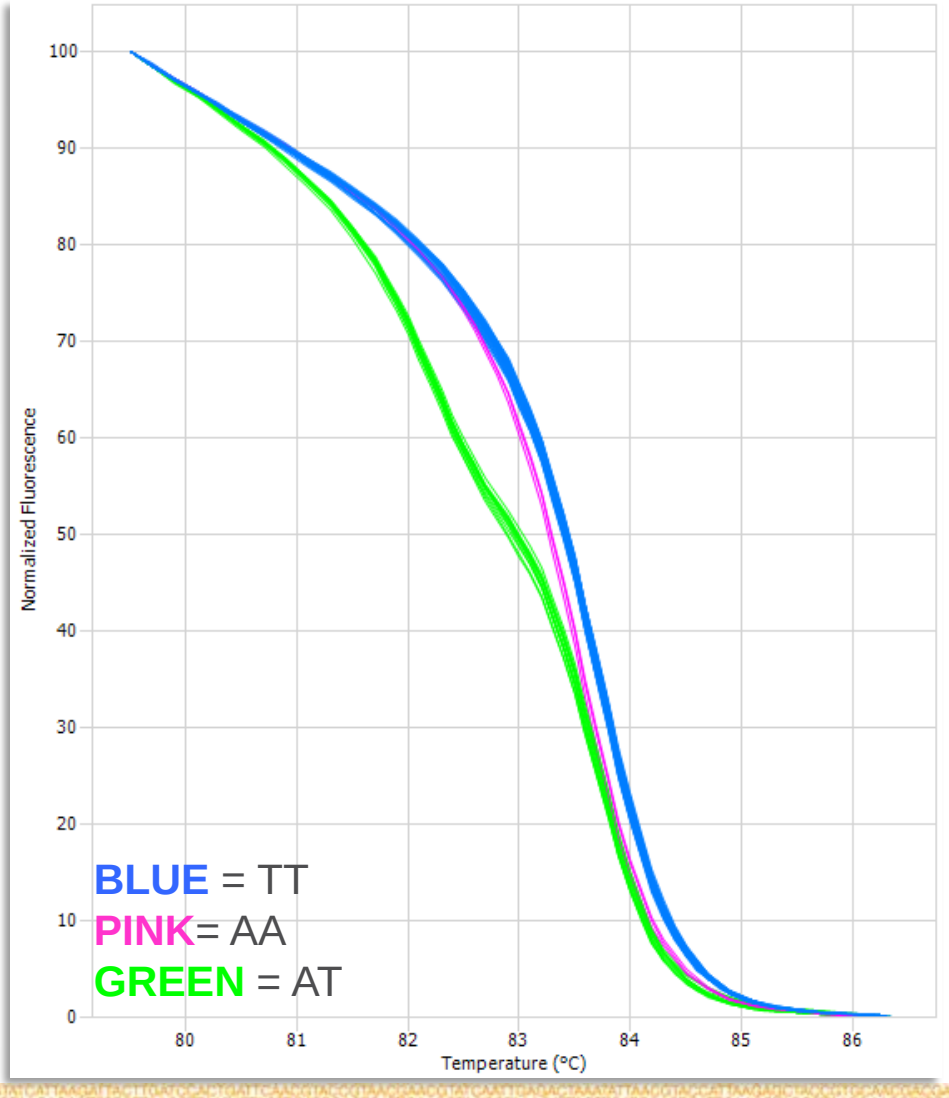
Primer and Amplicon Sequences (75 bp):

```
5′- CCAGGCCCTGCATGGA
5′- CCAGGCCCTGCATGGAGGAGGTGATGTGGGtGAACCAGGGTGACCGGCTGACATTCTCCACCTTCTTGAGCTCCT   84.08°C
5′- CCAGGCCCTGCATGGAGGAGGTGATGTGGGaGAACCAGGGTGACCGGCTGACATTCTCCACCTTCTTGAGCTCCT   83.95°C
                                                    TAAGAGGTGGAAGAACTCGAGGA -5′
```

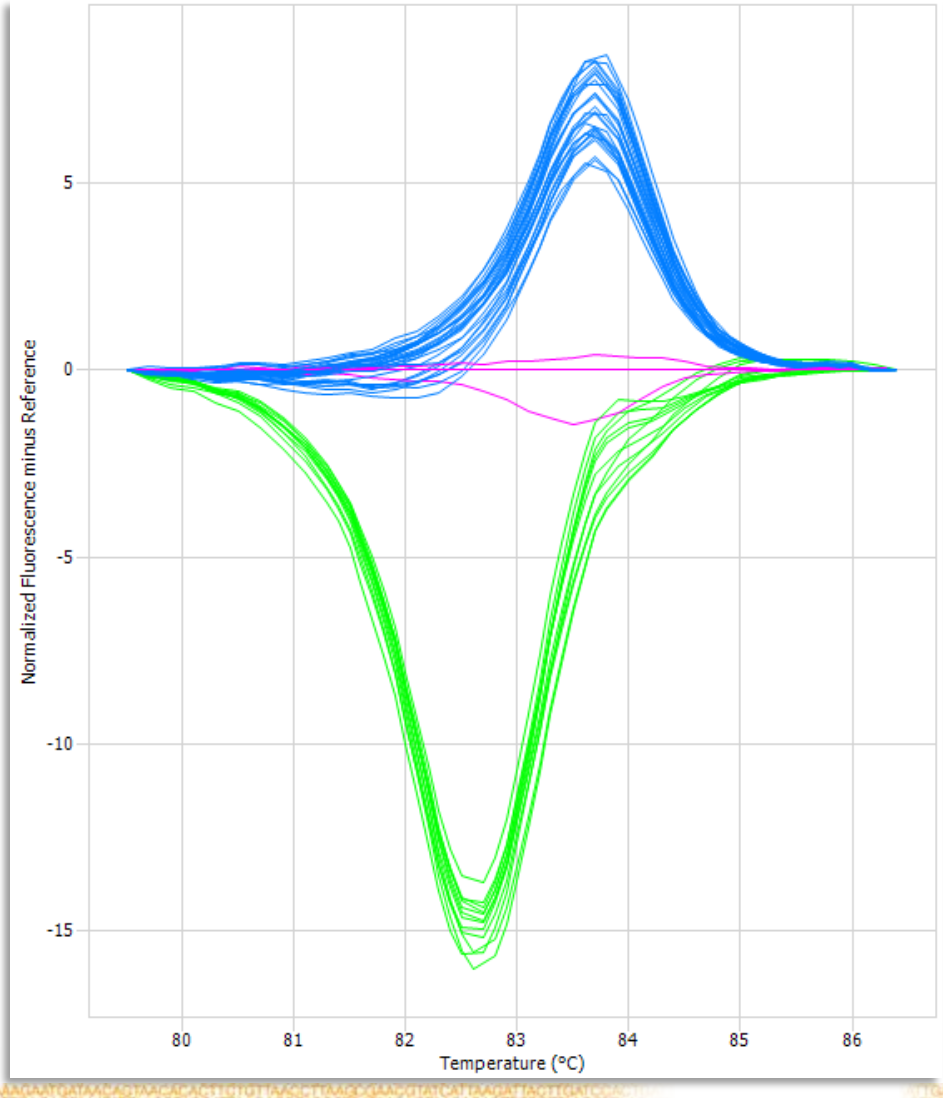
- 45 human genomic DNA samples

Class IV SNP Genotyping

Normalized Melt



Difference Plot



illumina®

Available HRM Master Mixes

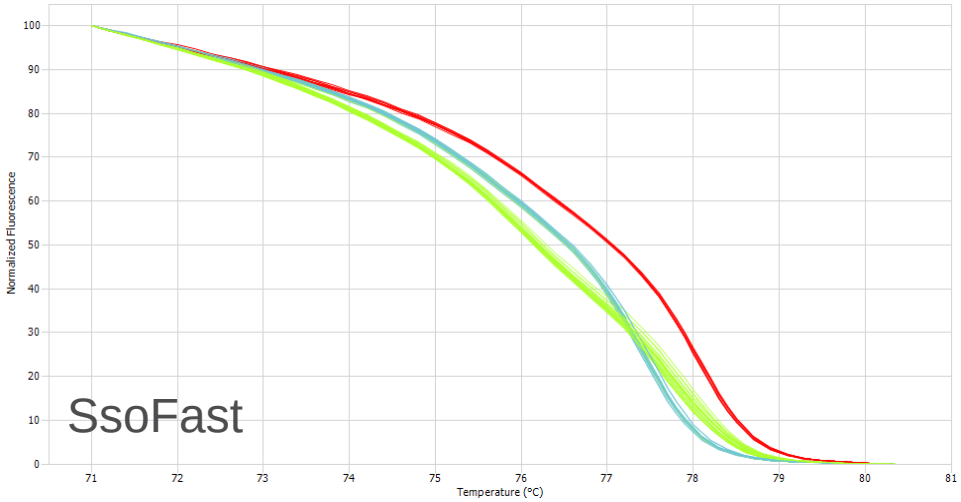
- Four commercially available HRM mixes evaluated on Eco system
  - BioRad SsoFast EvaGreen Supermix
  - Life Technologies EXPRESS SYBR® GreenER™
  - Life Technologies MeltDoctor™ HRM Master Mix
  - Roche LightCycler ® 480 HRM
- Genotyping of a Class II SNP
  - A/C polymorphism in solute carrier family 28 (sodium-coupled nucleoside transporter), member 2 (SLC28A2) gene (rs1060896)

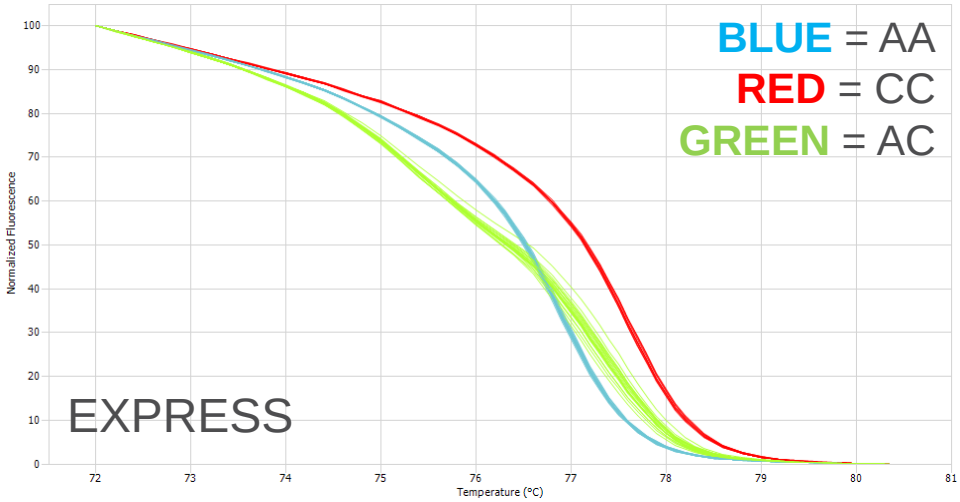
Primer and Amplicon Sequences (65 bp):

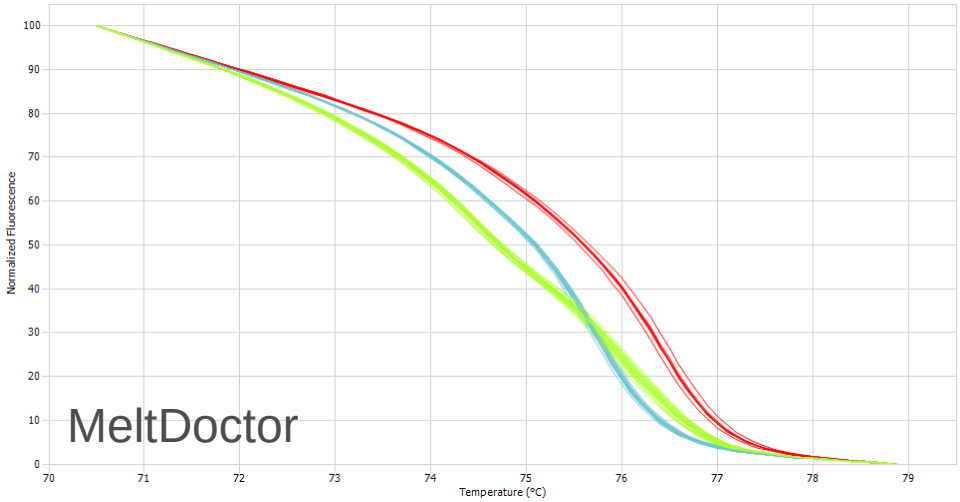
5′- **AAAGCAAGAAGTTTCTGCAAAACA**
5′- AAAGCAAGAAGTTTCTGCAAAACACACGCCAG**a**TTGTTCAAGAAGATCCTGTTGGGCCTGTTGTG **77.12°C**
5′- AAAGCAAGAAGTTTCTGCAAAACACACGCCAG**c**TTGTTCAAGAAGATCCTGTTGGGCCTGTTGTG **77.93°**
TAGGACAACCCGGACAACAC –5′

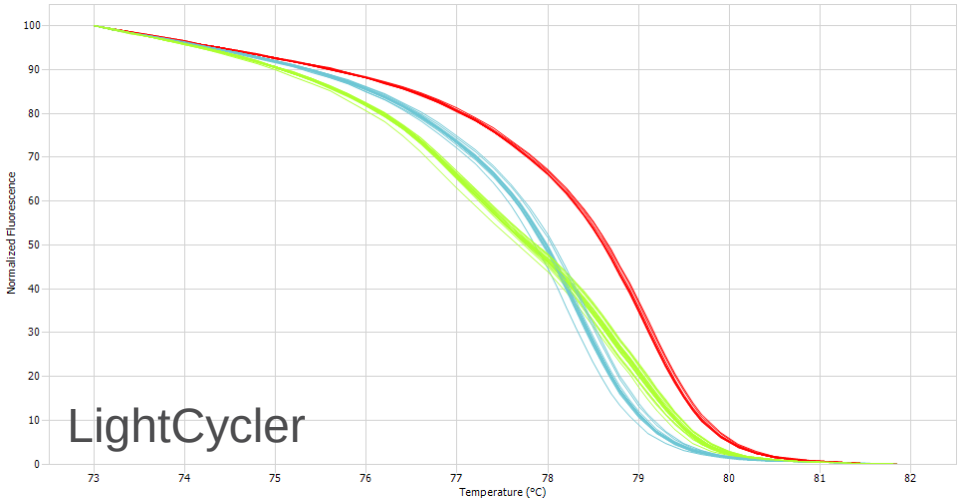
- 45 human genomic DNA samples

Normalized Melt Curves





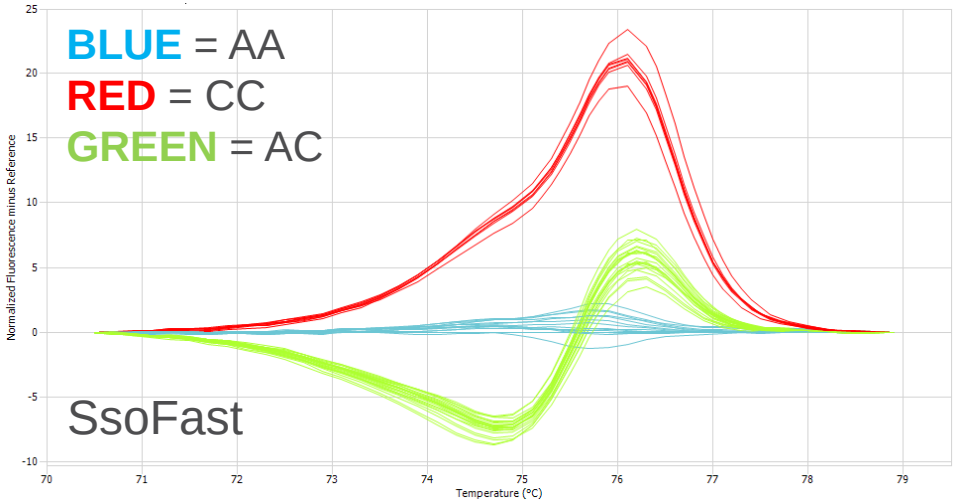


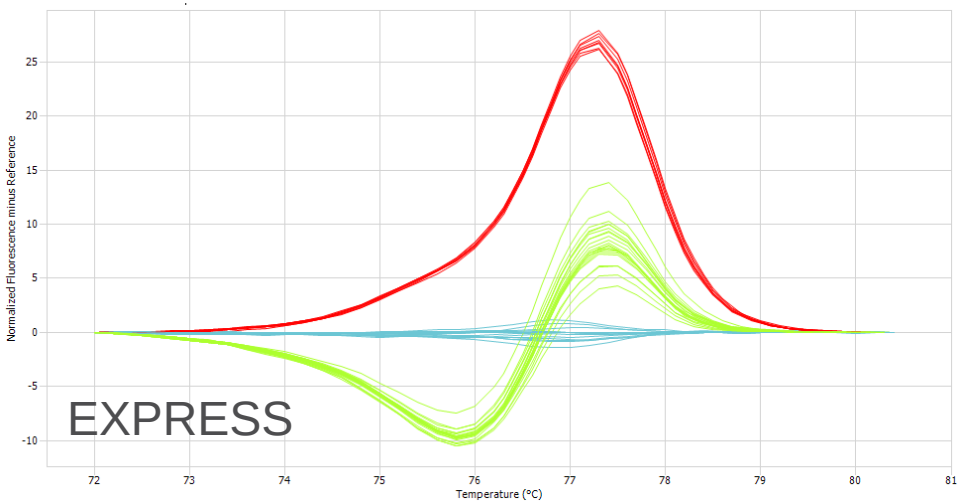


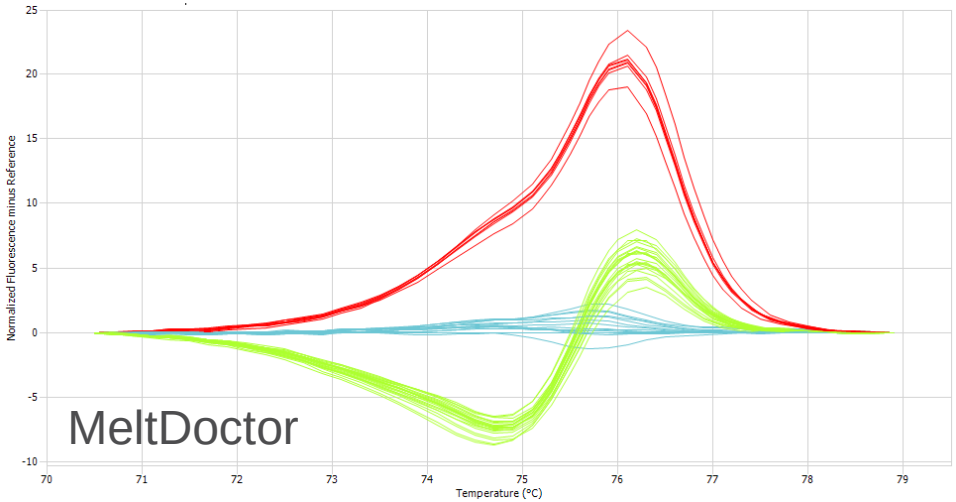
- Three genotypes clearly resolved by all mixes

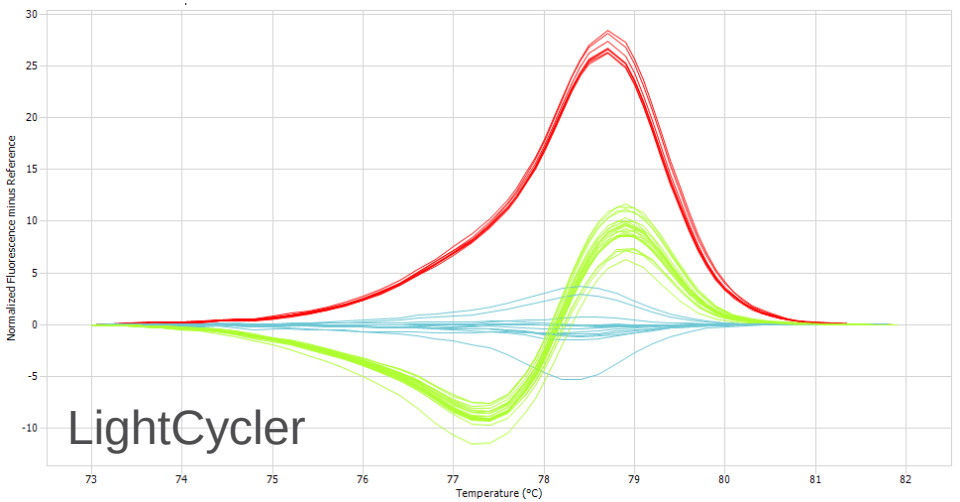
illumina®

Difference Plots







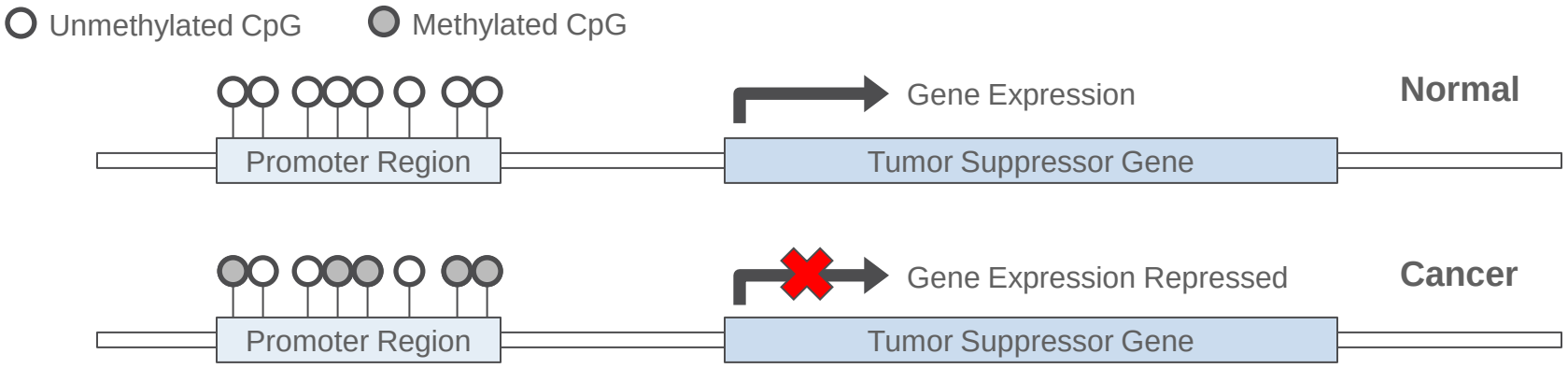


- Wild Type (AA) sample used a reference for difference plot

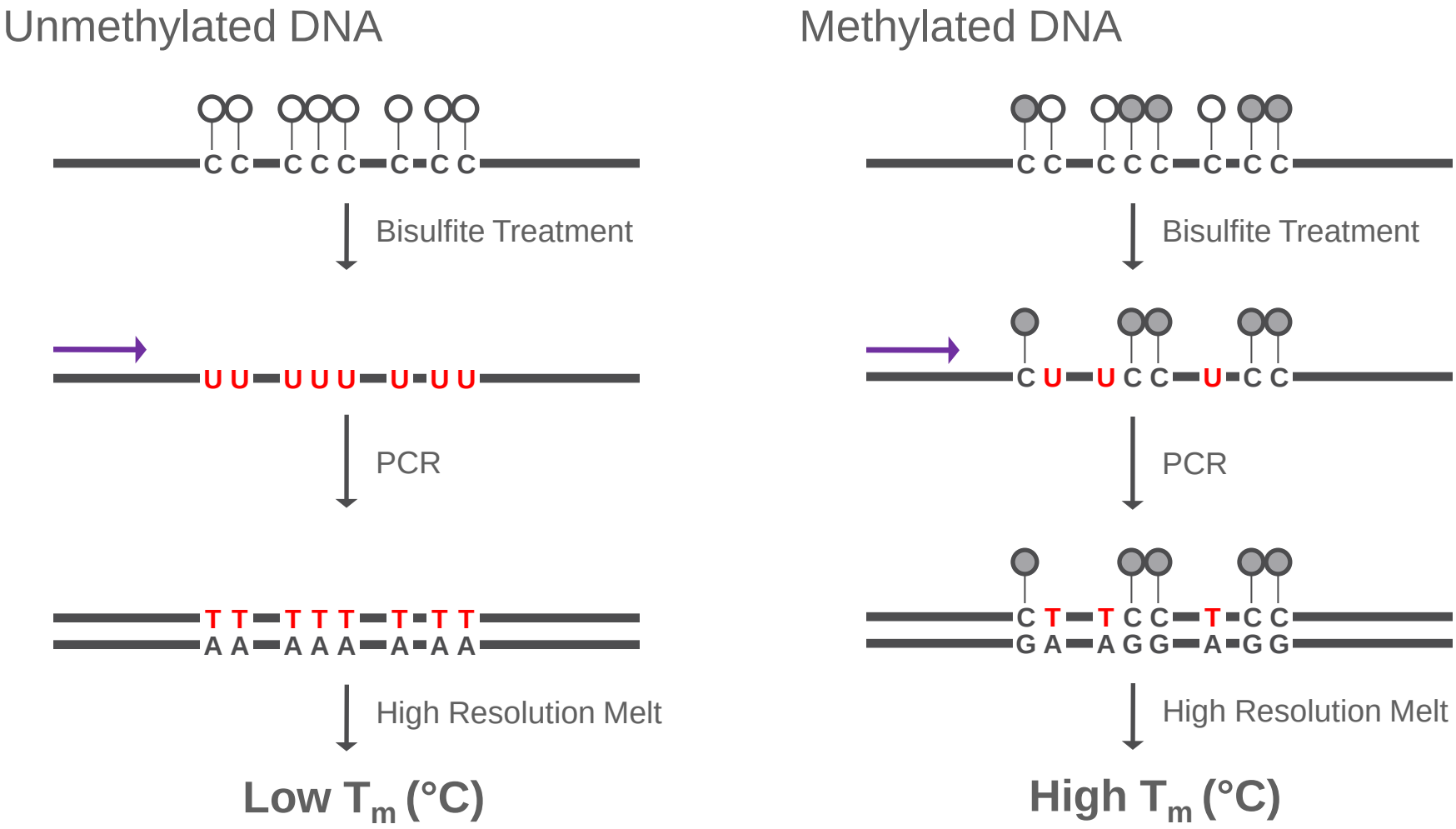
illumina®

Application – DNA Methylation Analysis

- DNA methylation of (CpGs) is a key epigenetic mechanism regulating gene expression



Methylation Analysis by HRM



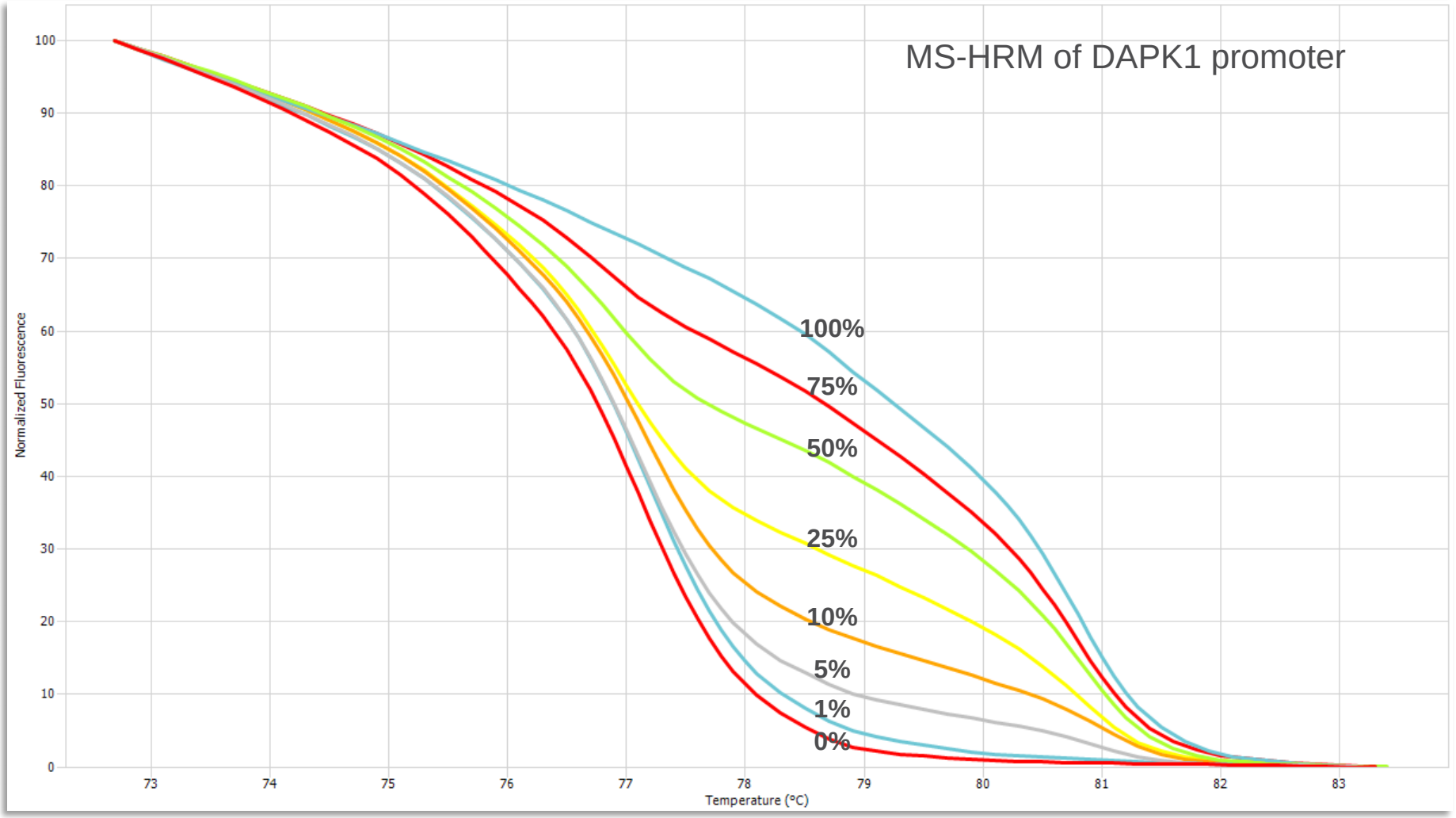
- Samples differentiated by differences in melting temperature

HRM can be Sensitive and Semi-Quantitative

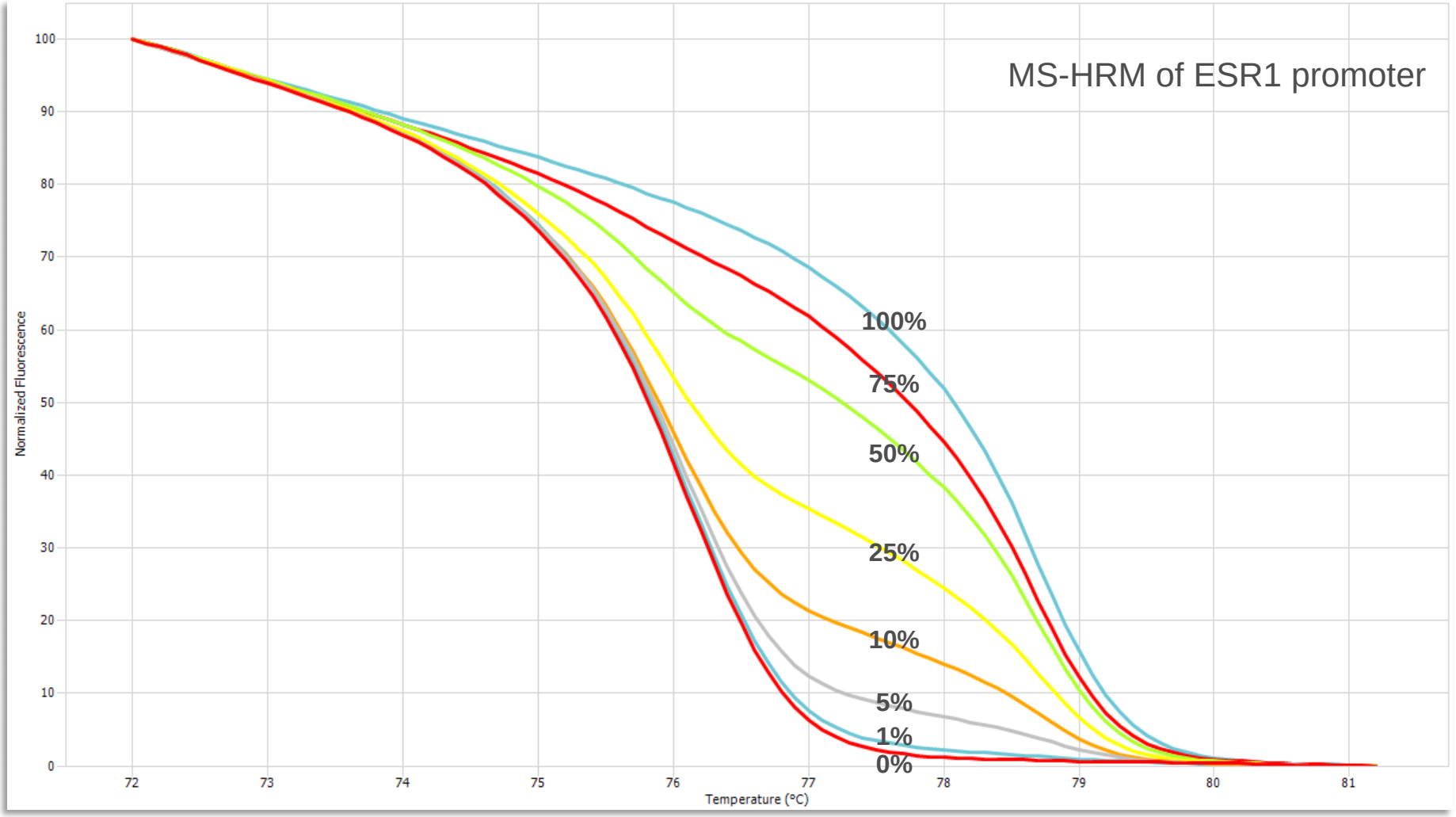
- Bisulfite converted methylated and unmethylated DNA (Qiagen) mixed in different ratios to form a ‘standard curve’

- Primers designed according to recommendations from Wojdacz et al (2009)
  - Limited number of CpGs included in primers to overcome PCR bias
  - 60°C annealing temperature

HRM can be Sensitive and Semi-Quantitative



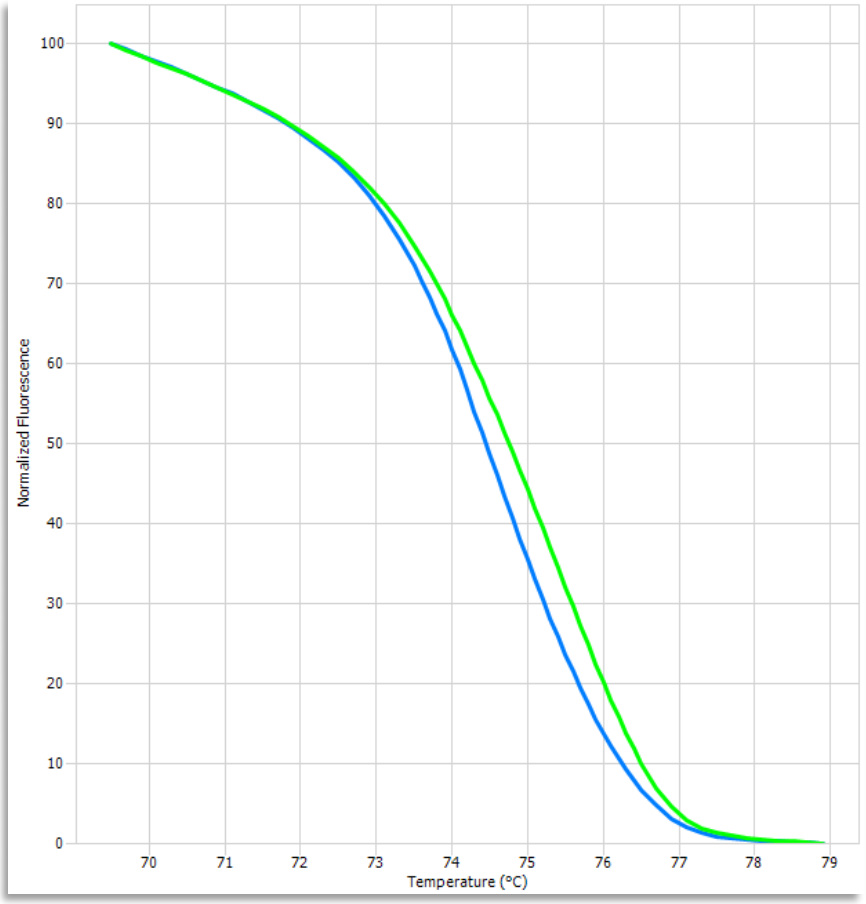
HRM can be Sensitive and Semi-Quantitative



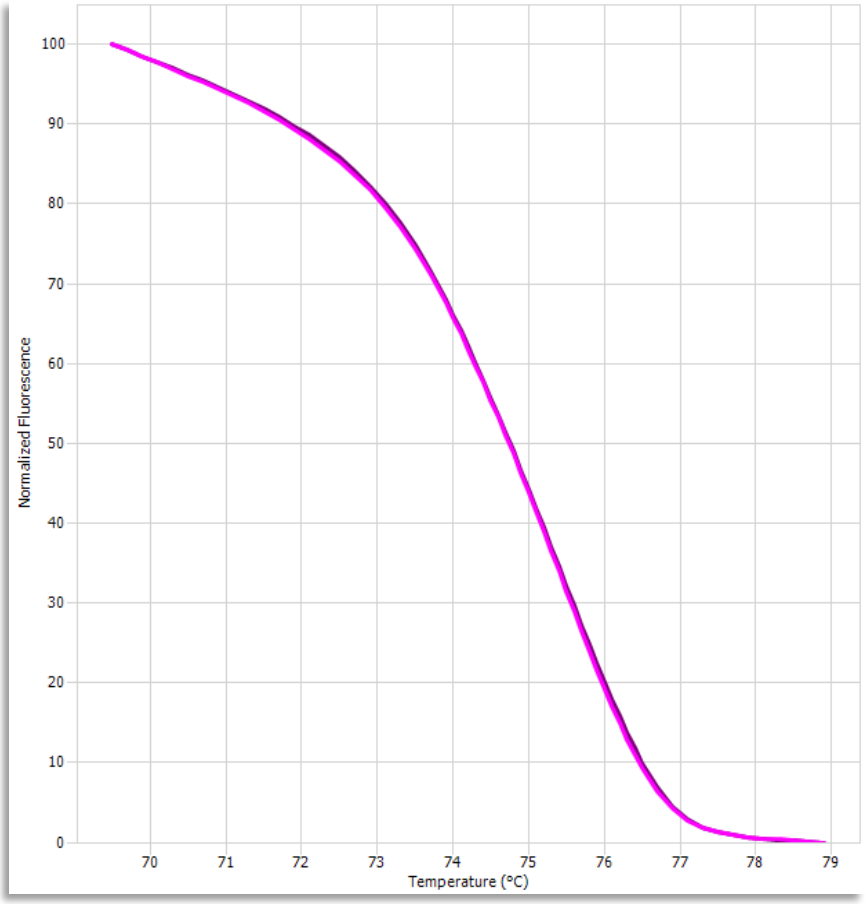
- Both assays able to detect 1% methylated DNA in a background of unmethylated DNA

Methylation Analysis of Tumor, Pseudotumor and Normal Tissue

Tumor sample: expected to be less methylated than the normal adjacent tissue
Pseudotumor sample: expected to have same methylation level as the normal adjacent tissue



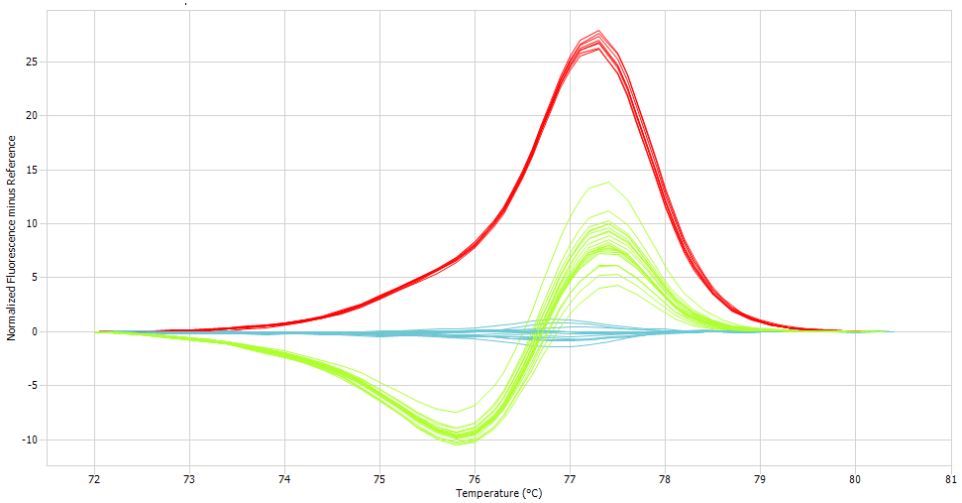
GREEN: Normal Adjacent Sample
BLUE: Tumor Sample

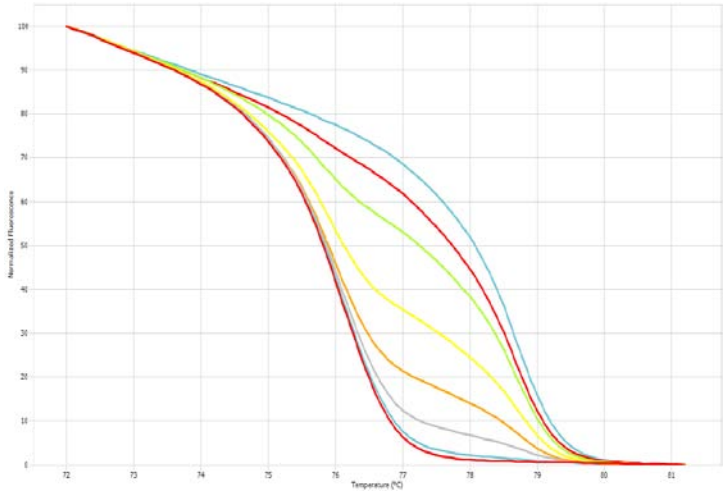


PURPLE: Normal Adjacent Sample
PINK: Pseudotumor Sample

Summary

- Requires high performance instrumentation
- Enables cost effective high value analysis
  - Genotyping
  - Methylation





illumina®

Acknowledgements

- The Eco team

McGill University

- Moshe Szyf, Ph.D.
- Barbara Stefanska, Ph.D
- Aurélie Bouzelmat