High Resolution Melts HRM

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Presented by Jennifer McMahon

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High Resolution Melts

Analysis of change in fluorescence as a PCR product is melted

- PCR amplify an amplicon with two primers and an intercalation dye and then melt the product – double stranded to single stranded
- Detect difference between a single base pair change.

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High Resolution Melt Specifications

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

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Rotor-Gene™ 6000

the world's only real-time rotary thermo-optical analyser with HRM capabilities

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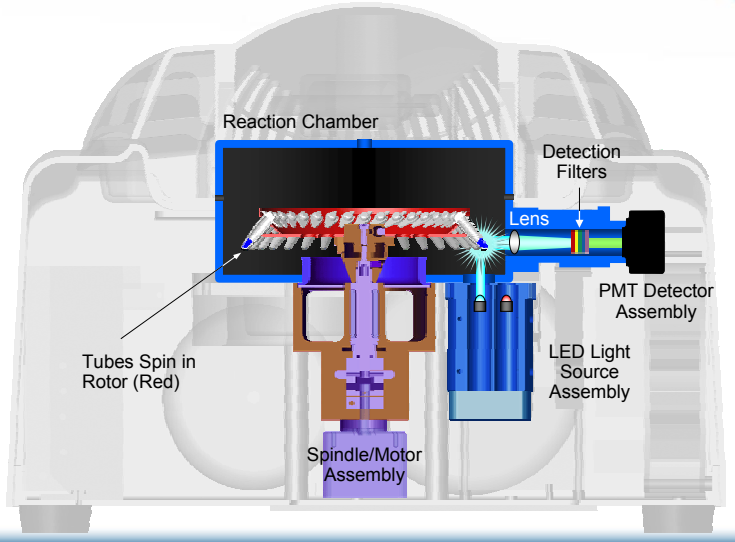
Rotor-Gene™ 6000

Cross-section of rotary optics



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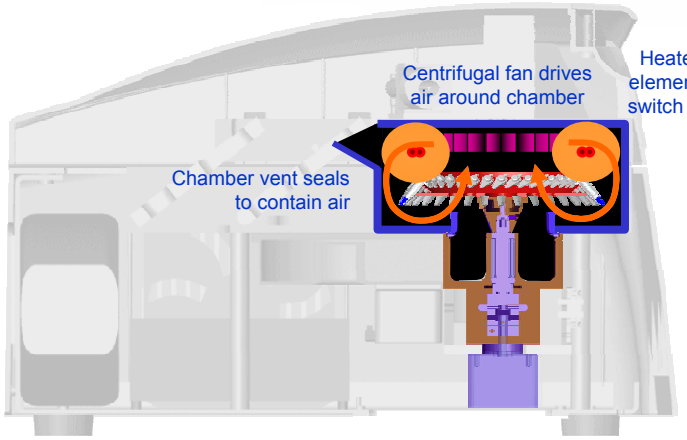
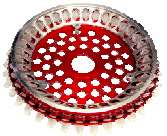
THERMAL UNIFORMITY-

-/+0.01C

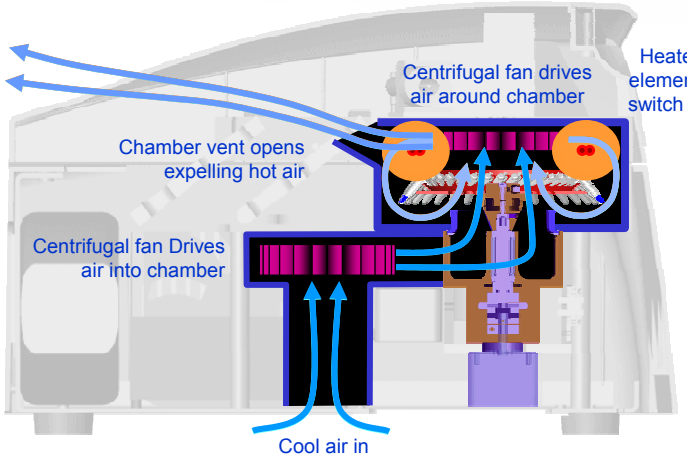
Rotor-Gene™ 6000
Heating mechanism

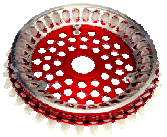




Note: holes in the rotor allow free airflow

Rotor-Gene™ 6000
Cooling mechanism





Note: holes in the rotor allow free airflow

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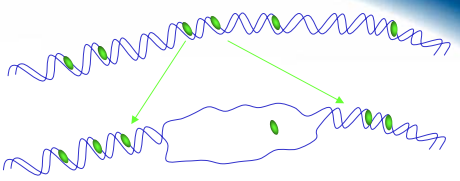
Intercalation Chemistries

SYBR™ Green I is toxic to PCR, so concentration used is very low

Unsaturated binding allows dye to relocate as melting begins

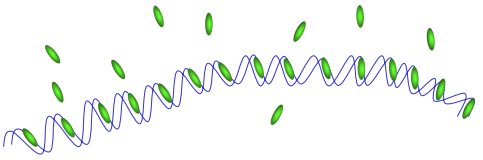


SYBR® Green I

Saturating dye technology for HRM -LCGreen™ I, EVA Green, Syto 9

Saturation dyes are less toxic, so concentration used can be high enough to allow all sites to be saturated

Saturation eliminates potential for dye relocation-ideal for HRM



LC Green™ I

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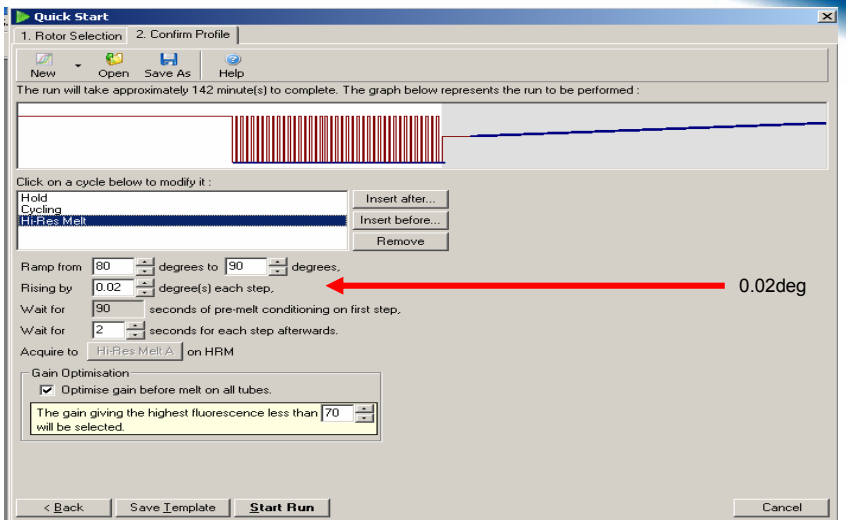
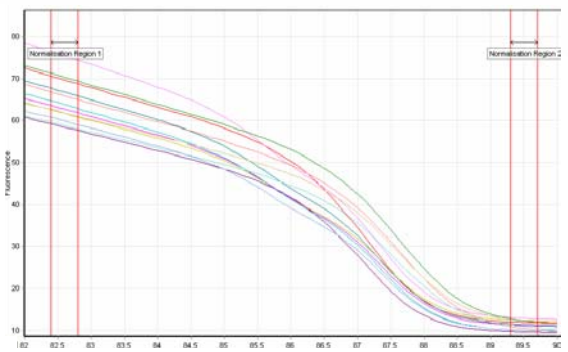
Setting up a Reaction

- Use standard PCR conditions as a starting point, typically 250nM primer, 1.5mM Magnesium chloride, 0.2mM dNTPs, 1.25 U Platinum Taq, 1.5µM SYTO 9, 50ng DNA
- Don't generally usually use real-time mix – decreases cost per assay
- Set up cycling and add HRM step at the end
- HRM step typically 0.1°C steps over 10 °C, HRM step takes around 20 minutes

HRM Profile



Data Acquisition



- Melting curves-normalized by selecting linear regions before and after the melting transition
- Two regions defined-upper 100% double stranded and lower single stranded baseline

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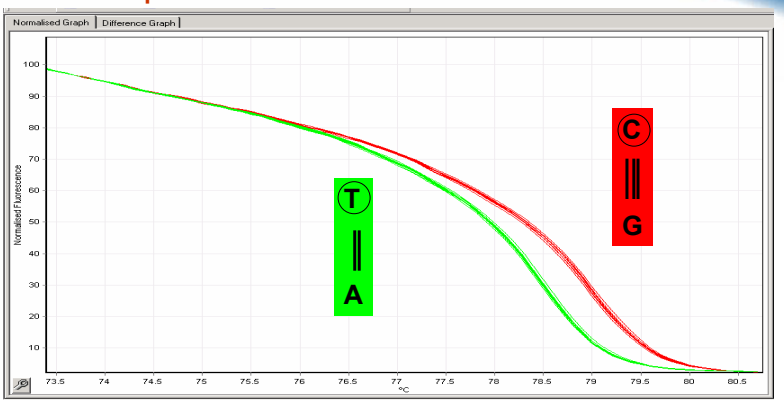
Homoduplexes C or T



Homozygotes represented by a single base change are differentiated by a difference in Tm melt.

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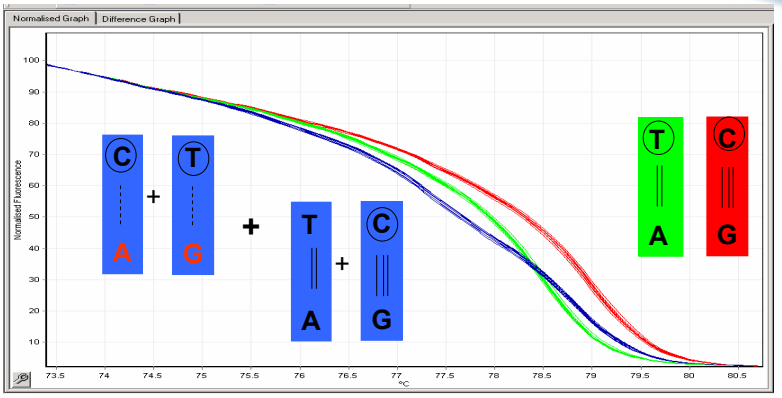
Heteroduplex C>T



Heterozygotes form heteroduplexes, the heterozygote (blue) trace is a mix of 4 duplexes

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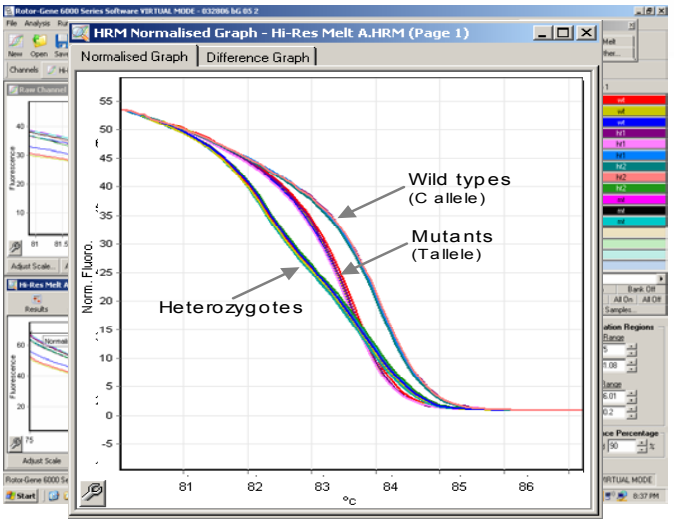
SOFTWARE: Normalised HRM data



- ACTN3 (R577X) (C—T).
- 10 replicates.
- 40 cycle fast (~34 min).

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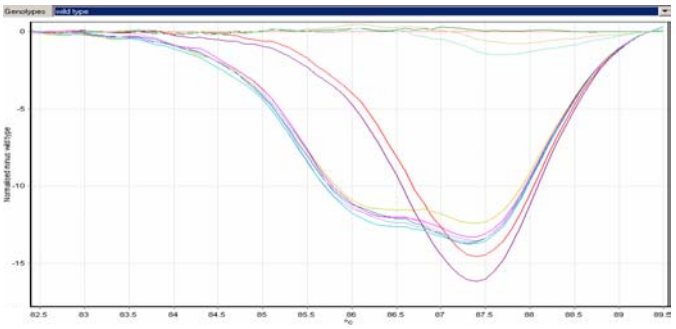
Difference Graphs



- Difference graph displays the difference between each sample and a given genotype control
- Allows a calculated percentage confidence relative to a known genotype

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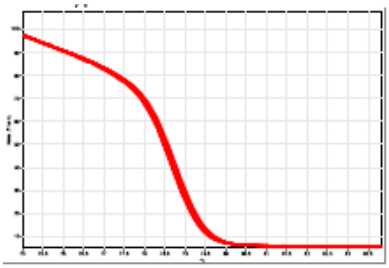
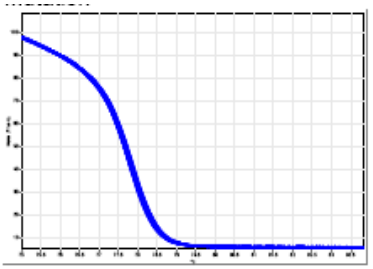
Confidence in HRM Results

Wildtype (72 Replicates)



Mean Tm 78.78 ±0.04%

Mutant (72 Replicates)



Mean Tm 77.90 ±0.04%

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Applications

- **SNP genotyping/ Allelic discrimination**
  - Identify Candidate Predisposition Genes
  - Association Studies-eg.comparing cases and controls, genotype to phenotype
  - Prevalence -within population or different sub groups
  - Loss of Heterozygosity
  - DNA fingerprinting
- **Mutation Discovery/Screening/Scanning**
- **Predictive Testing**
  - Penetrance/Linkage studies-variant track with disease within a family
- **Species Identification**

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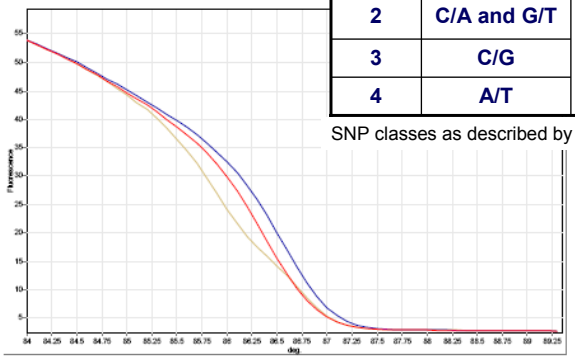
Genotyping
Class 4 SNP

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.

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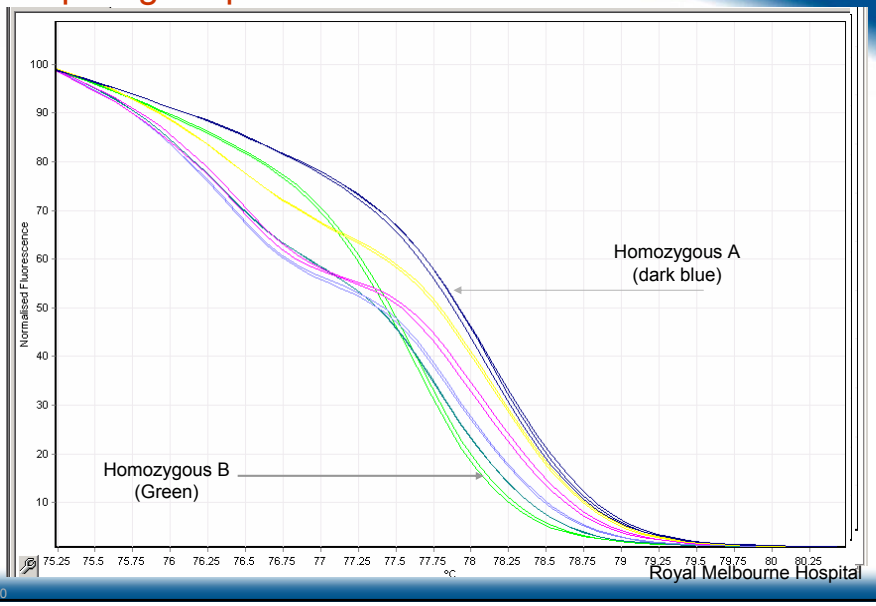
"Spiking" Experiments



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Tm Comparisons-Factor V G1691A

63bp **tctgaaaggttacttcaaggacaa**aatacctgtattccTtgcc**tgtccagggatctgctctta**

89bp **ggttacttcaaggacaaaatacctg**tattccTtgcctgtccagggatctgctcttacagattagaagta**gtcctattagcccagaggcg**

169bp **ttgaaggaaatgccccatta**tttagccaggagacctaacatgttctagccagaagaaattctcagaatttctgaaaggttacttcaaggac aaaatacctgattccTtgcctgtccagggatctgctcttacagattagaagtagtcc**tattagcccagaggcgatgt**

Amplicon size	Mutation	Wildtype	△Tm Homozygote's
63bp	77.50 ± 0.04	78.18 ± 0.01	0.68 ± 0.05
89bp	79.46 ± 0.02	80.03 ± 0.02	0.57 ± 0.04
169bp	81.49 ± 0.02	82.21 ± 0.02	0.72 ± 0.04

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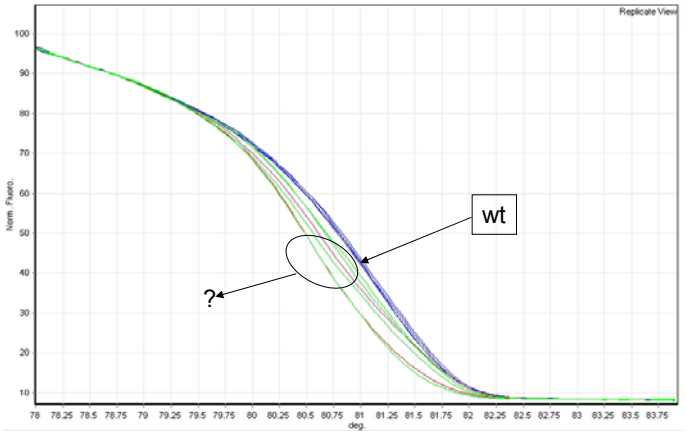
Sensitivity-Somatic Mutation Discovery

189 bp product 37% GC content



Detect small quantities of mutant DNA in a background of wildtype DNA species-sensitivity 5%
No homozygous spiking necessary

PMCI-Melbourne

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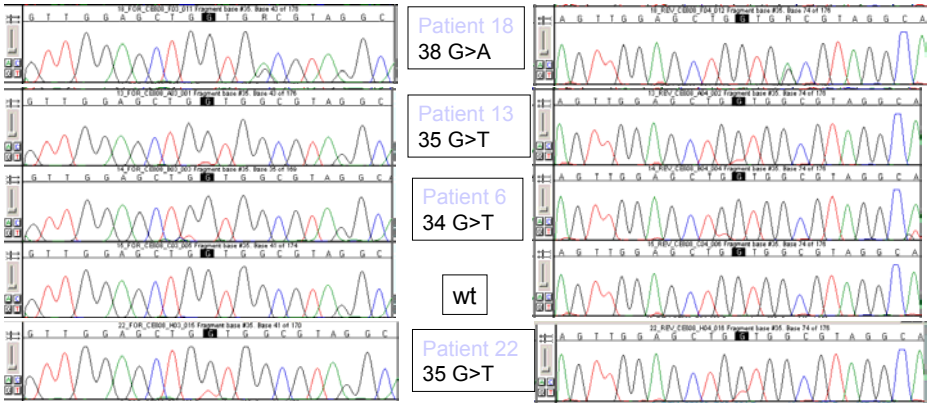
Sequencing –Somatic variants

Sequence directly of the product-product column purified and not consumed

Forward 3’ Reverse 5’



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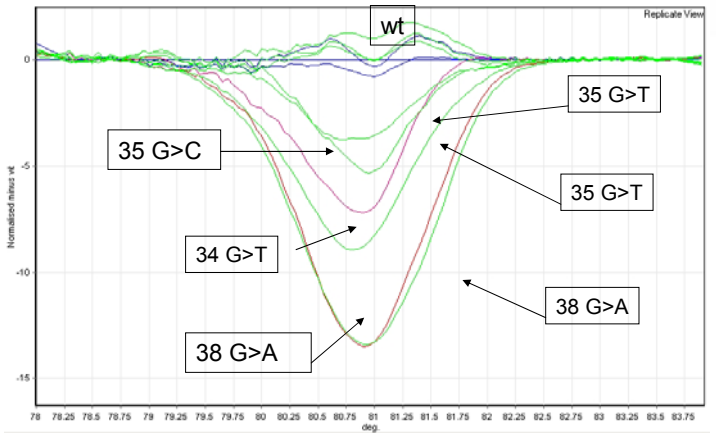
Difference Graph



White *et al.* 2006 report

Evaluation of High Resolution Melt Analysis for Mutation Scanning

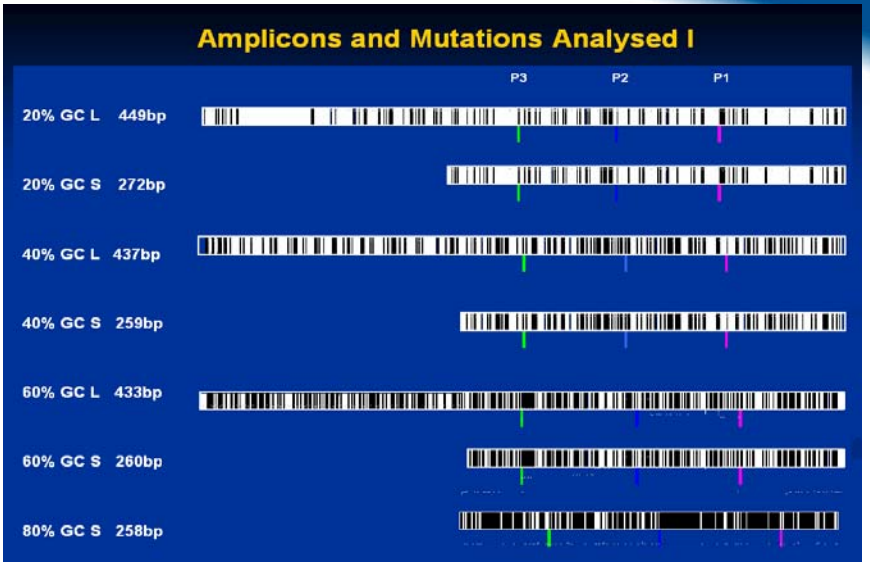
Helen White, GR Taylor, GL Potts, NCP Cross, C Taylor

National Genetics Reference Lab (Wessex), Salisbury

Regional Genetics Service and CR-UK Mutation Detection Facility, Leeds

http://www.ngrl.org.uk/Wessex/downloads.htm

White *et al.* 2006 report



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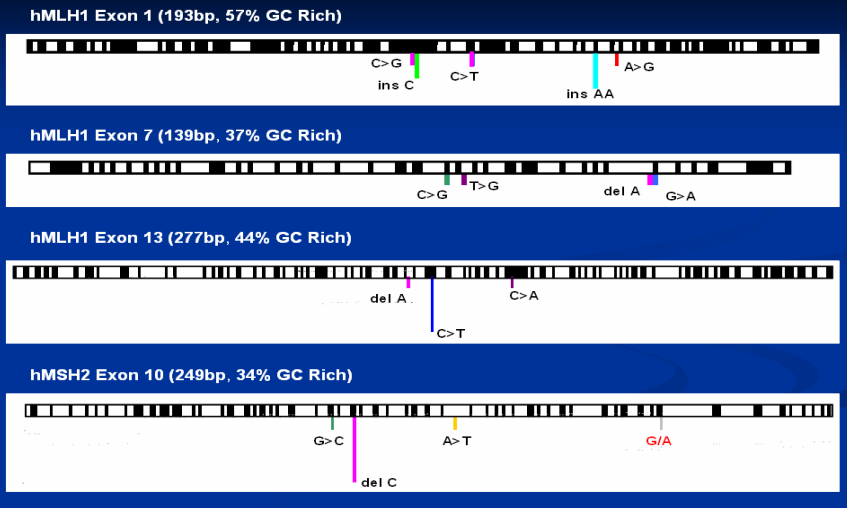
White *et al.* 2006 report

Amplicons and Mutations Analysed II



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White *et al.* 2006 report

Sensitivity and Specificity

Sensitivity = true positive / (true positive + false negative)

Specificity = true negative / (true negative + false positive)

	RotorGene 6000		HR-1		LightScanner 384 well			
					High Sensitivity		Normal Sensitivity	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
20L	100	82.6	89.6	97.8	87.5	100	75	100
20S	100	100	100	91.3	100	100	100	100
40L	100	100	95.8	97.8	100	100	75	100
40S	100	100	95.8	97.8	100	100	71	100
60L	100	90.9	95.8	84.4	-	-	-	-
60S	100	100	100	97.7	100	95.7	69.6	100
80S	100	87	100	93.5	100	87	98.5	87
hMLH1 x1	100	96.7	100	94.2	100	80	80	90
hMLH1 x7	100	100	100	95.2	100	90.5	71.4	96.8
hMLH1 x13	100	98.2	100	85.5	100	96.4	50	100
hMSH2 x10 Combined	100	96.4	100	96.3	100	80	100	90.9

White *et al.* 2006 report

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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

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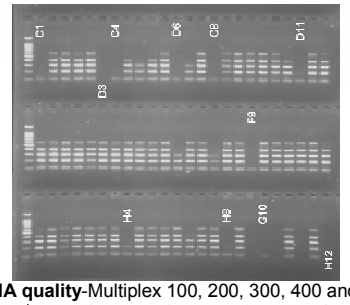
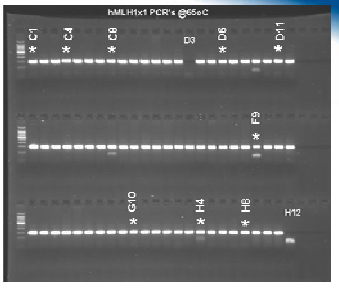
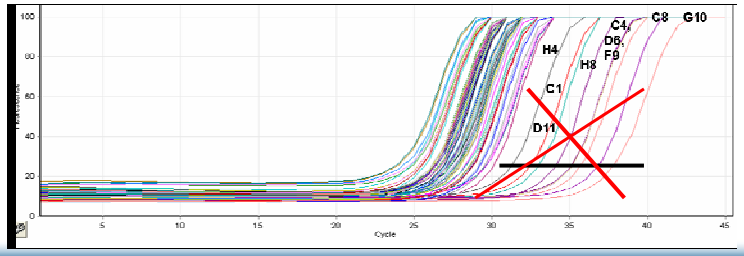


DNA quality-Multiplex 100, 200, 300, 400 and 600pb product



Amplification of 193bp product



Poor Quality DNA in = poor results out!

Important to view your data Real Time to check DNA quality

Guidleines:

Assess the CT values - integrity of your DNA
Assess the amplification efficiency
Assess the derivative plot melt curves-is there one product? Is the PCR optimized? Primer-dimer issues?

Using the Real Time data allows you to make OBJECTIVE decisions about the changes observed

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
- Newly identified genes-little information

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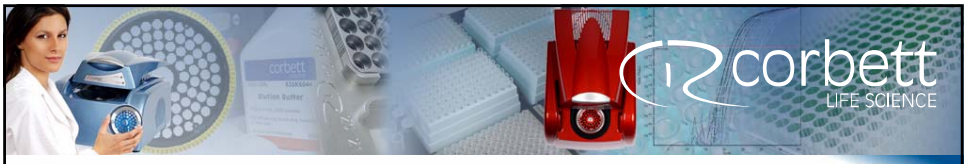
Summary

- Simple, fast, cost effective method for gene scanning and detecting a single-base change in your sample
- Rapid cycle PCR with HRM analysis set up at one time
- NO labeled probes, cheap intercalation dye
- NO Post-PCR processing with additional reagents such as sequencing, DHPLC, RFLP
- Excellent sensitivity and specificity - capable of detecting BOTH heterozygous and homozygous changes
- Costs less than competing technologies
- Sequence directly off the product- sample not consumed
- Detect from a pool of 10 samples -1/20 alleles, 5% sensitivity
- Auto call software
- Scanning and genotyping can be performed simultaneously in the same reaction



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