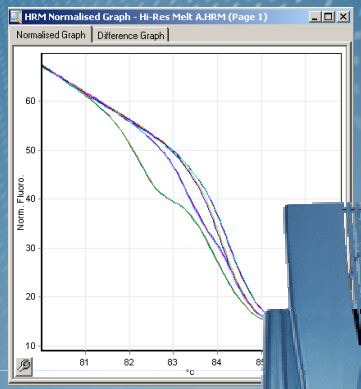
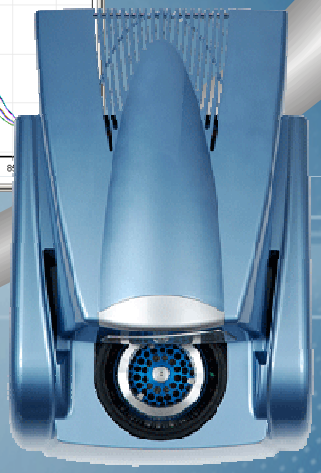
HRM™

high-resolution melt





HRM APPLICATIONS

Andrea A Tesoriero

corbett
LIFE SCIENCE

www.corbettlifescience.com

High Resolution Melt (HRM)

detailed monitoring of changes in fluorescence as a PCR products are melted

- PCR amplify an amplicon with two primers and an intercalation dye and then melt (dissociate) the product – double stranded to single stranded

- Can detect even a single base pair change

HRM Specifications

- **Instrument requires:**
  - high-intensity + high sensitivity optics
  - high-speed data capture
  - very precise temperature control and resolution

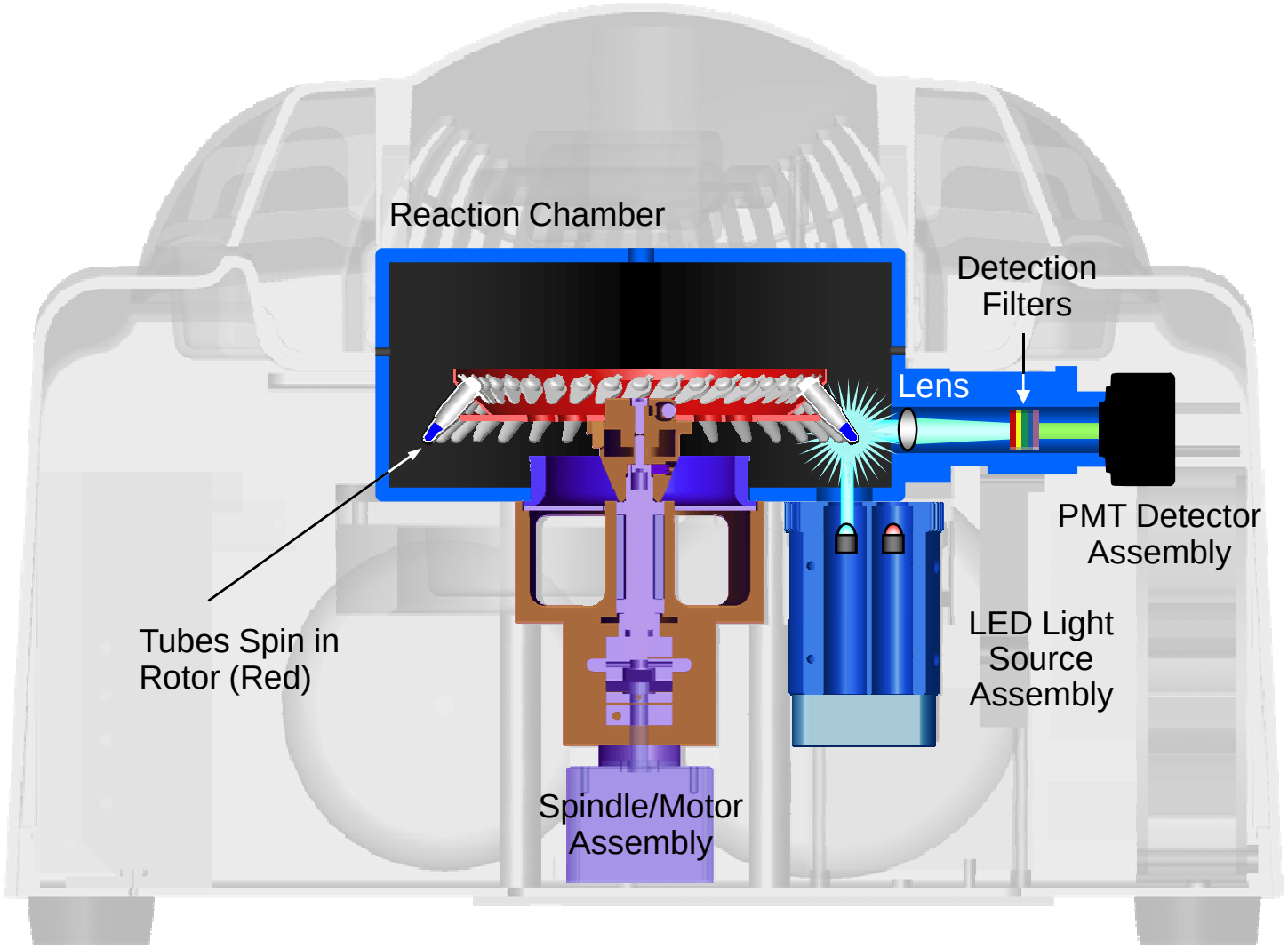
Rotor-Gene™ 6000

The world's only real-time rotary thermo-optical analyzer

An ideal platform for HRM

Rotor-Gene™ 6000

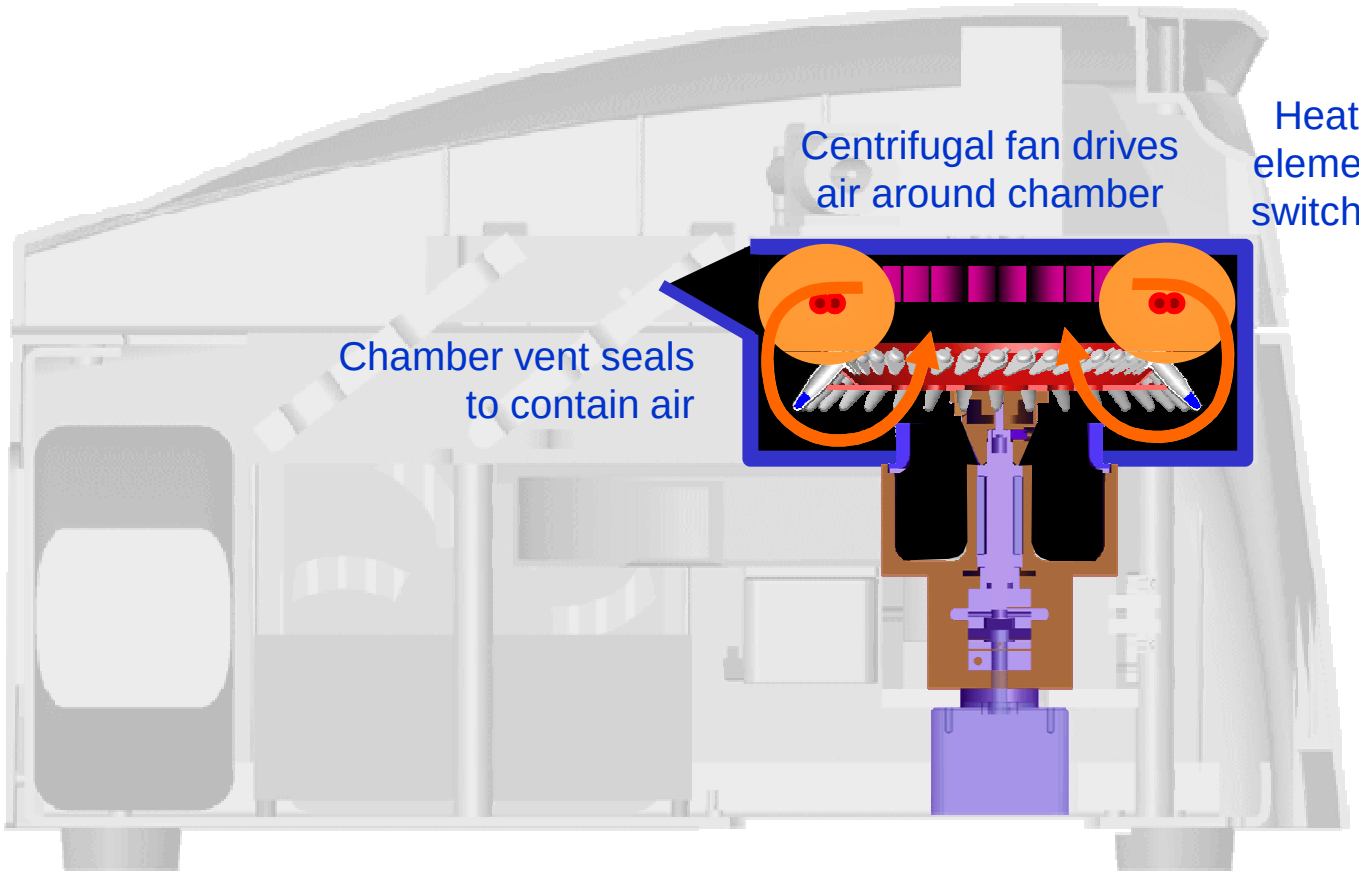
Cross-section of rotary optics

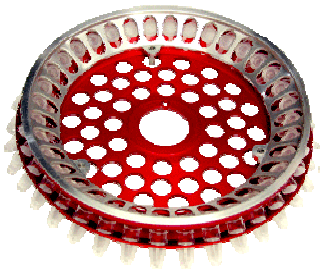


Thermal Control
±0.01

Rotor-Gene™ 6000

Heating mechanism

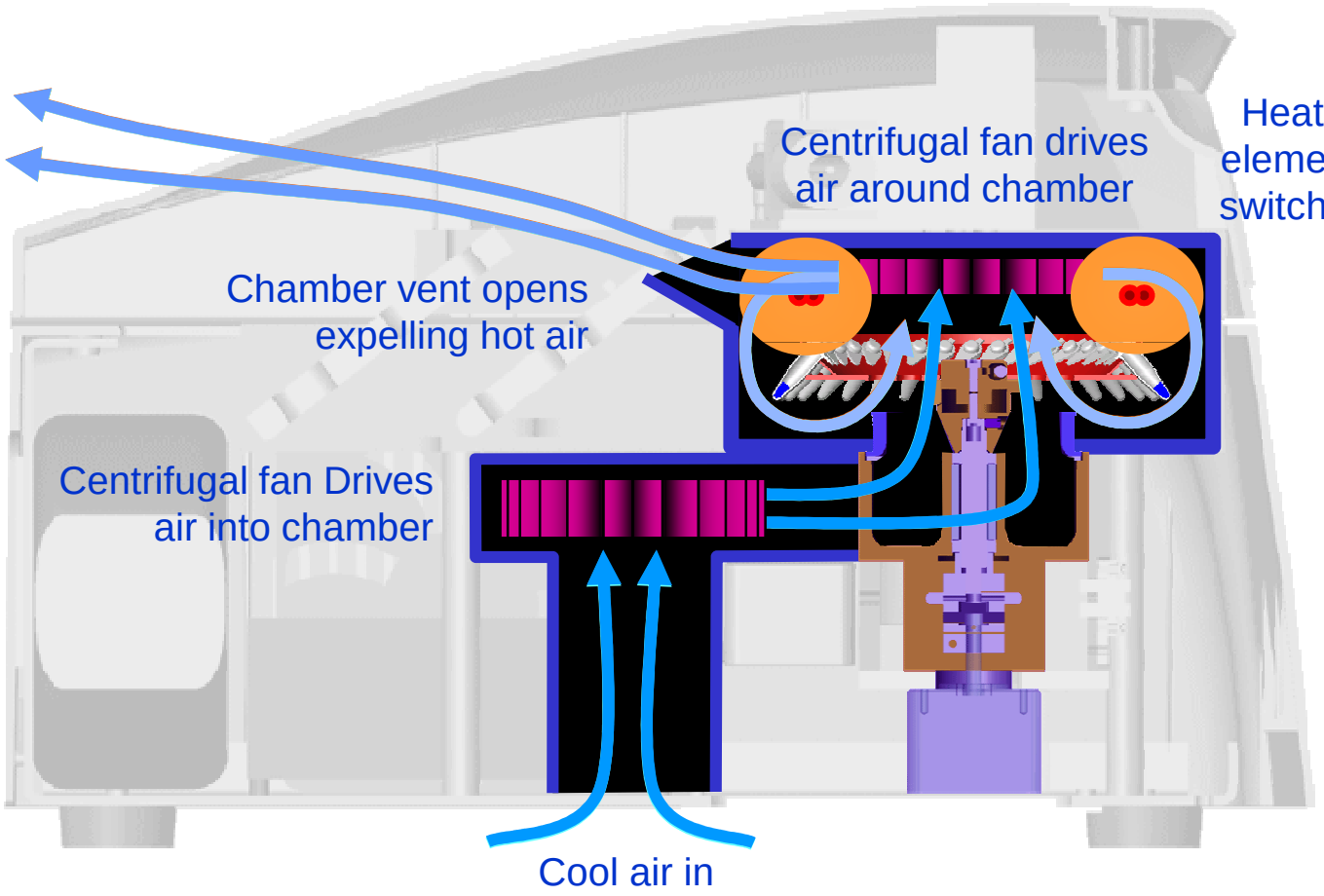


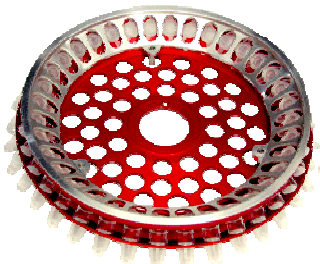


Note: holes in the rotor allow free airflow

Rotor-Gene™ 6000

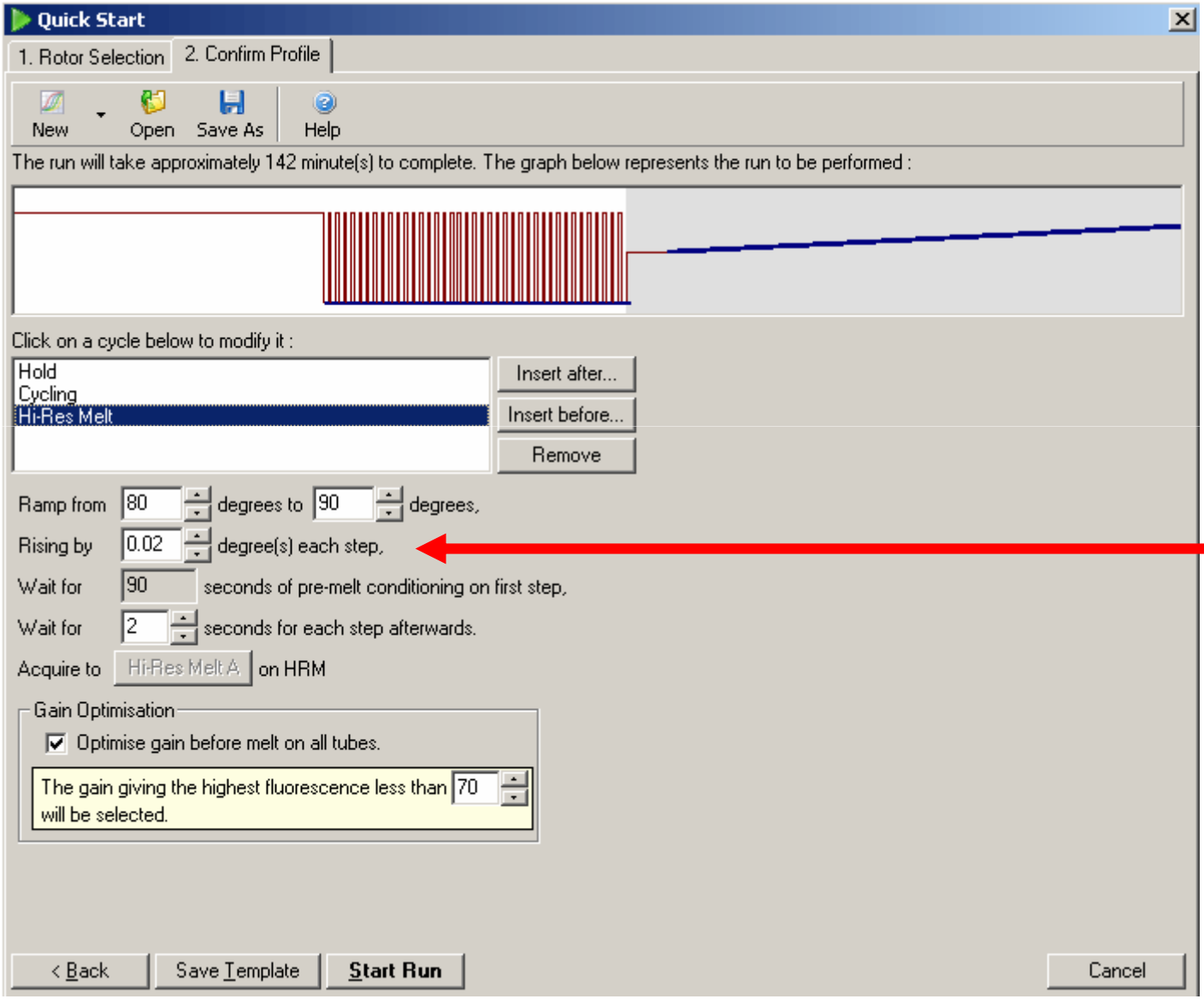
Cooling mechanism





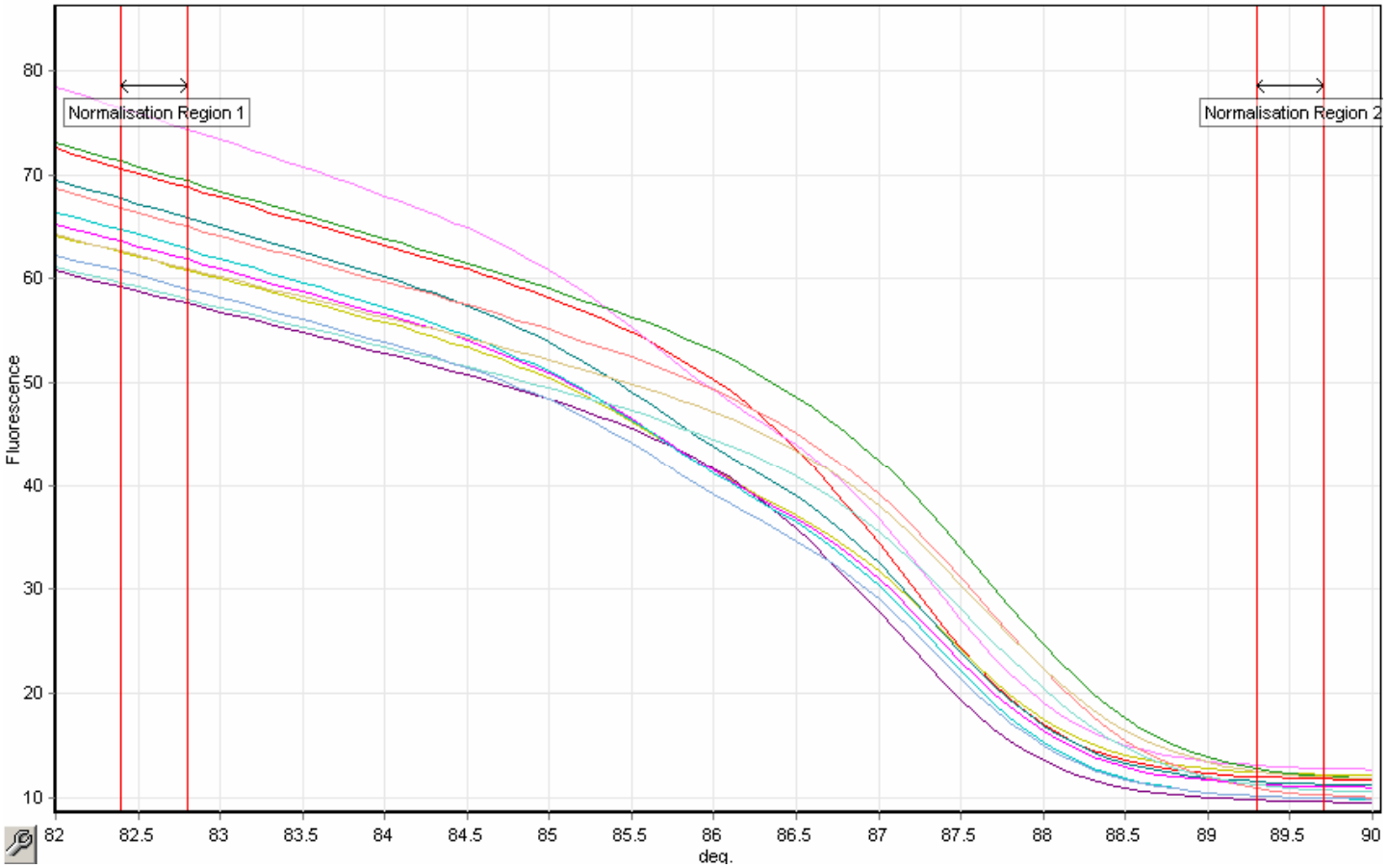
Note: holes in the rotor allow free airflow

HRM Profile



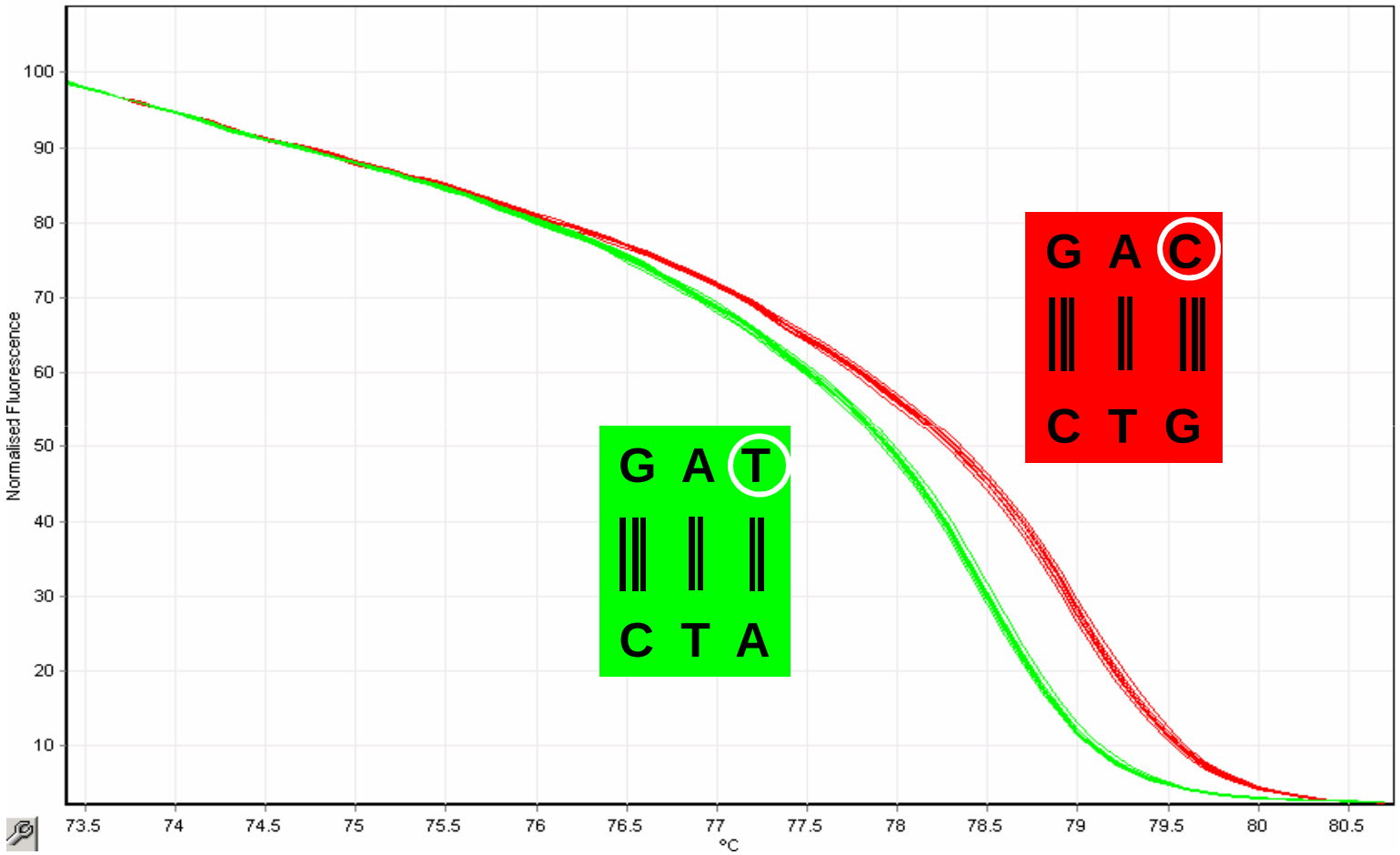
0.02℃

Data Acquisition



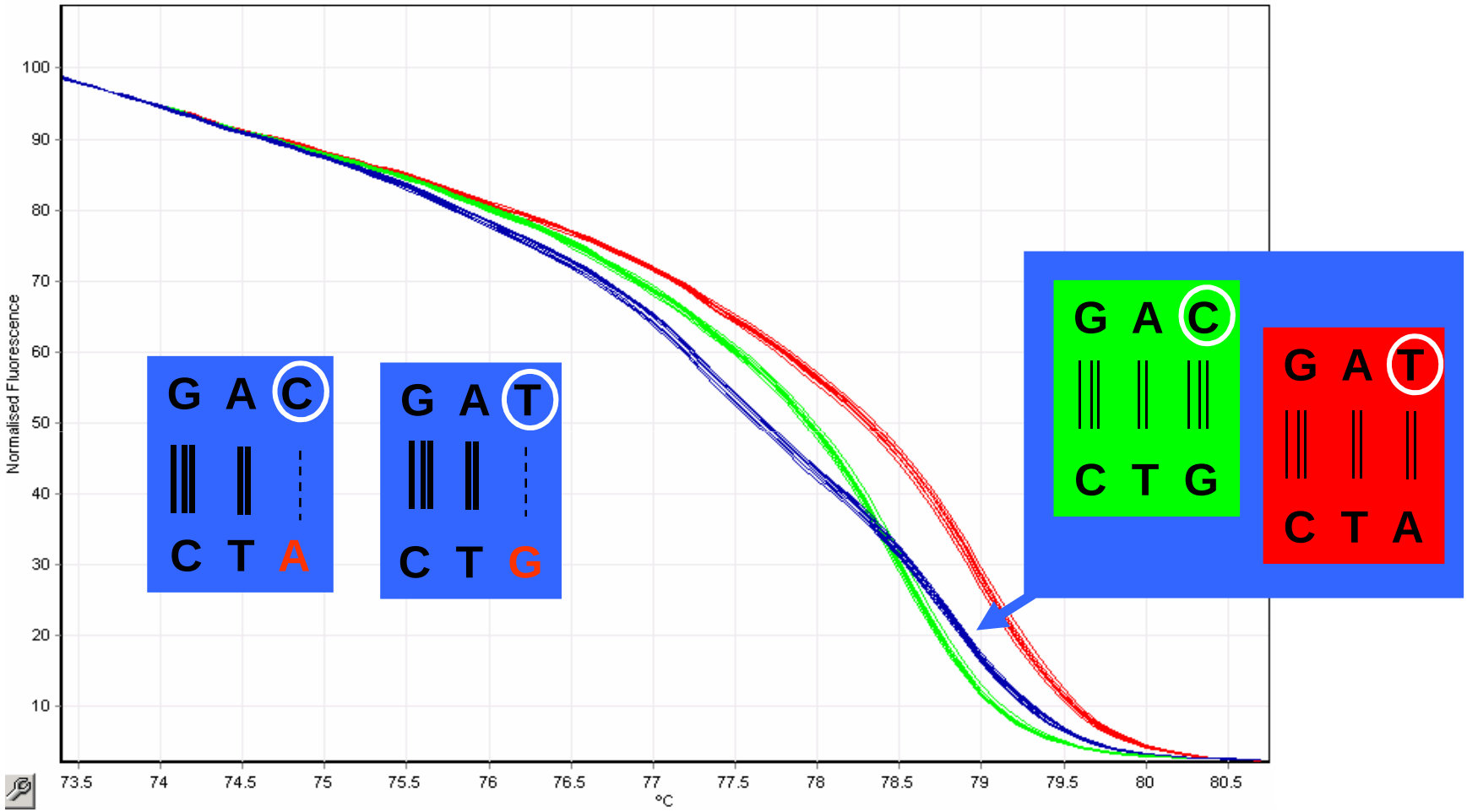
- Melt curves normalized—by selecting linear regions before & after the melt transition
- Two regions defined—upper 100% double stranded / lower single stranded baseline

Normalized Data: Homoduplexes C or T



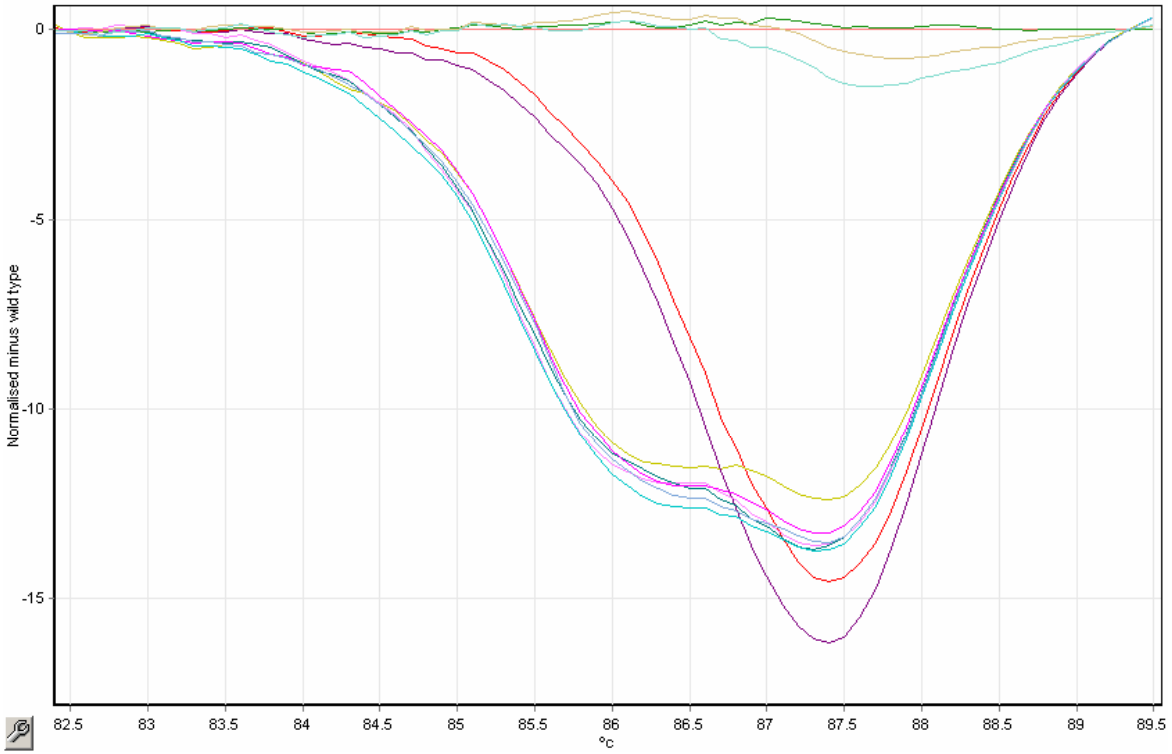
Homozygotes represented by a single base change are differentiated by a difference in T_m

Heteroduplex C>T



Heterozygotes form heteroduplexes, which result in lower melt curves and two transition points

Difference Graphs



- Difference graph displays each sample plot subtracted from a given genotype control plot
- Calculates a percentage confidence relative to a known genotype

HRM Summary

- Simple, fast, cost effective method for gene scanning and detecting a single-base change in your sample
- Closed system
- NO labeled probes
- Has excellent sensitivity and specificity is capable of detecting BOTH heterozygous and homozygous changes
- Costs less than competing technologies
- Sequence directly off the product—sample not consumed
- Auto call software
- Scanning & genotyping can be done simultaneously in the same reaction

SNP's

Detection of alpha-thalassemia-1 Southeast Asian type using real-time gap-PCR with SYBR Green 1 and high Resolution Melting analysis. Pornprasert S, Phusua A, Suanta S, Saetung R, Sanguansermsri T,*Eur J of Haematol. 2008.*

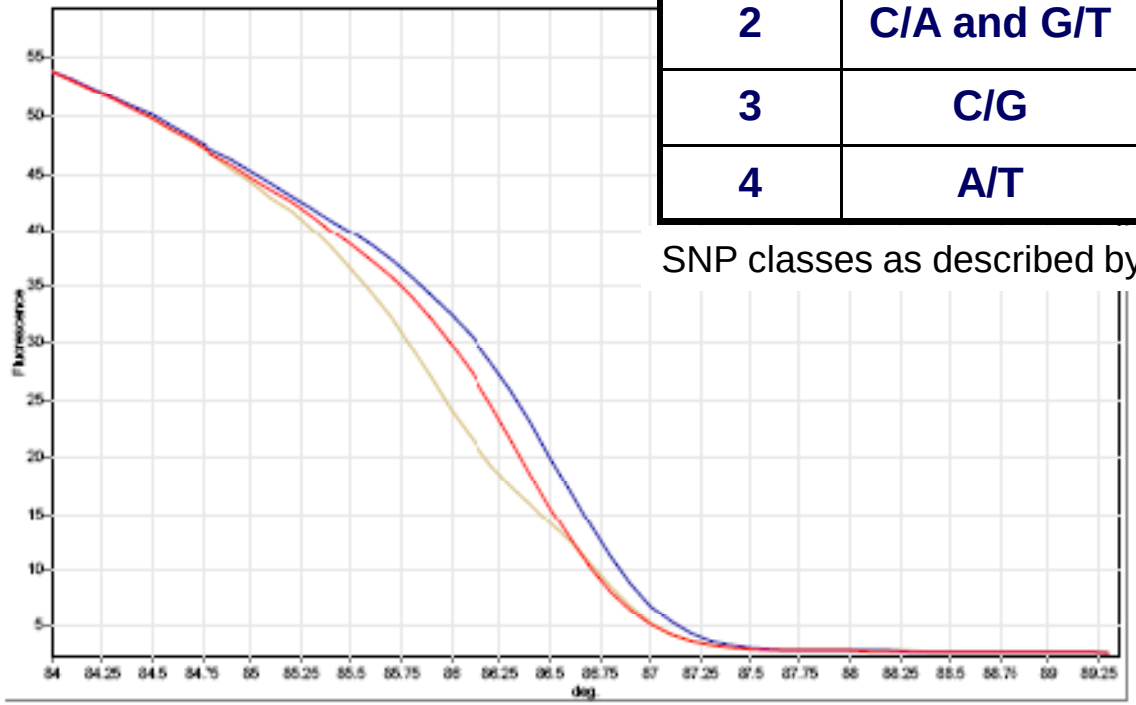
Direct Genotyping of Single Nucleotide Polymorphisms in Methyl Metabolism Genes Using Probe-Free High-Resolution Melting Analysis. L S. Kristensen and A.Dobrovic *Cancer Epidemiol Biomarkers Prev 2008;17(5) May 2008*

High-resolution melt analysis for the detection of a mutation associated with permethrin resistance in a population of scabies mites C Pasay, L Arlian, M. Morgan, D Vyszenski-Moher, A. Rose, D Holt, S.Walton and JMcCarthy *Medical and Veterinary Entomology (2008)* ***22, 82–88***

Genotyping
Class 4 SNP

SNP Class	Base Change	Typical Tm Shift	Rarity (in humans)
1	C/T and G/A	Large>0.5oC	64%
2	C/A and G/T	↓	20%
3	C/G	Very Small >0.2oC	9%
4	A/T		7%

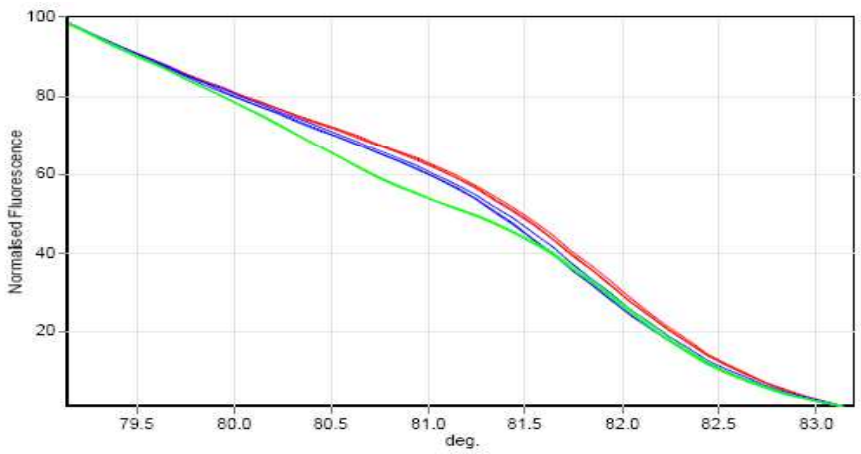
SNP classes as described by Venter *et al* 2002

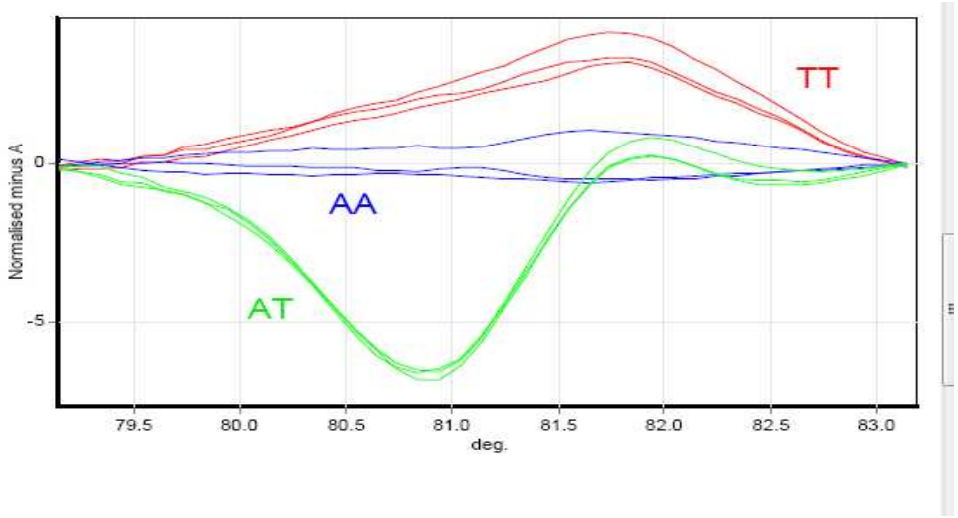


Example of a class 4 SNP on the Rotor Gene (MCT A1470T)
The rarest and most difficult SNP to discriminate.

Class 4 SNP using Sybr Green 1

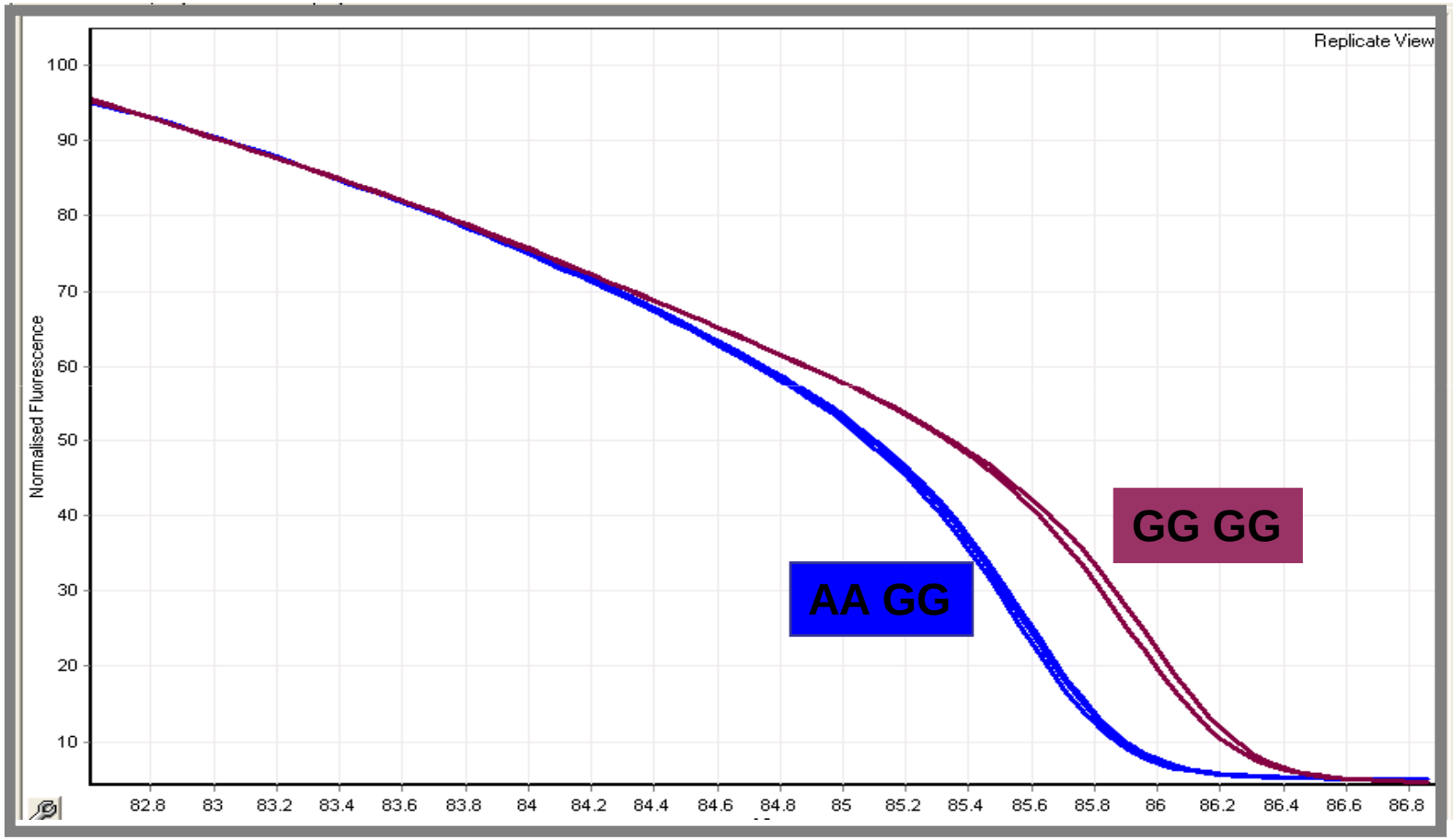
- SYBR can be used at non-saturating concentrations, for the most demanding HRM applications.





- Extra sensitivity of the Rotor-Gene may be a major reason we do not require saturating dye levels

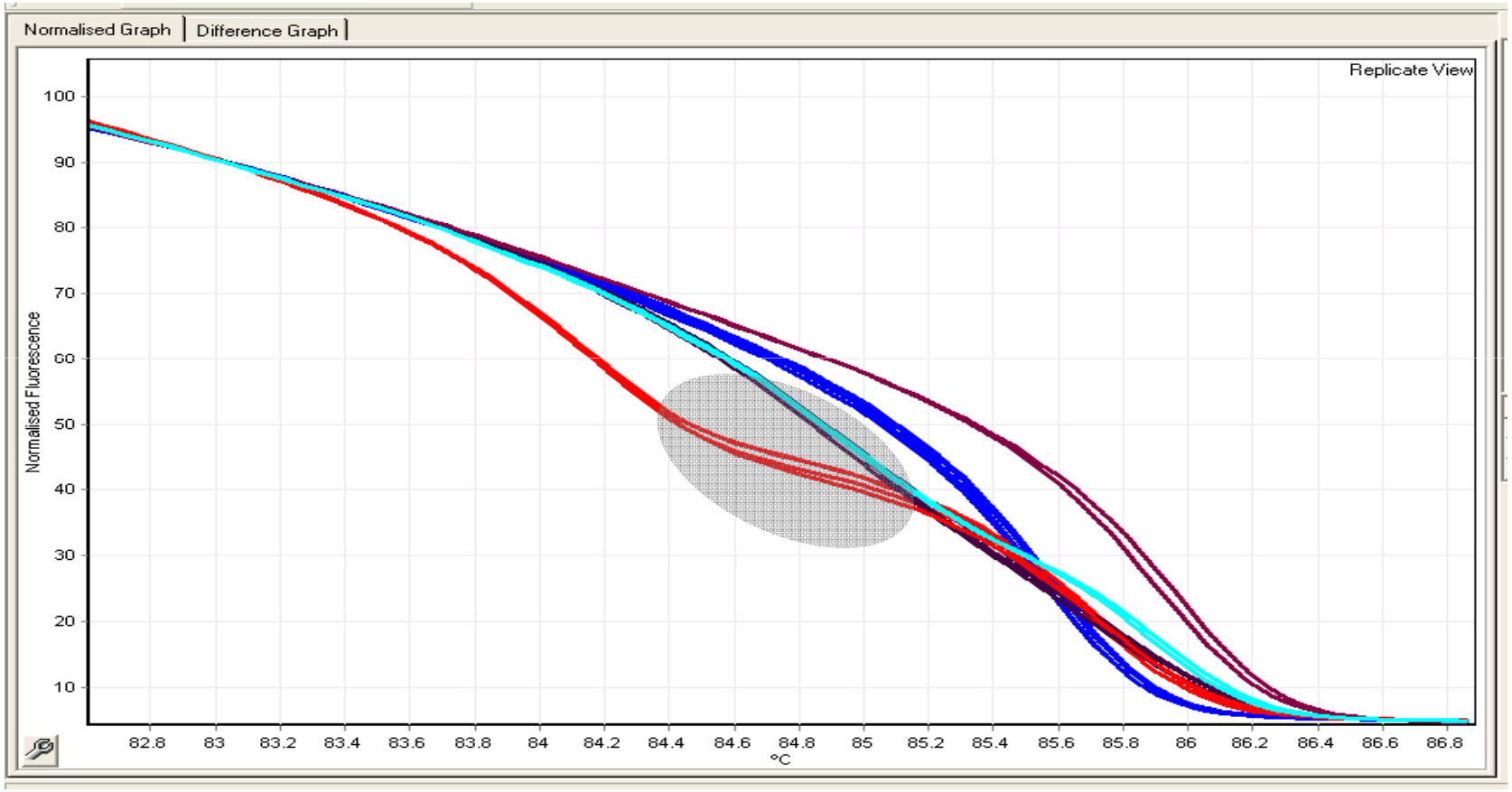
Adjacent SNP's Homozygosis'



Homozygotes represented by a 2bp base changes are differentiated by a difference in Tm by about 0.3deg

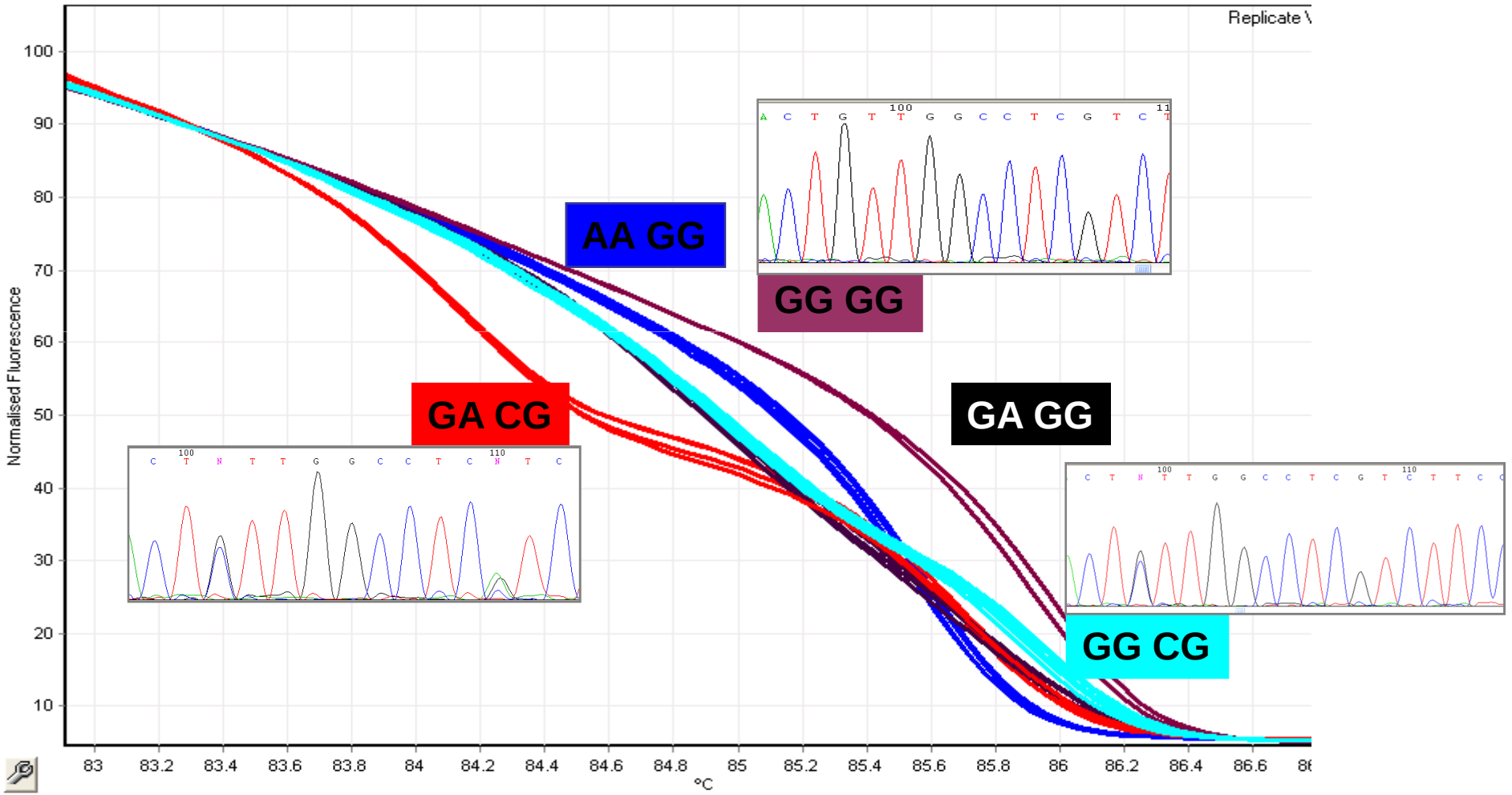
Data Courtesy of Howard Florey Institute, J.Rubio, C. Jenson

Adjacent SNP's Heterozygous



Heterozygotes form heteroduplexes, which result in lower melt curves and multiple transition points

Nomalised Data



Sequences were performed directly from HRM product, variants are 10bp apart

Complex Melts -HLA Compatability

Relationship	04	11
Patient	●	●
Maternal Aunt 1	●	●
Paternal Aunt		●
Maternal Aunt 2		●

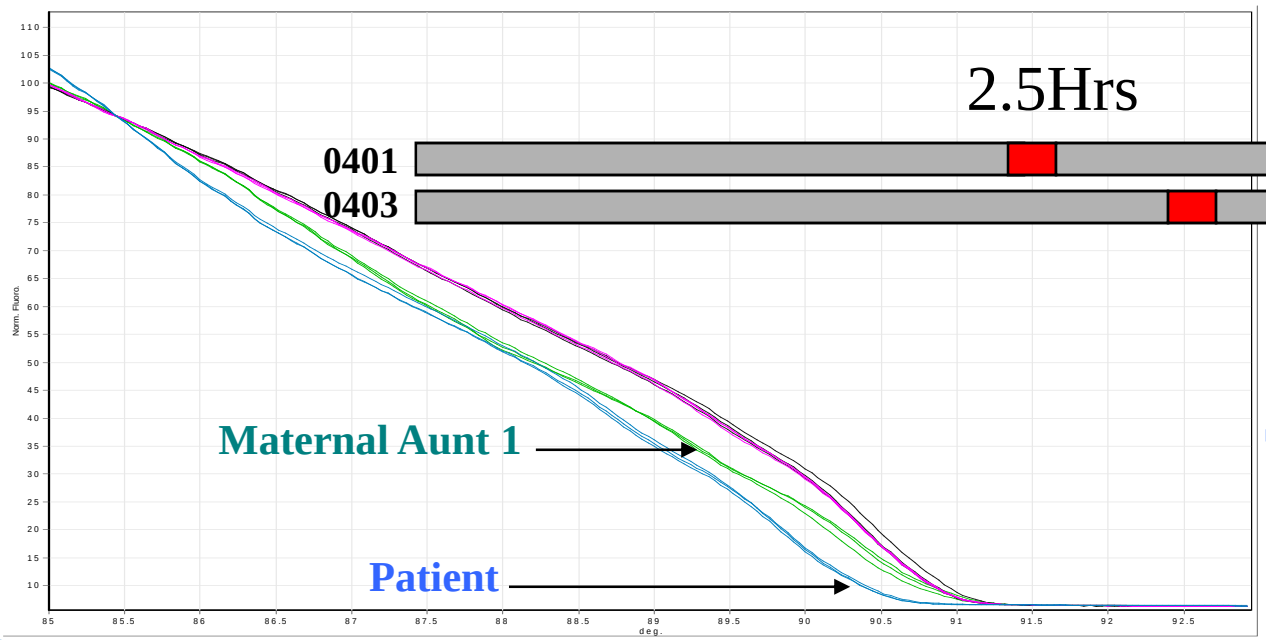
2 days

→ 20 probes

} SS sequencing →

04	11
0403	1143
0401	1104
	X
	X

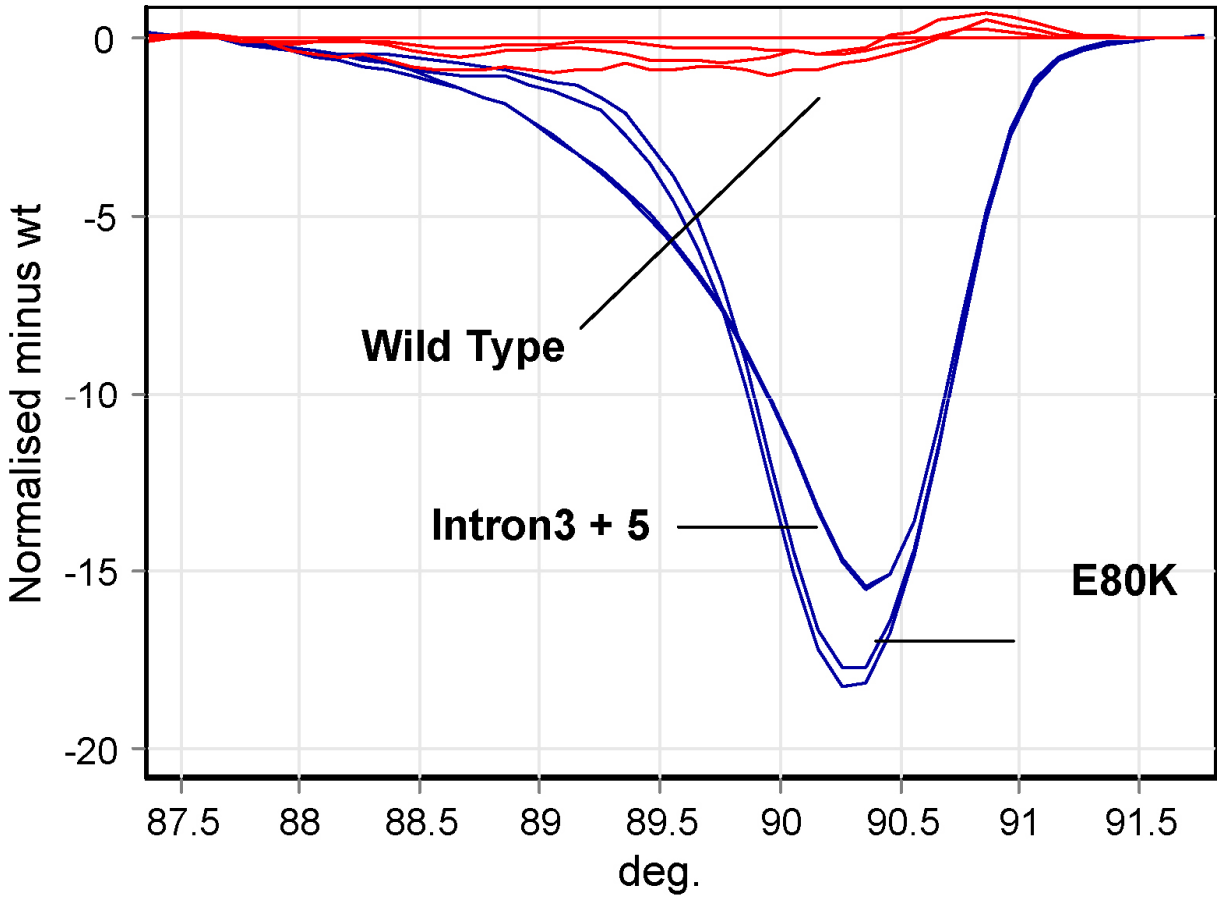
2 days



→ No match

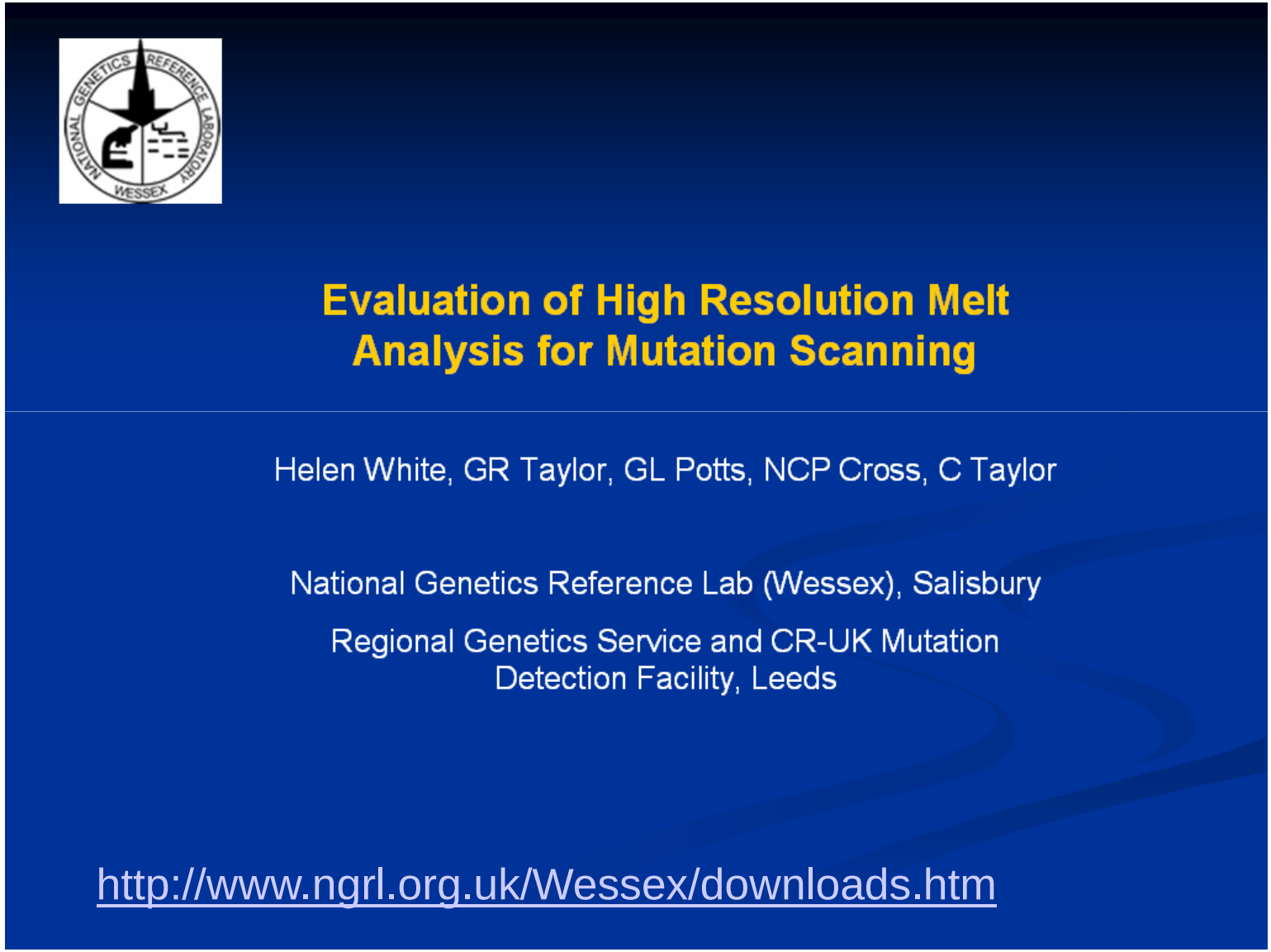
Mutation Detection

Genomic Mutations in LDLR gene- 23 fragments

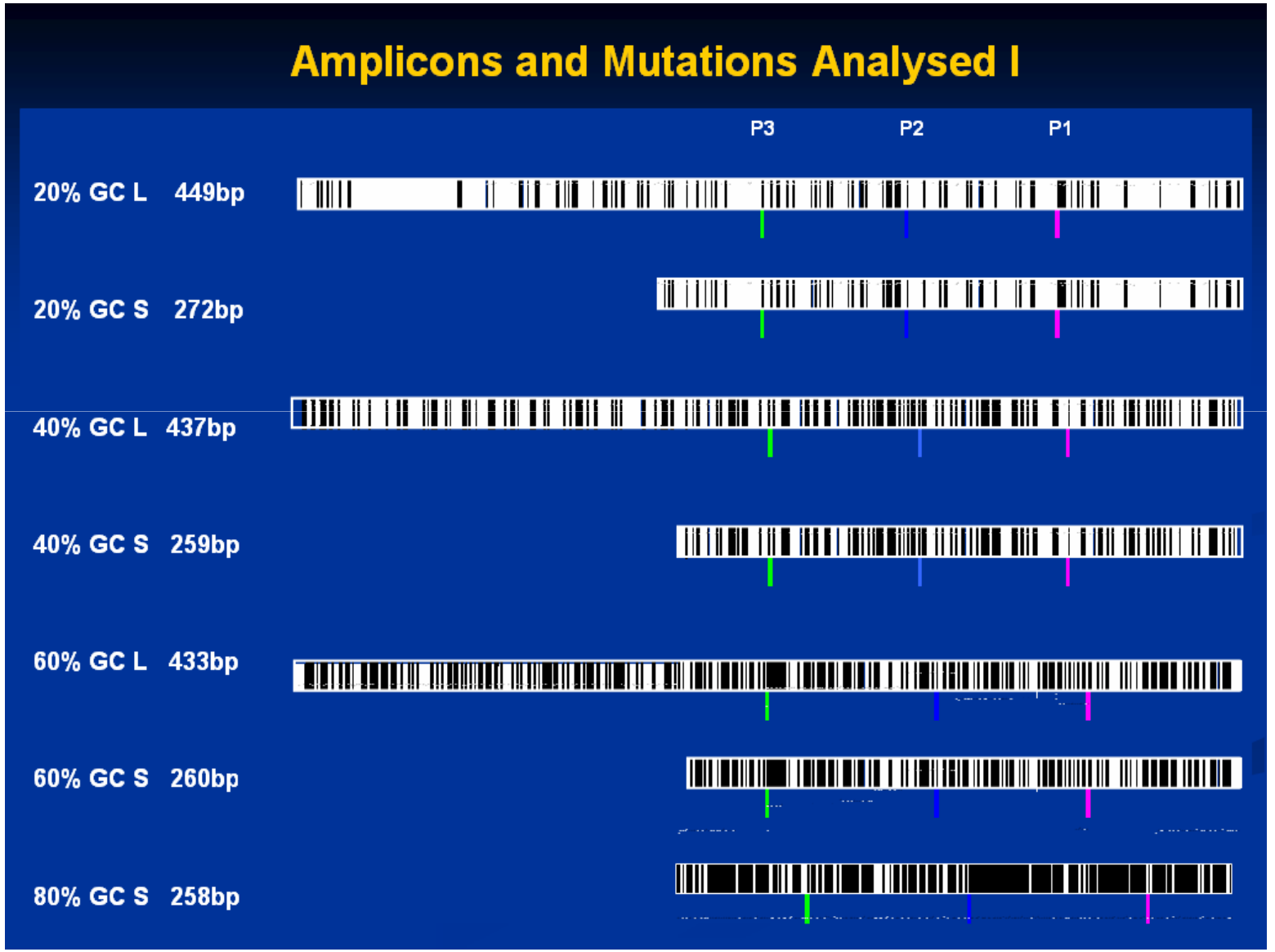


Atherosclerosis 194 (2007) 279–286. **Development of an affordable, sensitive and rapid screening method for mutation detection in UK FH subjects** C Hubbart, R Whittall, M Scartezini, S Humphries

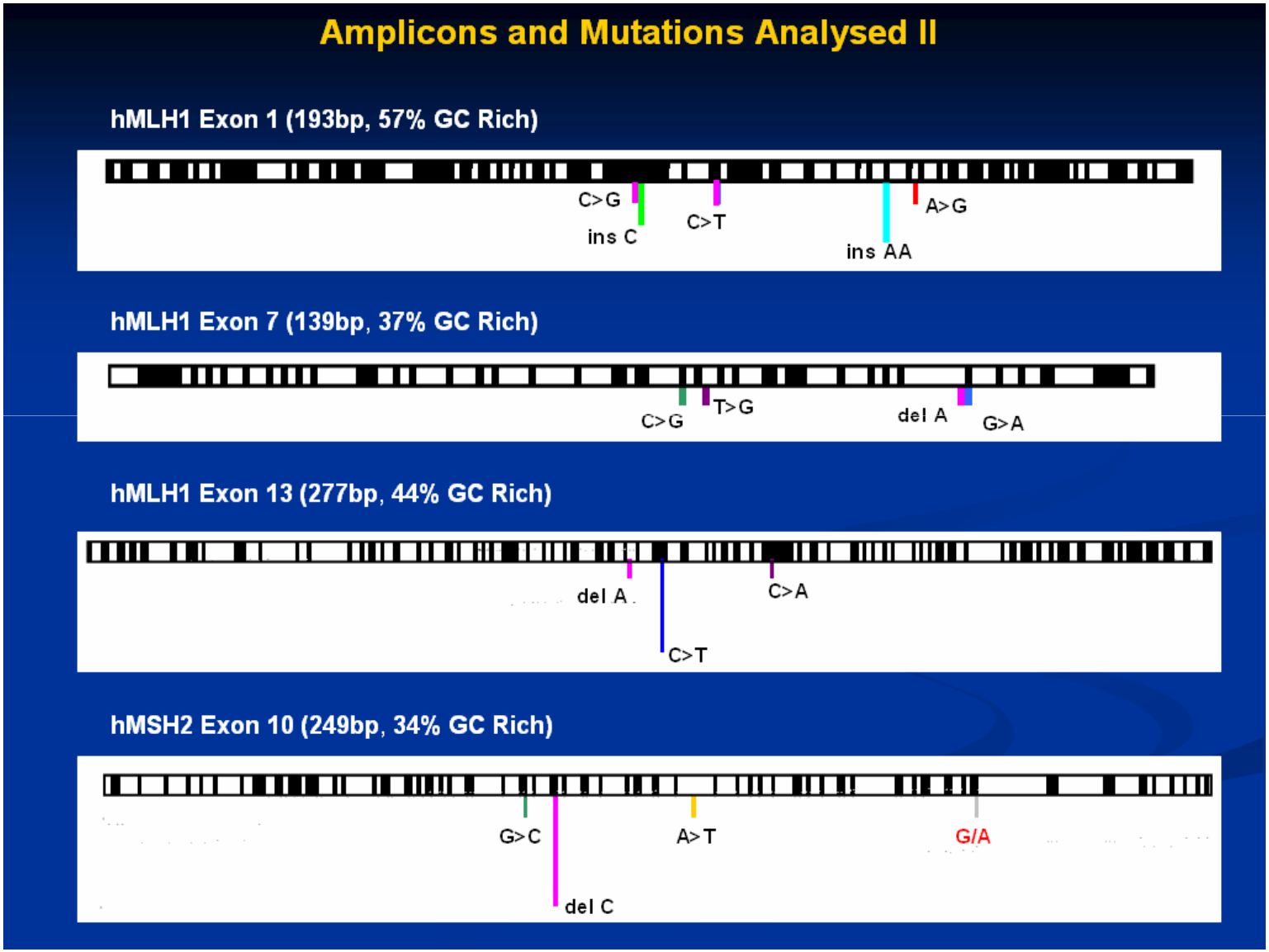
White *et al.* 2006 report



White *et al.* 2006 report



White *et al.* 2006 report



White *et al.* 2006 report

Summary

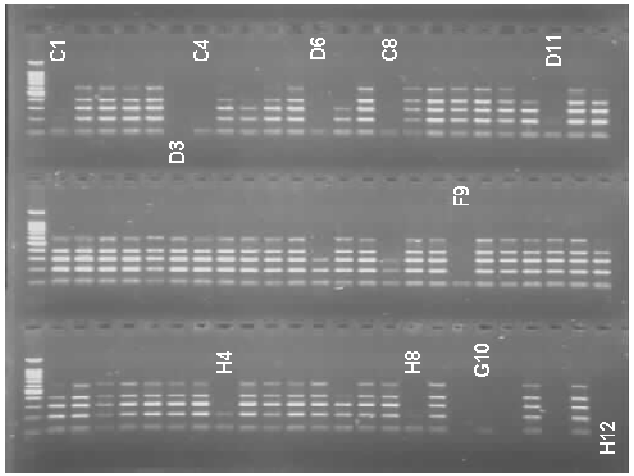
- 624 samples analysed in total
- 11 amplicons tested: 139bp – 449bp with GC contents of 22-79%
- Mutations included all possible point mutation base substitutions and 1 and 2bp insertions and deletions
- The same PCR reaction was analysed using the HR-1 and 384 well LightScanner (Idaho Technology) and RotorGene 6000 (Corbett Research)

Sensitivity and Specificity of mutation detection for each platform were:

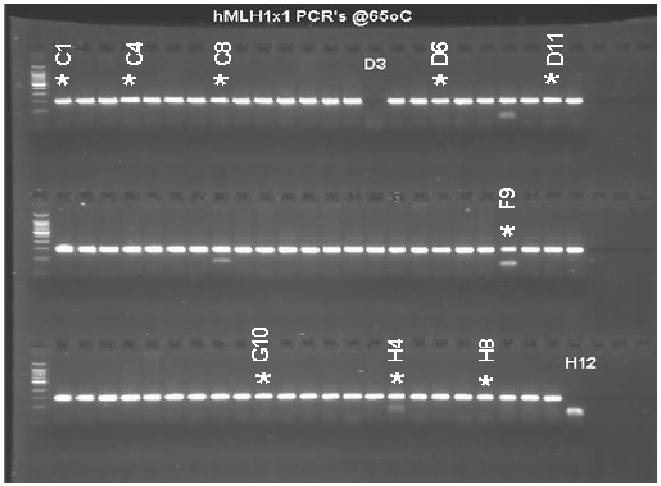
	Sensitivity	Specificity
RotorGene 6000	100.0	95.3
HR-1	98.4	95.0
LightScanner 384 well (High)	99.0	88.0
LightScanner 384 well (Normal)	83.9	95.3

DNA Quality—late amplification

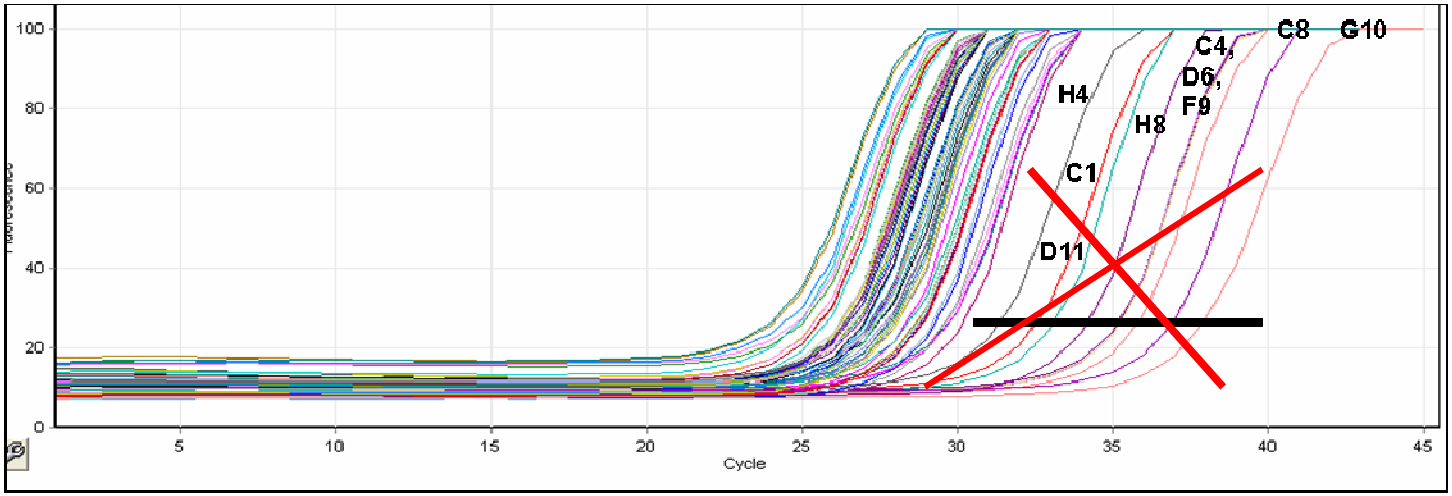
White *et al.* 2006 report



DNA quality-Multiplex 100, 200, 300, 400 and 600 bp product



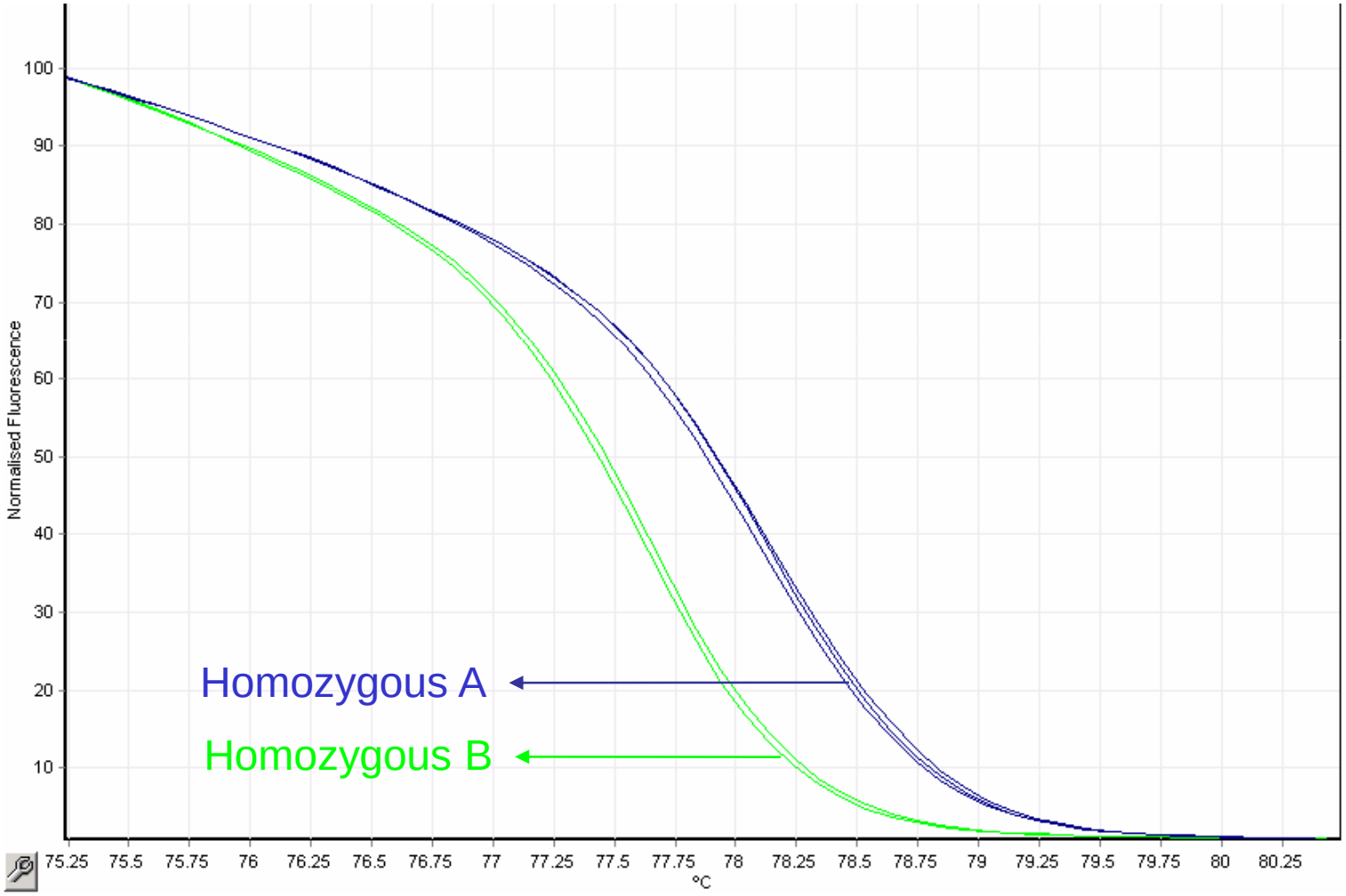
Amplification of 193 bp product



Sensitivity

“Spiking” Experiments

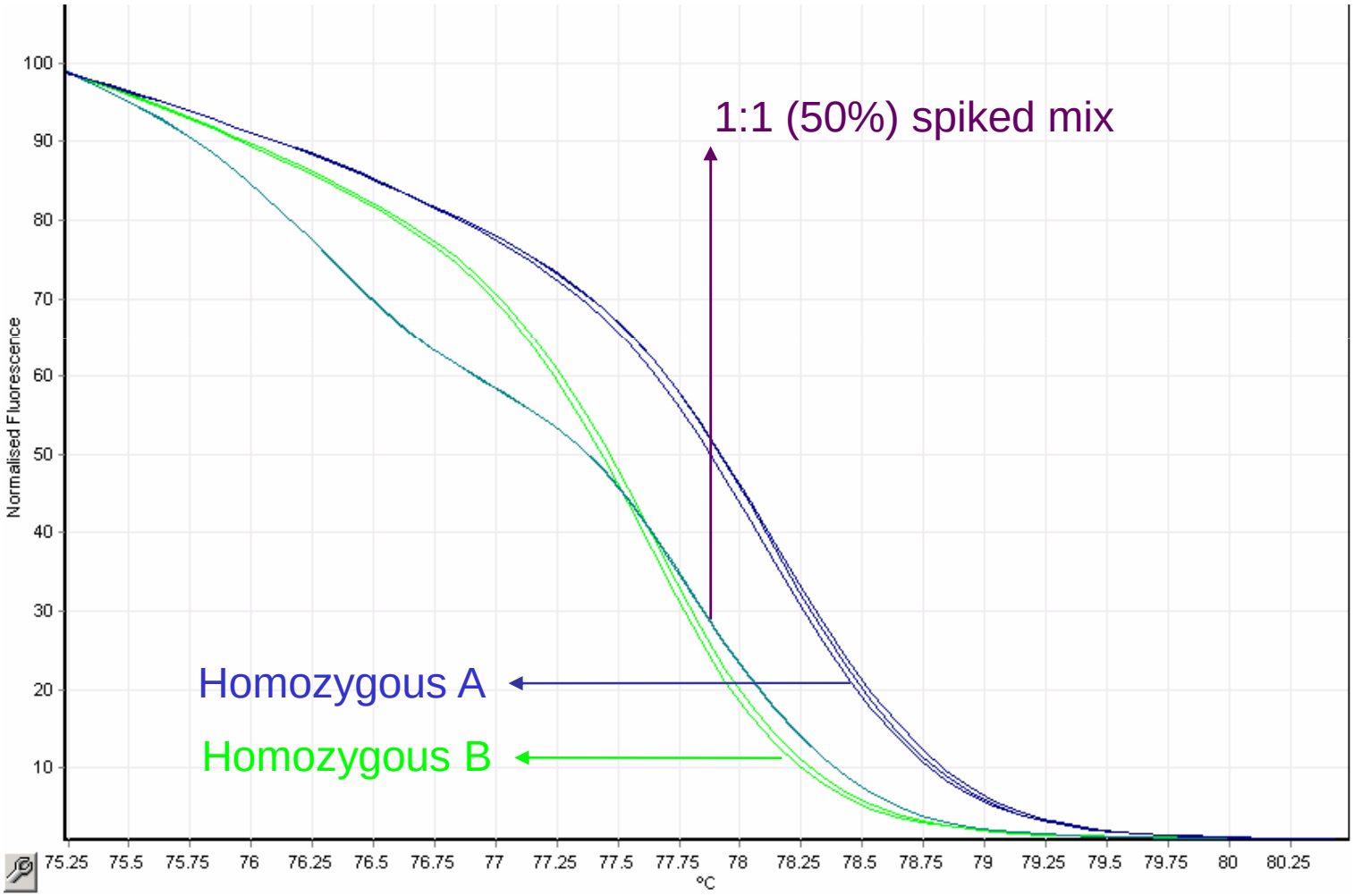
-applicable for acquired mutations and pooling samples



Royal Melbourne Hospital

“Spiking” Experiments

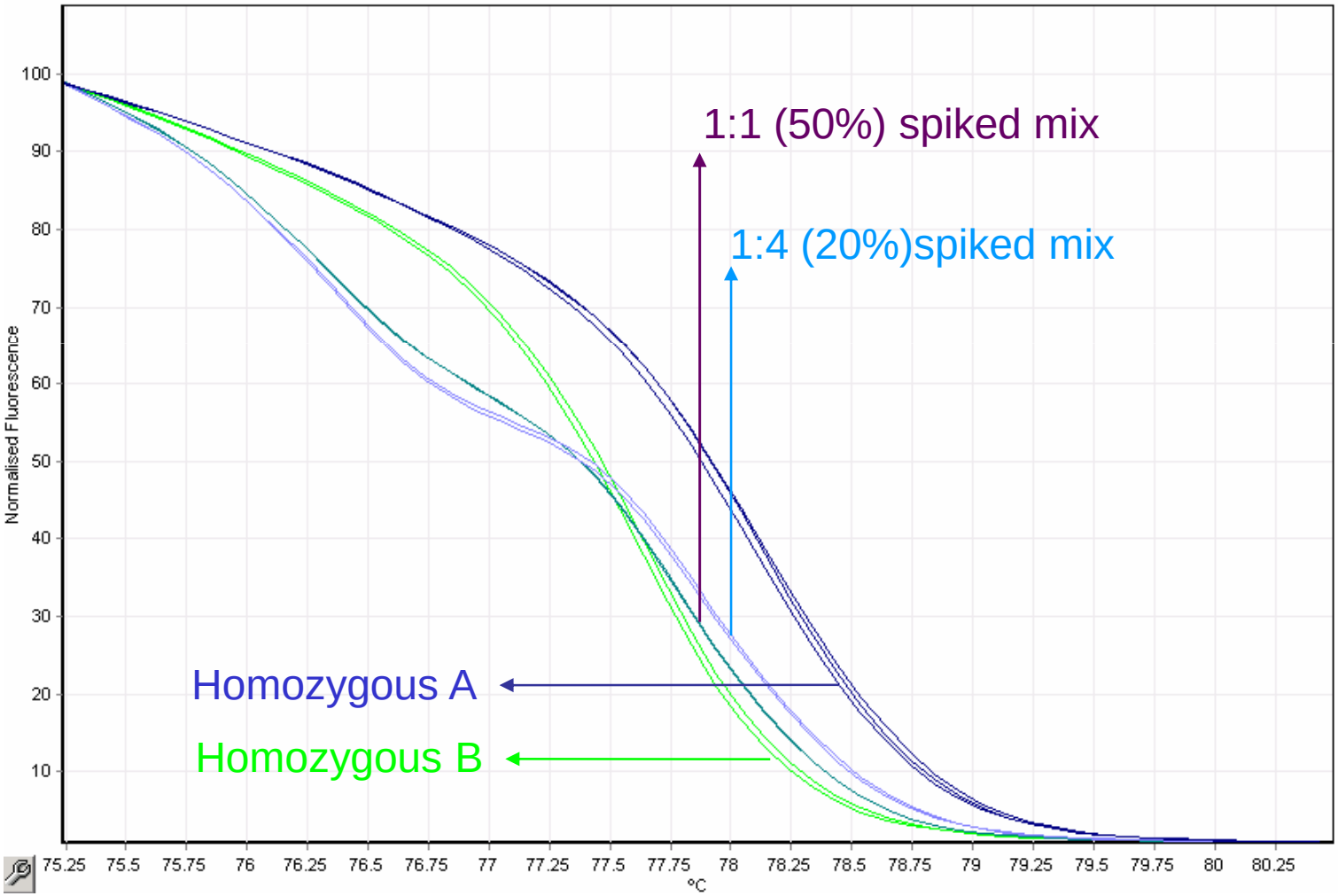
-applicable for acquired mutations and pooling samples



Royal Melbourne Hospital

“Spiking” Experiments

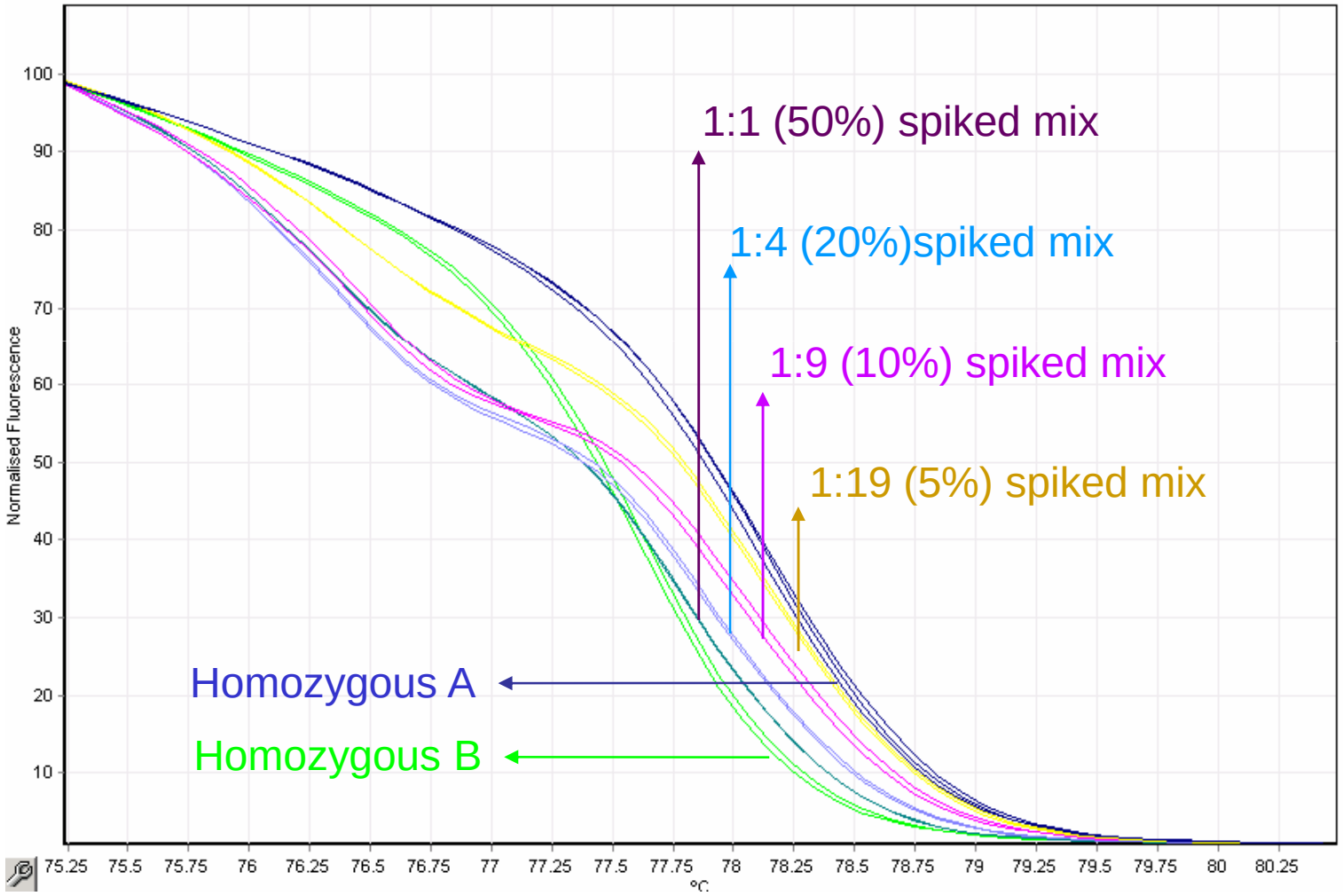
-applicable for acquired mutations and pooling samples



Royal Melbourne Hospital

“Spiking” Experiments

-applicable for acquired mutations and pooling samples



Courtesy Royal Melbourne Hospital-Hematology

Acquired Mutation Detection

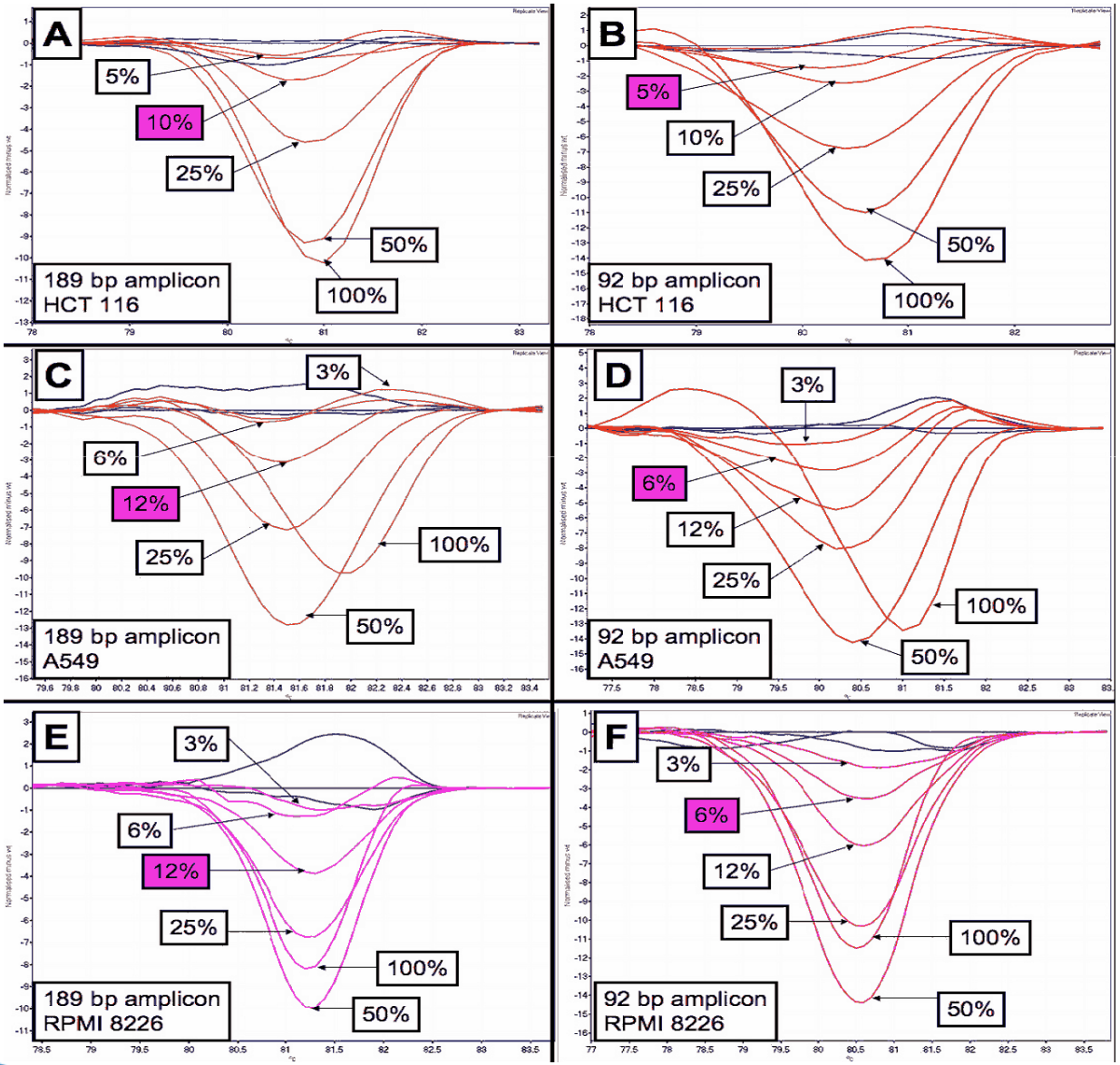
High resolution melting for mutation scanning of *TP53* exons 5-8
M Krypuy, A Ahmed, D Etemadmoghadam, S Hyland, Australian Ovarian Cancer Study Group, J Brenton, S Fox, A deFazio, D Bowtell and A Dobrovic *BMC Cancer* 2007, 7:168

High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer
M.Krypuy, G.Newnham, D.Thomas, M.Conron, A.Dobrovic *BMC Cancer. 2006 Dec 21;6(1):295*

Detection of the transforming AKT1 mutation E17K in non-small cell lung cancer by high resolution melting . H Do, B.Solomon, P.Mitchell, S.Fox ,A.Dobrovic *BMC Research Notes 2008, 1:14*

Sensitivity

Krypuy *et al.* 2006 report



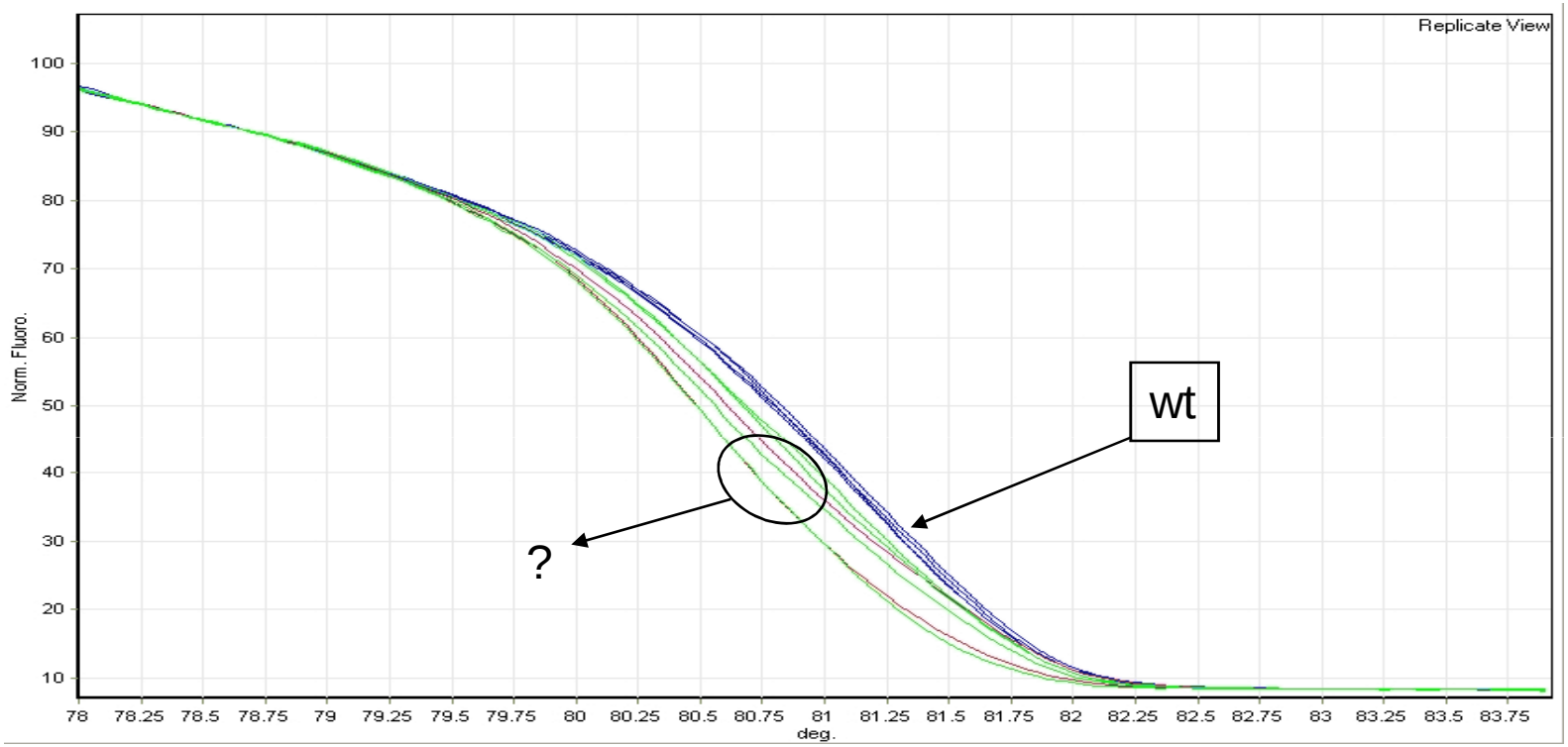
38 G>A
Heterozygous
92bp 5% cellular ratio
1:19 dilution
Represents 2.5% allele ratio
1 out of 39 alleles

34 G>A
Homozygous

35 G>C
hypotriploid
possible 2:1 mutant

Somatic Mutation Discovery KRAS

Exon 2 189 bp product 37% GC content-

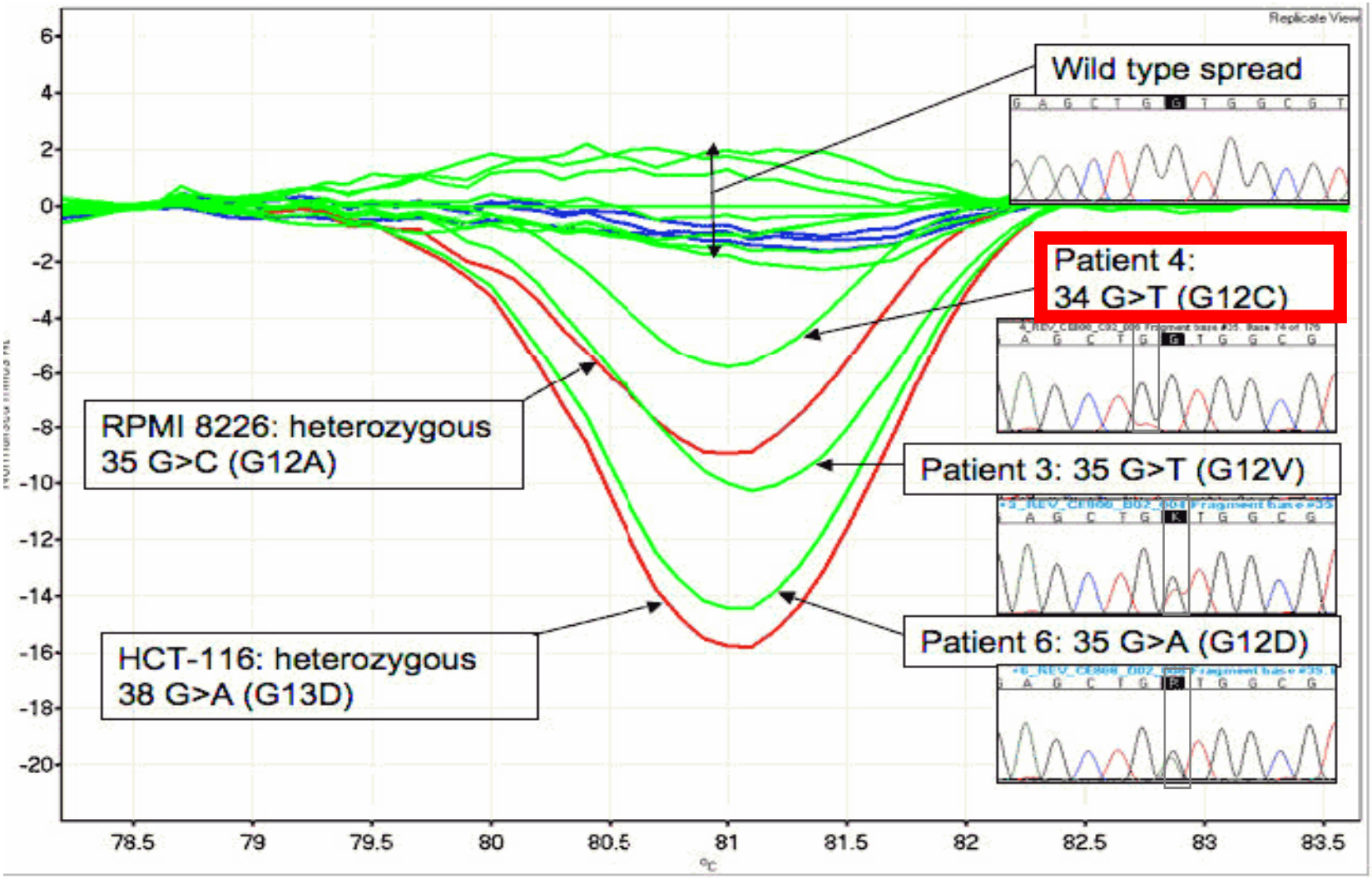


- Detected small quantities of mutant DNA in a background of wildtype DNA
- Confirmed 9/30 positive clinical samples
- No homozygous spiking necessary
- The heteroduplex shape is not used to determine differences, rather the deviation from wildtype, as the shape is compromised due to the proportion of normal DNA

Peter MacCallum Cancer Centre Melbourne

Difference Graph

Krypuy *et al.* 2006 report



This paper concludes that HRM will allow

"*confident screening for mutations in samples that have at least 10% tumor cells*"

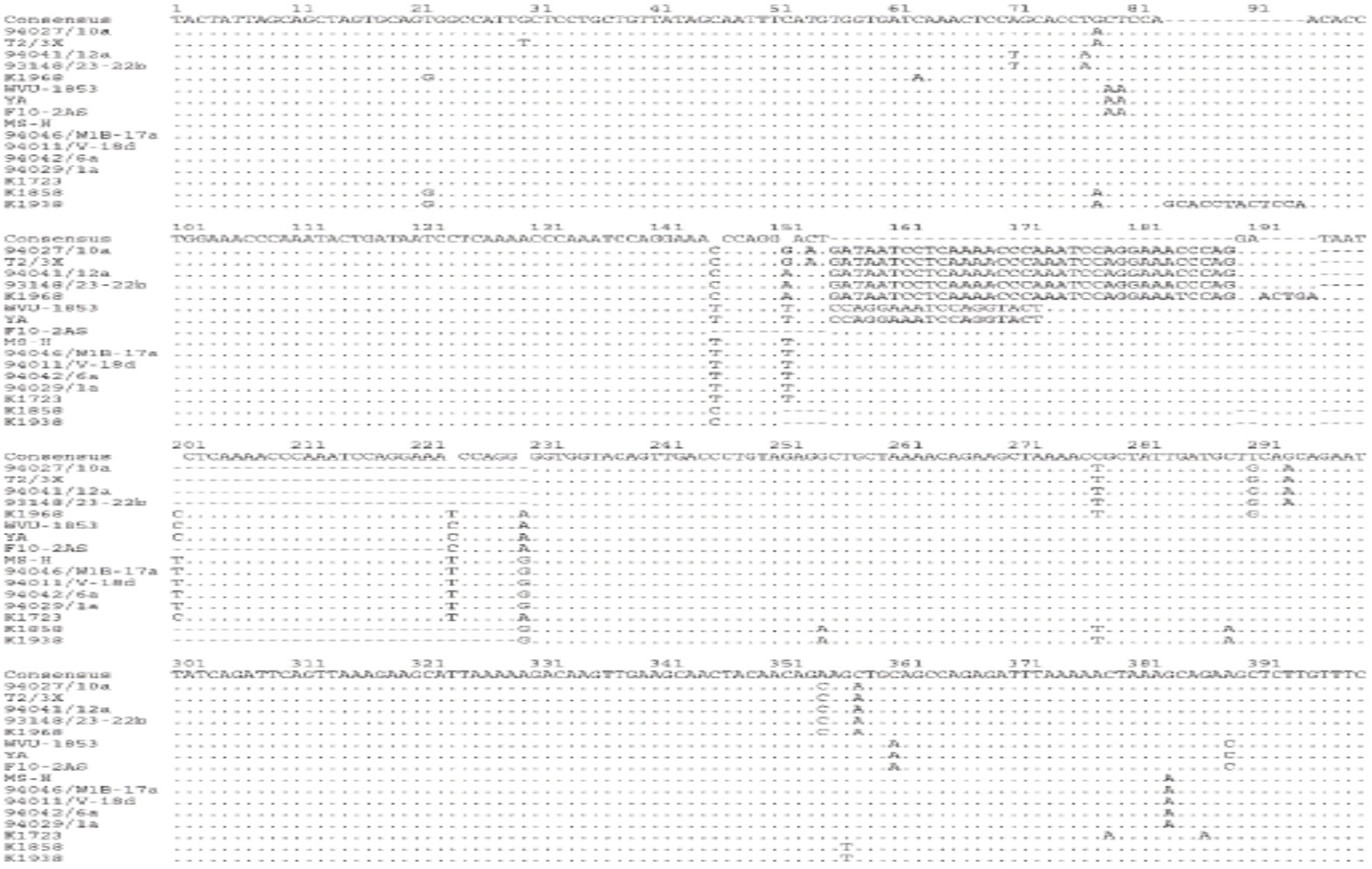
Krypuy *et al.* 2006 report

Species Identification

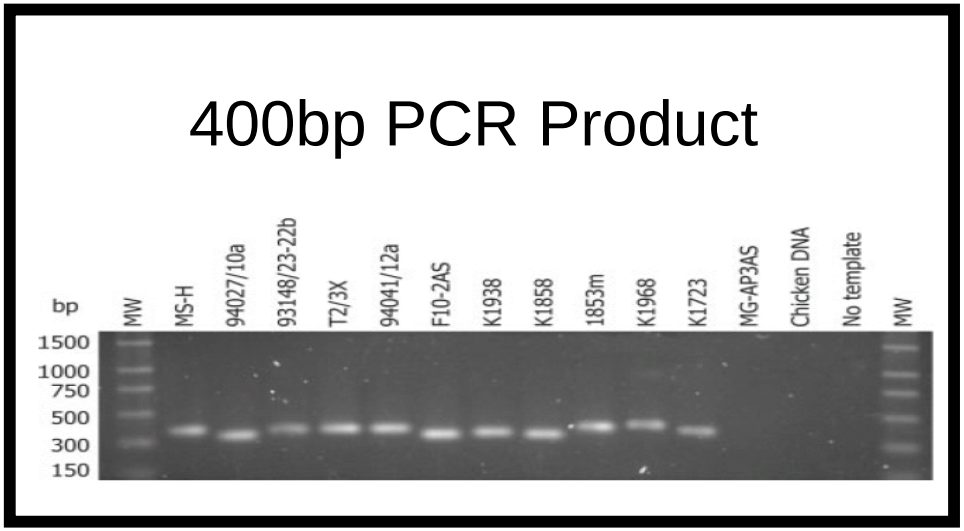
Classification of Mycoplasma synoviae strains using single-strand conformation polymorphism and high-resolution melting-curve analysis of the vlhA gene single-copy region Nathan Jeffery, Robin B. Gasser, Penelope A. Steer and Amir H. Noormoham ***Appl Environ Microbiol. 2007; 73: 3431-3436***

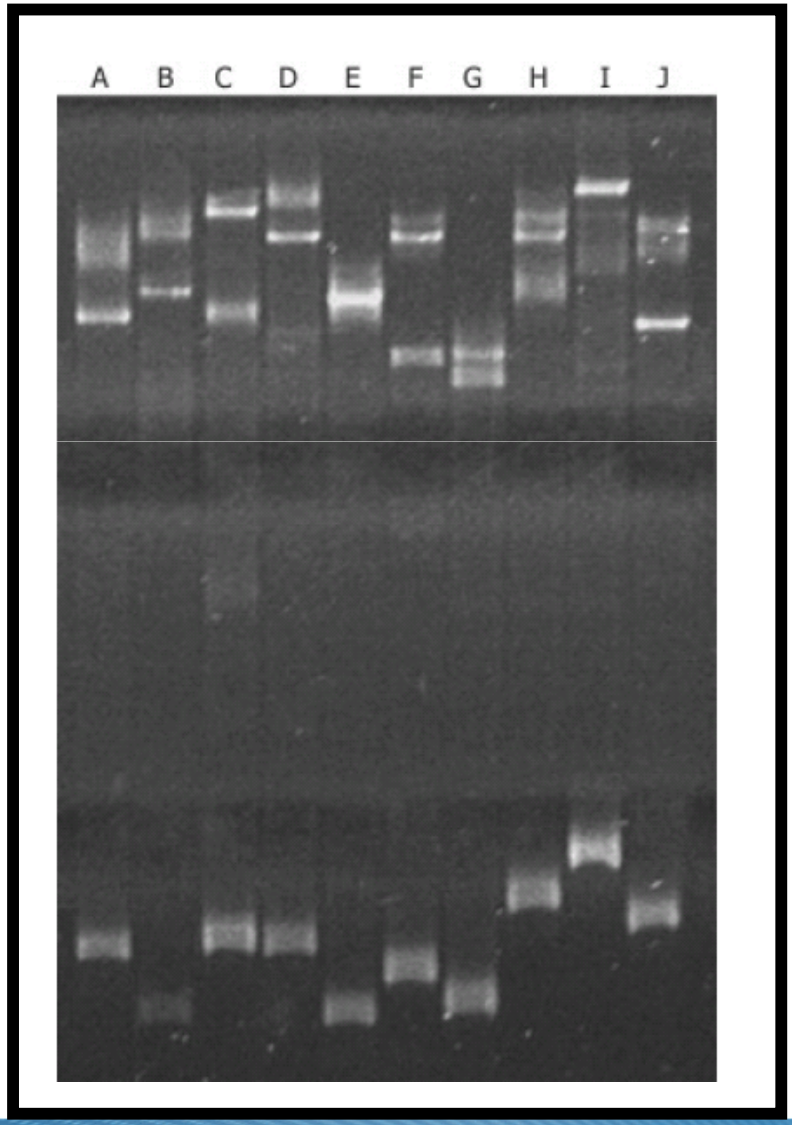
High-Resolution DNA curve analysis of the clustered regularly interspaced short palindronic-repeat locus of Campylobacter jejuni. Price EP, Smith H, Huygens F, Giffard **PM *Microbiology (2007), 153, 2679–2688***

Strain Sequence Alignment

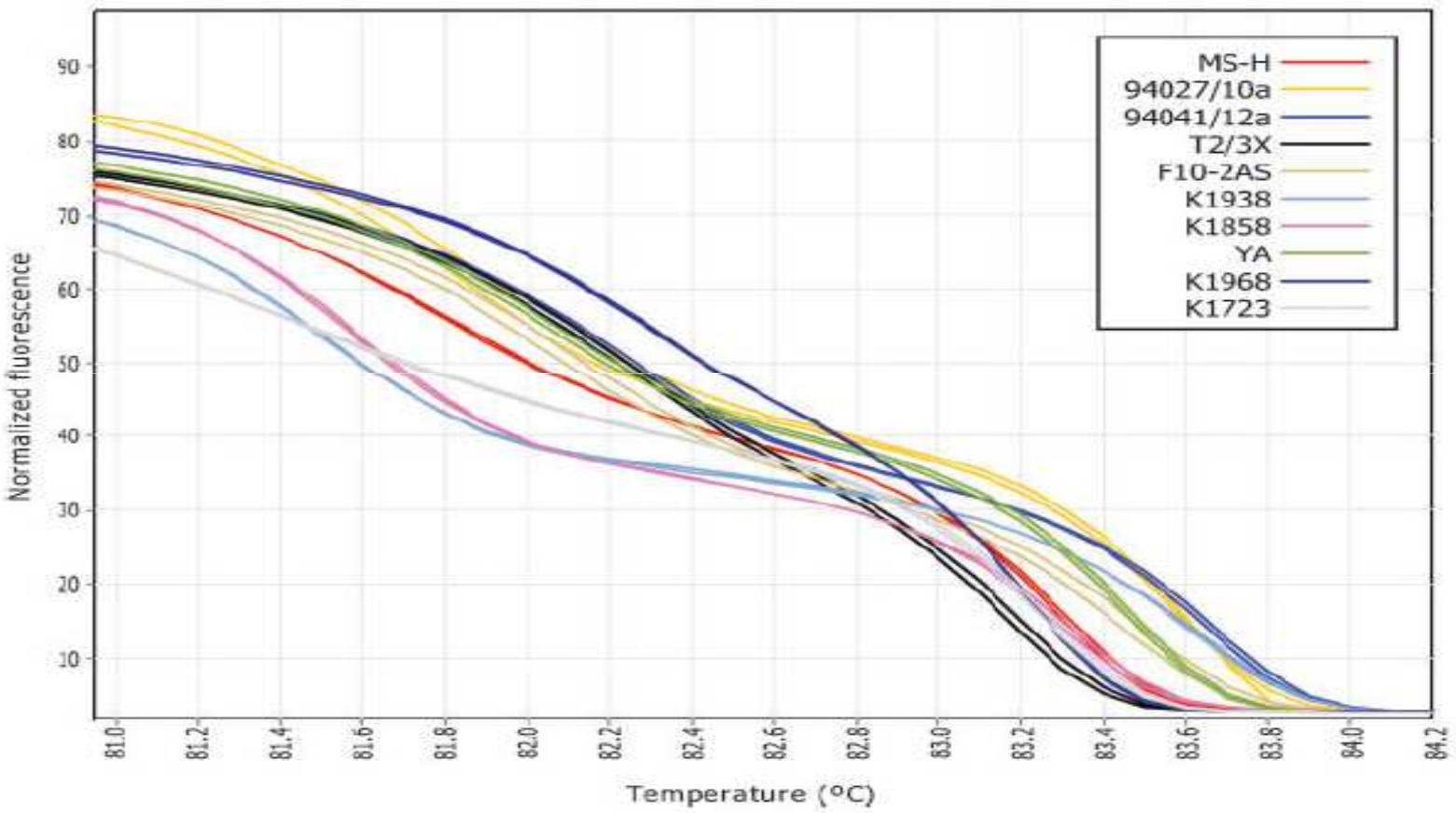


SSCP


400bp PCR Product
bp
MW
MS-H
94027/10a
93148/23-22b
T2/3X
94041/12a
F10-2AS
K1938
K1858
1853m
K1968
K1723
MG-AP3AS
Chicken DNA
No template
MW
1500
1000
750
500
300
150


A
B
C
D
E
F
G
H
I
J

Normalised HRM



~400 bp fragment, 35 different M. synoviae isolates

Epigenetics

Methylation-sensitive high resolution melting (MS-HRM): A new approach for sensitive and high-throughput assessment of methylation.

Tomasz K. Wojdacz, Alexander Dobrovic

Nucleic Acids Research Feb 2007

"***The simplicity and high reproducibility…***
…makes MS-HRM the method of choice"

What is methylation?

- Addition of a methyl group to cytosine DNA in CpG island
- Bisulphate treatment converts non methylated cytosine bases to uracil and methylated remain unaffected
- Uracil converted to thymine by PCR

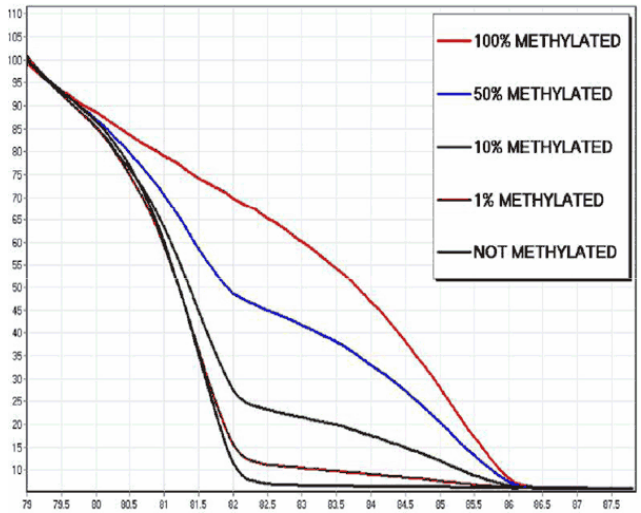
Non methylated	GACATCG → GATATTG
Methylated	GACAT^{m}CG → GATATCG

- Methylation acts to “turn off” transcription
- Markers for; Early events in cancer
  Monitoring progression
  MGMT predictive markers treatments

MGMT MS-HRM

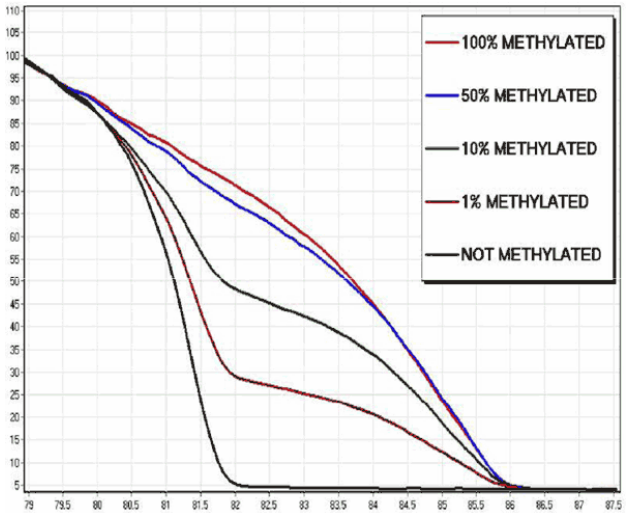
Wojdacz *et al.* 2007 report

Effect of annealing temperature on sensitivity

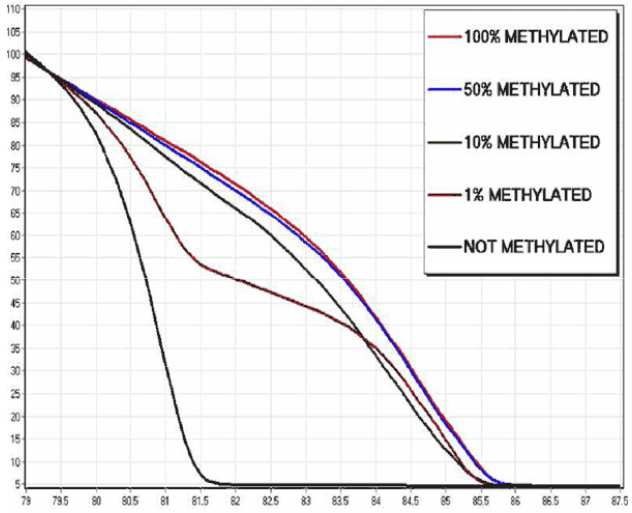


60℃

Discrimination at higher end



62℃



63℃

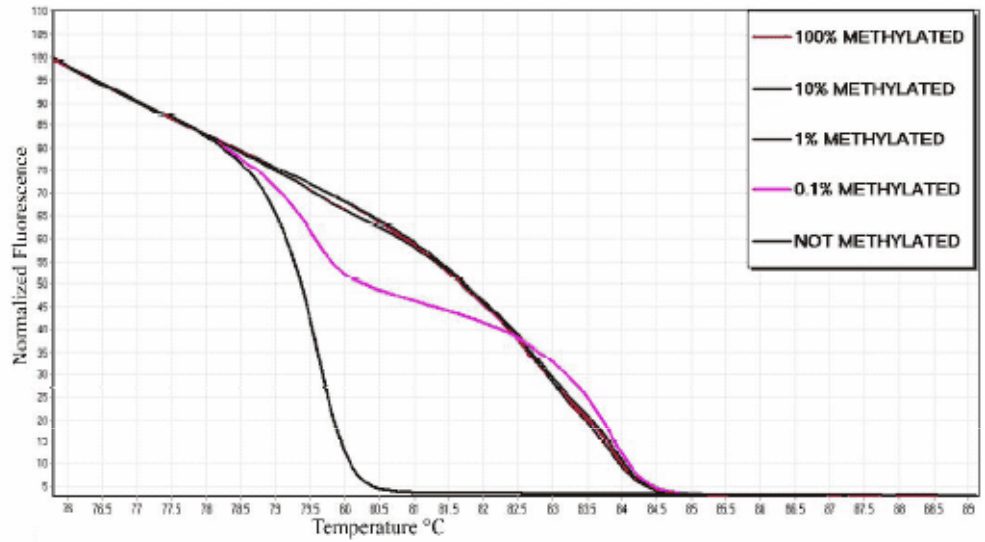
Discrimination at lower end

increasing temperature

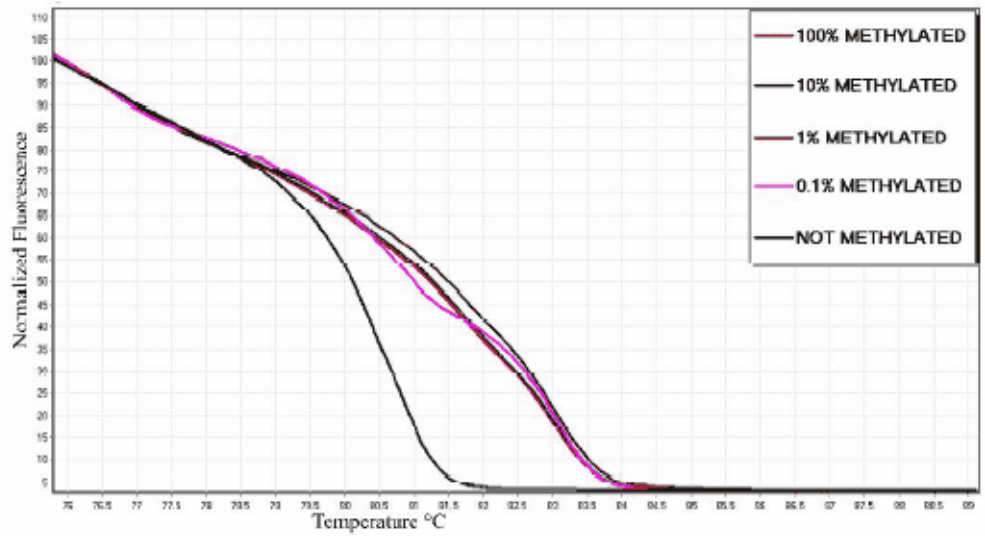
Low level methylation
0.1% MGMT sensitivity MS-HRM

Wojdacz *et al.* 2007 report

12 CpG's/ 109bp

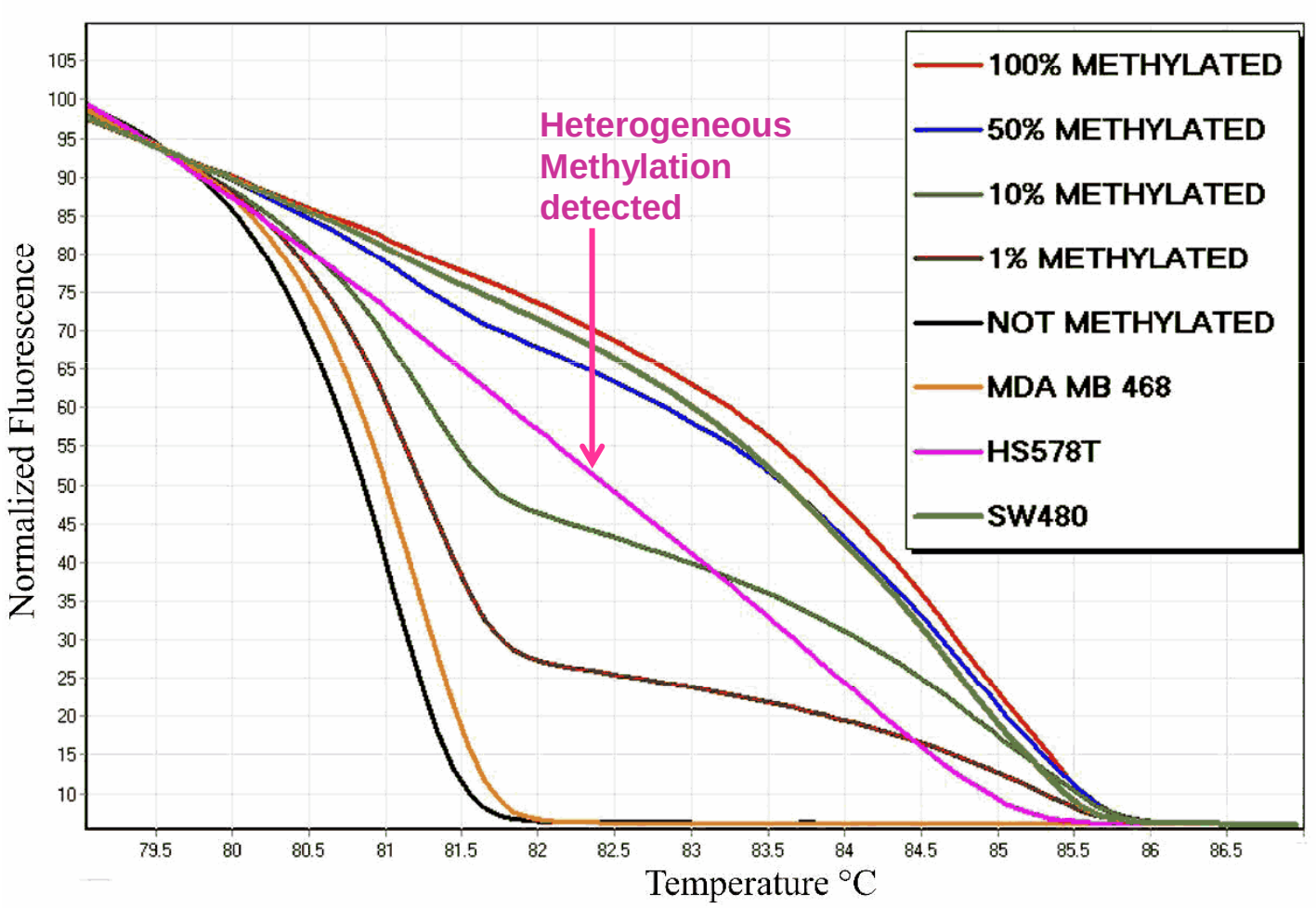


5CpG's/ 94bp



Heterogeneous methylation

Wojdacz *et al.* 2007 report



MS-HRM is a sensitive, reliable,

fast and cost effective

"***…method of choice***"

Wojdacz *et al.* 2007 report

Genomic Imprinting

Rapid detection of methylation change at H19 in human imprinting disorders using methylation-sensitive high-resolution melting.
Wojdacz TK, Dobrovic A, Algar EM. *Hum Mutat. 2008 May 12*

Methylation-Sensitive High-Resolution Melting-Curve Analysis of the SNRPN Gene as a Diagnostic Screen for Prader-Willi and Angelman Syndromes.H White, V Hall, and N Cross.*Clinical chemistry September 2007*

Promoter Methylation

BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1 tumour phenotype. C Snell, M Krypuy, E Wong, M Loughrey, A Dobrovic
Breast Cancer Research 2008

Applications

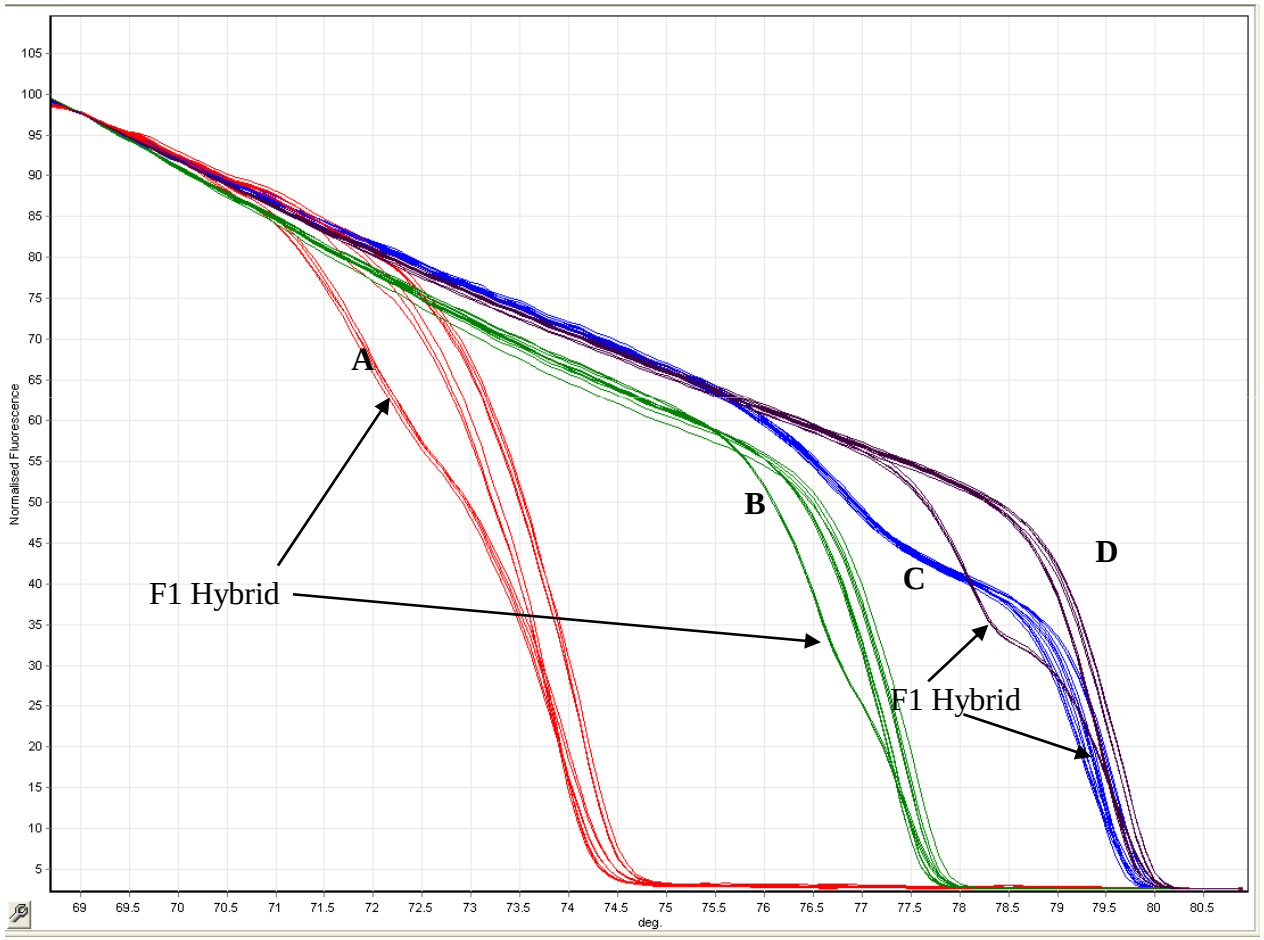
- Detect small quantities of mutant DNA in background of wildtype DNA species
- Important in somatically acquired mutations
- Pooling samples—up to 10 samples
- Simple for diseases that cause no heterogeneity-like Factor V Leiden, haemochromotosis, sickle cell anemia, etc
- Appropriate for disorders where the mutational spectrum is wide—mismatch repair genes
- Newly identified genes—little information
- Species identification
- Epigenetics

www.corbettlifescience.com

corbett
LIFE SCIENCE

www.corbettlifescience.com

Singleplex HRM



Multiplex HRM