



The University of  
**Nottingham**

**Mutation Screening by high resolution melting  
(HRM) analysis: a simple and robust method for  
mutation detection in formalin-fixed and  
paraffin embedded tissue**

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## Objectives

- Mutation screening: a brief background.
- Development of HRM mutation screening protocol.
- Mutation detection: a comparison of real-time PCR with QMC-PCR / High Resolution Melting.

## Mutation in Cancer

- The importance of germline and somatic (tumour specific) mutations screening in cancer is increasing as well due to increase in the number of treatment options and differences in cancer behaviour of the same histological type
  - Implications
  - Diagnostic
    - BCR-ABL oncogene in CML
  - Prognostic
  - Predictive of response to specific therapies
    - NSCLC with EGFR Kinase domain mutation has dramatic response to TKI, Gefitinib (N Engl J Med 2004; 350: 2129–39)
    - The presence of KRAS mutations in colorectal cancers abrogates tumour response to anti-EGFR therapy (Virchows Arch 2008; 453(5):417-31)
- New criteria that comprise “profiling” the mutations present within a tumour, in addition to those used by pathologist are required for more consistent classification / staging of tumours and to allow bespoke tailoring of therapies.

## Mutation Detection Techniques

### Considerations

- Template Quality: Frozen section/good quality; FFPE/usually compromised
- Precision: Sensitive (less false negative); Specific (less false positive)
- Sample type: Heterogeneous sample (tumour)/low abundance mutant cell needs a highly sensitive technique
- Mutation type: Known mutation/ specific method; unknown mutation/ unbiased method
- Cost

## Mutation Detection Techniques

How can we detect mutations?

➤ A wide choice of techniques can be used for mutation detection. The techniques fall into two categories:

### 1- Screening technologies

Advantages:

- All mutations (known & unknown), including novel mutations may be detected/unbiased
- Technology is available in many molecular genetics labs

Disadvantages:

- Sensitivity tends to be lower than targeted methods/false negative?
- Experienced operators needed
- labour intensive

## Mutation Detection Techniques

### 2- Targeted technologies

#### Advantages:

- Only mutation (known) assayed for may be detected, less time consuming/Specific
- Sensitivity tends to be higher than screening technologies

#### Disadvantages:

- Mutations not assayed for may be missed/biased
- Expensive

## Mutation Detection Techniques

➤ Sequencing:

- Order of nucleotides is identified during synthesis of a complementary strand to the DNA sample.

➤ Sequencing-based technologies:

Direct Sequencing:

- individual nucleotides can be detected using a dye (dye termination sequencing)
- “Gold Standard”.
- Needs good quality template; the use of FFPE samples can introduce sequencing artefacts.

*Clin Cancer Res 2006; 12: 4403s–4408s*

- Reasonably expensive: pre-screening will reduce the downstream sequencing.
- Relatively lower limit of detection (false negative results); low abundance mutations in heterogeneous samples needs cell enrichment by macro- or microdissection.

## Mutation Detection Techniques

### ➤ Pyrosequencing:

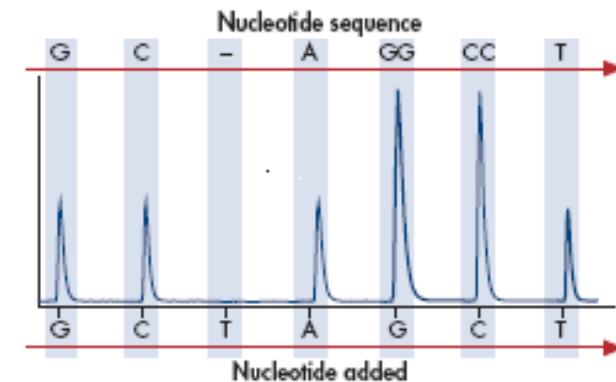


Is sequencing by synthesis, the activity of the enzyme can be detected during synthesis using chemiluminescence.

Known Hot-spots are amplified using specific primers where one primer is biotin-labelled

The products are hybridised onto beads

Sequencing takes place on the beads



## Mutation Detection Techniques

➤ Sequencing:

Nested PCR/Sequencing:

Reduce non-specific products; dilute out PCR inhibitors.

*(Clinical Cancer Research 2005; 11:5878-5885)*

Mutant enriched (ME) PCR/Sequencing:

A combination of PCR and restriction enzyme digest/ suitable for heterogeneous samples (e.g. Tumour).

*(Clin Cancer Res 2006; 12: 43-48)*

Cold PCR/Sequencing:

Cold PCR has been recently described as modifications of the standard PCR protocol which can enrich for mutant alleles by inserting a step of melting at a specific critical denaturation temperature ( $T_c$ ).

*(Nat Med 2008; 14:579-584)*



## Mutation Detection Techniques

### Alternative methods:

- Protein Truncation Test (PTT)
- Single Stranded Conformation Polymorphism Analysis (SSCP)
- Heteroduplex Analysis;
- Melt-curve analysis
- MALDI-TOF



## High Resolution Melting Analysis HRMA

### Basics

- PCR plus dsDNA saturating intercalation dye.
- Detailed monitoring of changes in fluorescence as a PCR products are melted with increasing temperature.
- HRM distinguish double stranded DNA from single stranded DNA by a change in fluorescent signal intensity in the presence of dsDNA dye.
- Base change causes change in melting temperature ( $T_m$ ) of DNA.

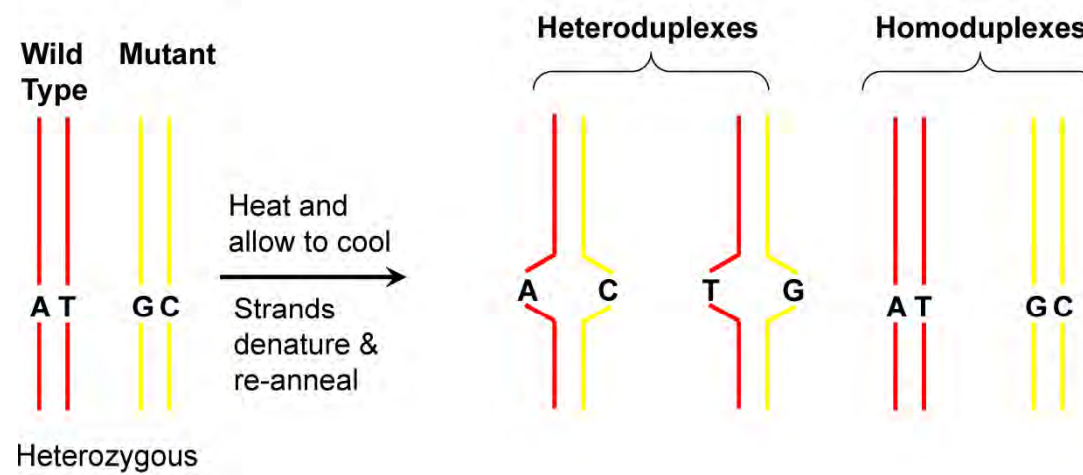
Base Change	$T_m$ shift
C>T & G>A	Large >0.5°C ↓ Small >0.2°C
C>A & G>T	
C>G	
A>T	

- Can detect even a single base change.



## High Resolution Melting Analysis HRMA

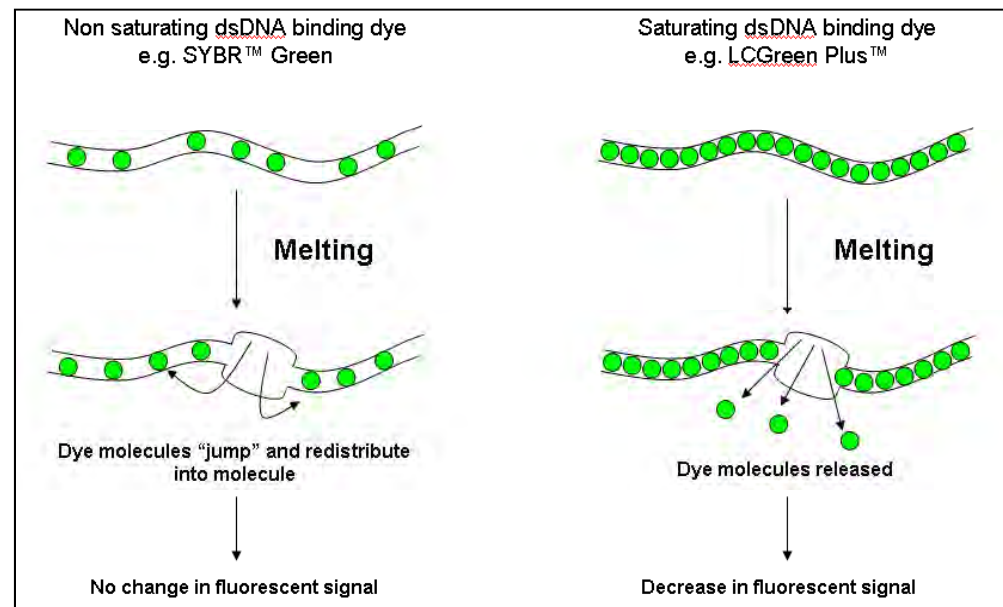
### Heteroduplex formation



## High Resolution Melting Analysis HRMA

dsDNA intercalation dyes/Full saturation vs Partial saturation

### Dye saturation model



*Clinical Chemistry 2003; 49: 853-860*

## High Resolution Melting Analysis HRMA

### Advantages

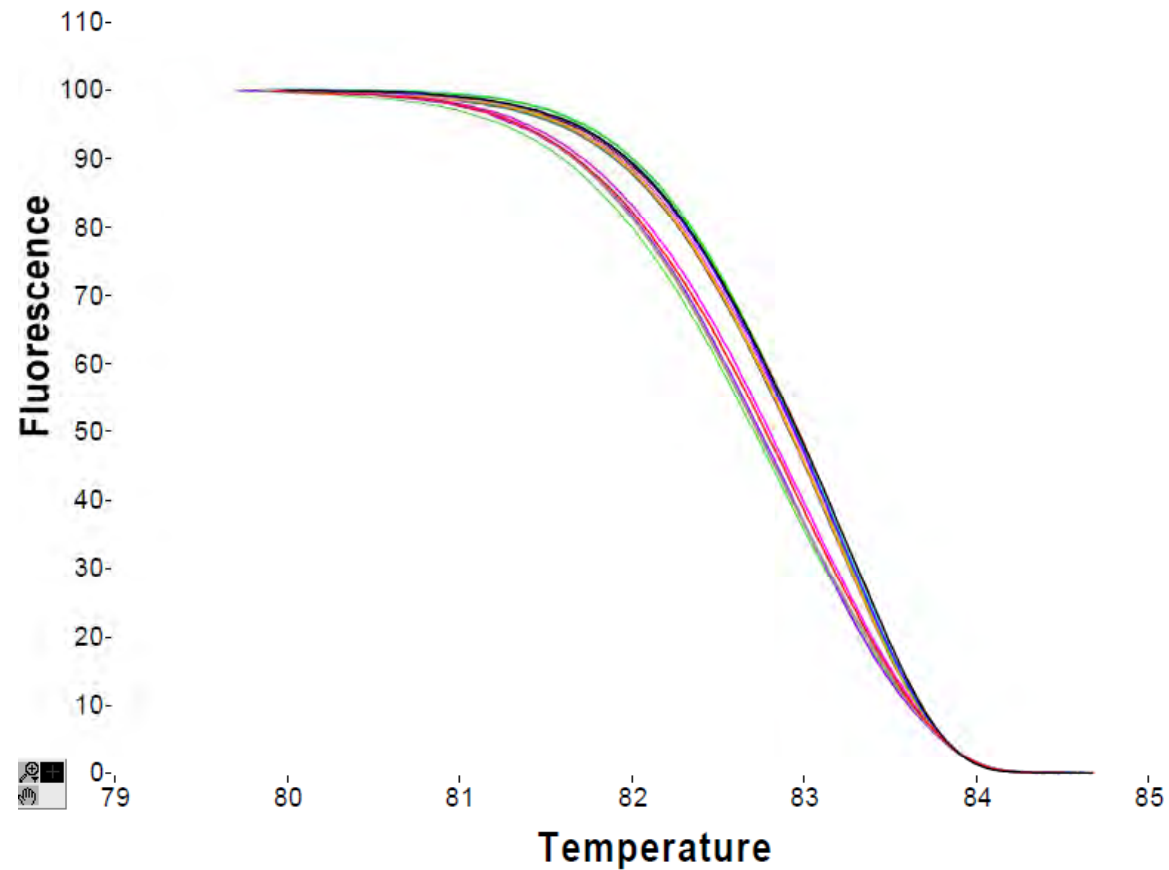
- Simple, fast, cost effective method for gene scanning and detecting a single-base change
- Closed system
- Has excellent sensitivity and specificity
- Sequence directly off the product, sample not consumed
- NO labelled probes
- Auto call software
- Scanning & genotyping can be done simultaneously in the same reaction



## High Resolution Melting Analysis HRMA

Analysis of Melting curve (HR1™ Idaho technology)

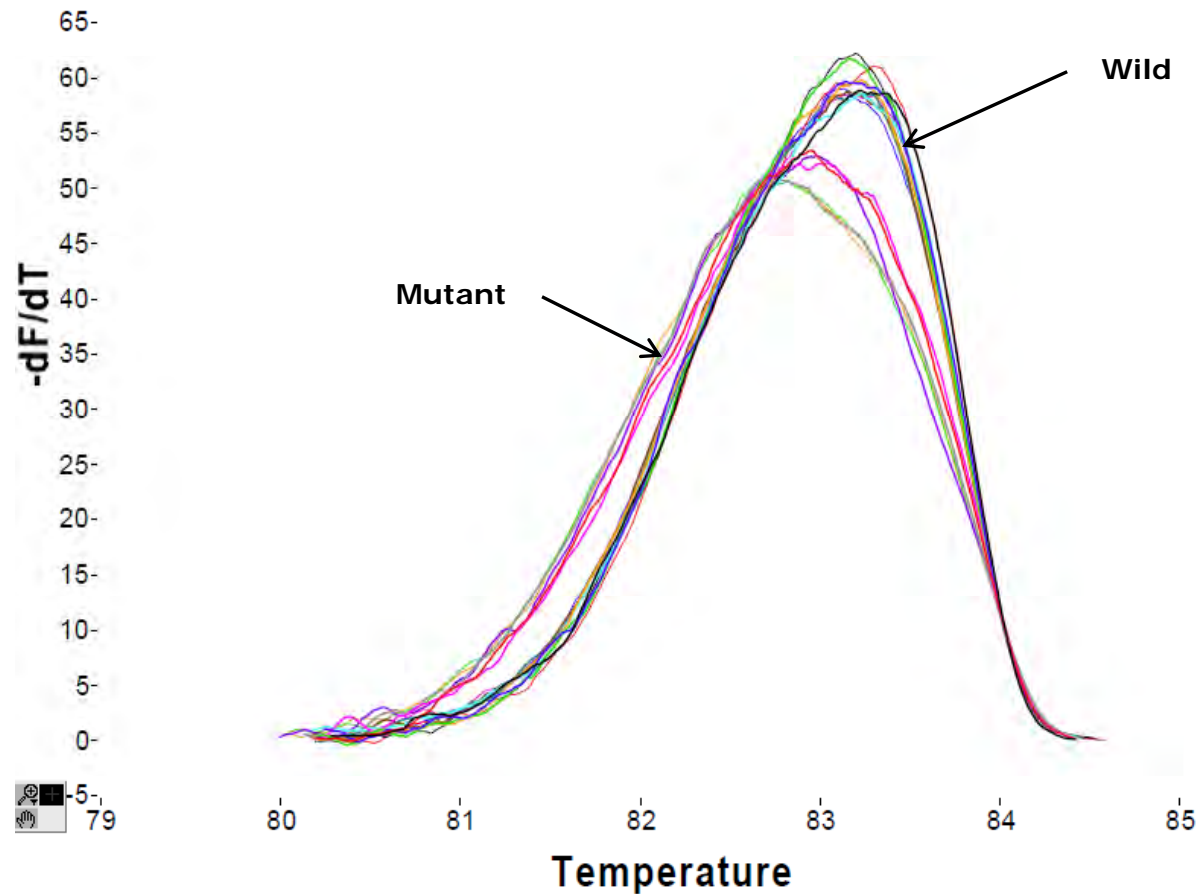
1- Melt curve





## High Resolution Melting Analysis HRMA

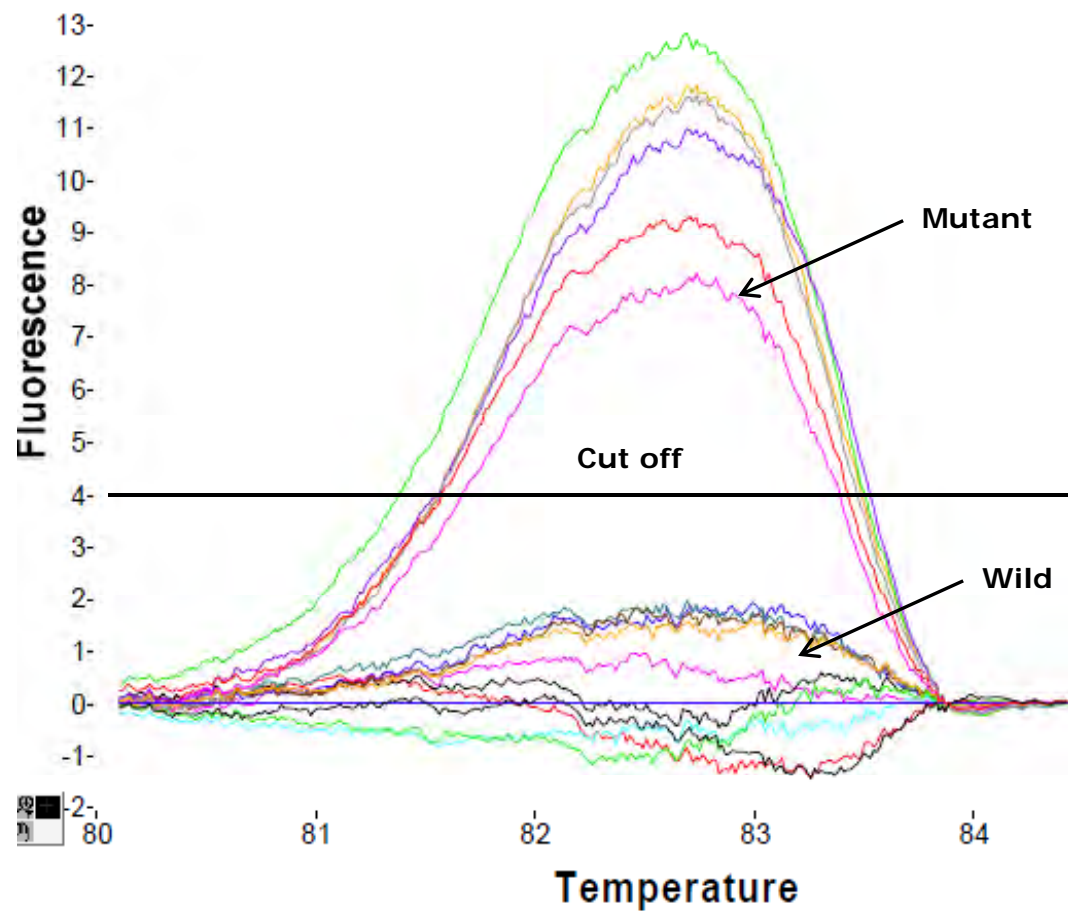
### 2- Normalised and temperature shifted Derivative Plot





## High Resolution Melting Analysis HRMA

### 3- Normalised and temperature shifted Difference Plot





## **Quick-multiplex-consensus (QMC)-PCR followed by high-resolution melting: a simple and robust method for mutation detection in formalin-fixed paraffin-embedded tissue**

Wakkas Fadhil, Salih Ibrahim, Rashmi Seth, et al.

*J Clin Pathol* 2010 63: 134-140  
doi: 10.1136/jcp.2009.070508

## Screening of Colorectal Cancer Clinical Samples

### Quick Multiplexed Consensus (QMC) PCR protocol

- Consisting of a Pre-Diagnostic Multiplex (PDM) reaction followed by a Specific Single Diagnostic (SSD) reaction.
- Ten mutation hotspots in (KRAS, BRAF, PIK3CA, and CDC4) were screened
- 29 colorectal cancer cell lines and 43 clinical sample (FFPE) were tested.
- 100% Sensitivity and Specificity in cell lines as compared to direct sequencing.
- 100% sensitivity and 71% specificity in FFPE tissue as compared to direct sequencing (sequencing false negative?)



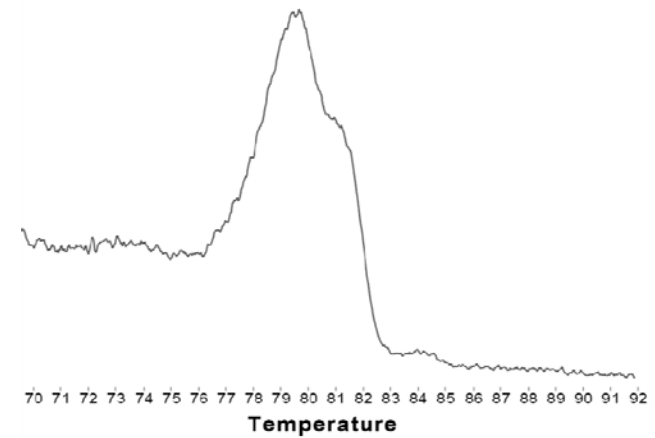
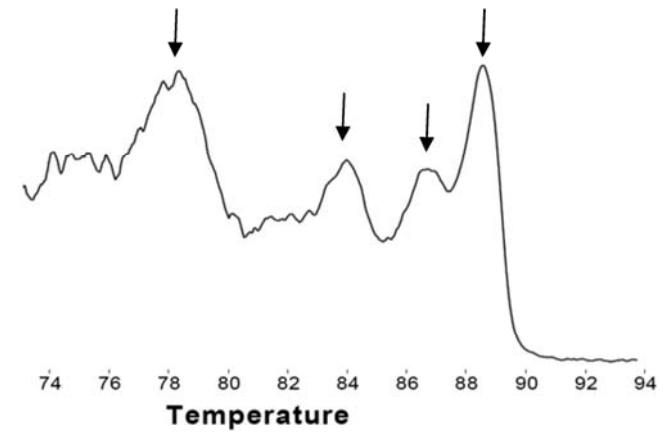
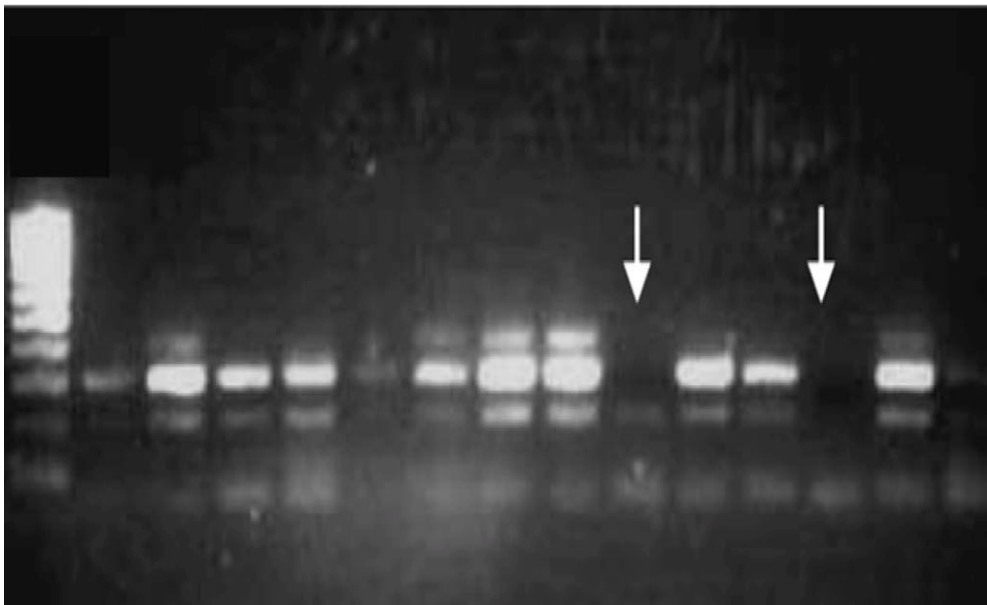
## Screening of Colorectal Cancer Clinical Samples

Sample quality assurance

Multiplex PCR/

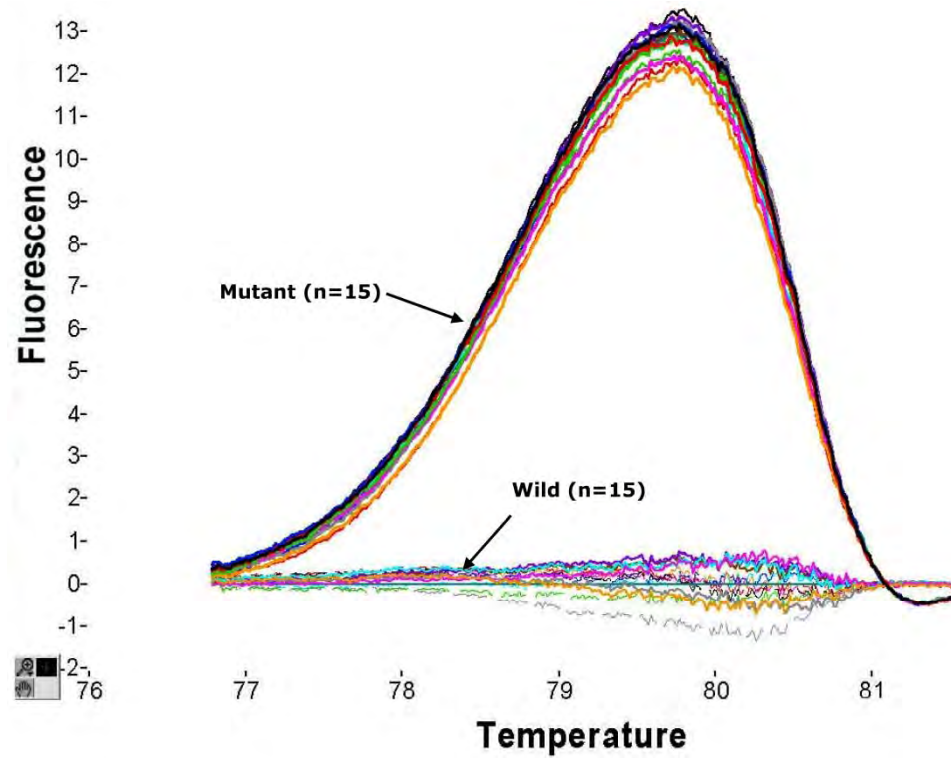
4 primer pairs for GAPDH gene

100-200-300-400 bp amplicons



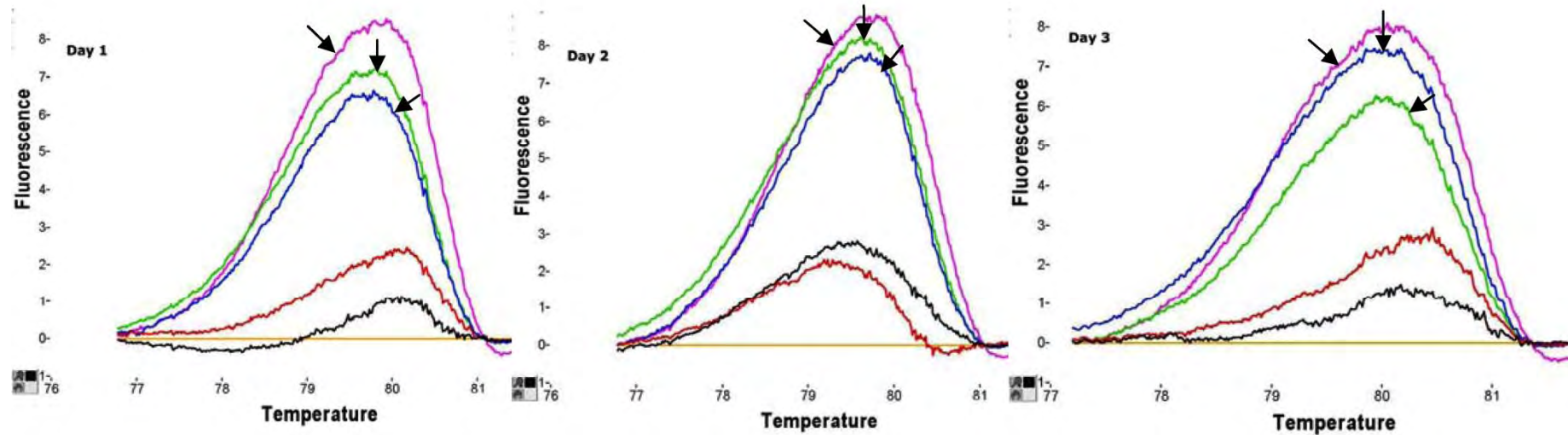


Assay accuracy  
Intra-Assay variation



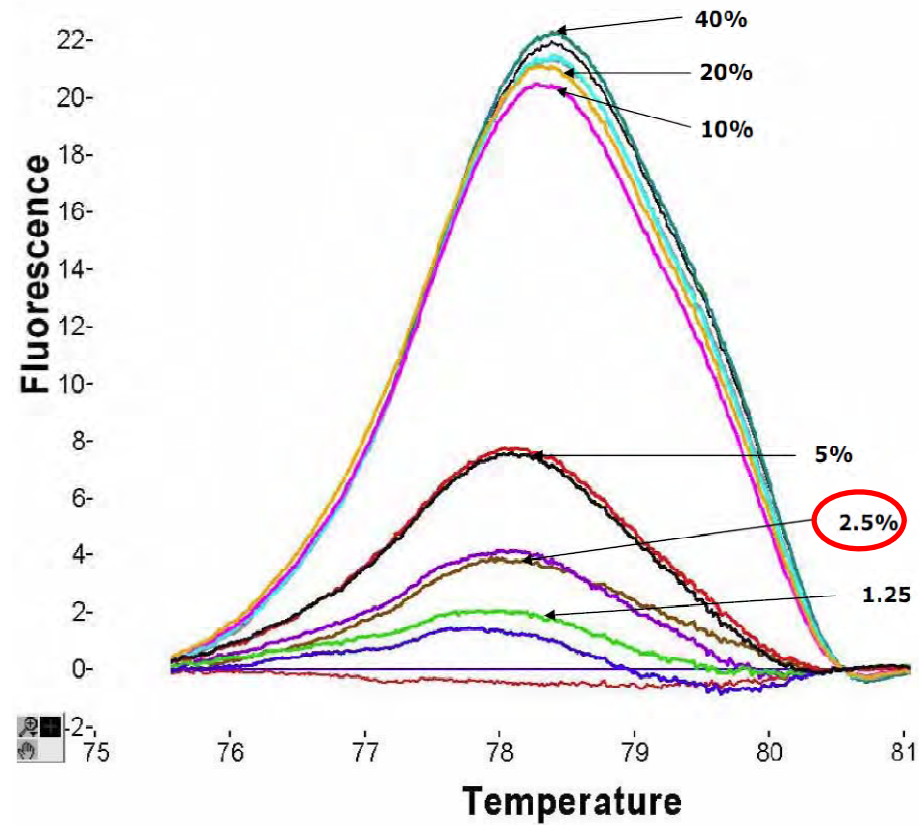


Assay accuracy  
Inter-Assay variation



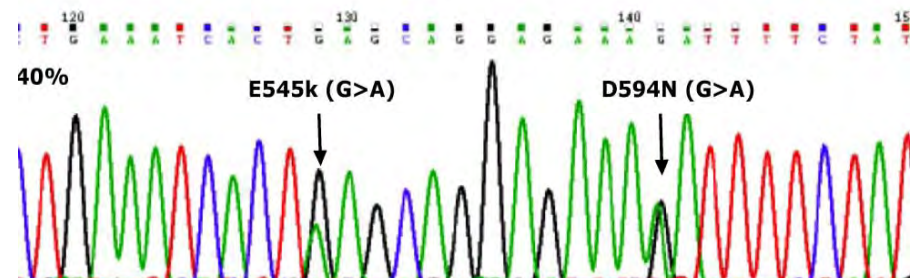
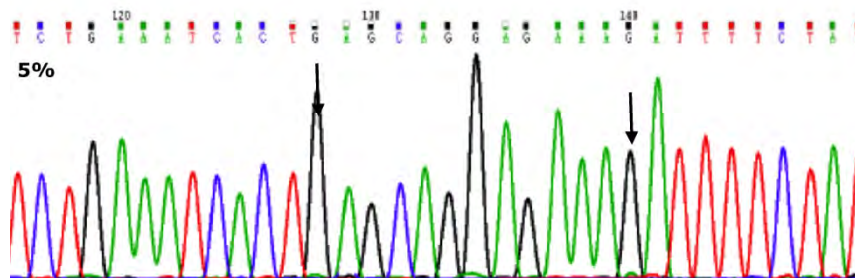
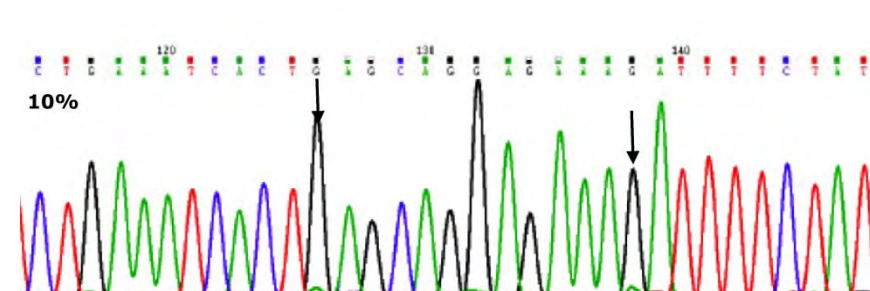
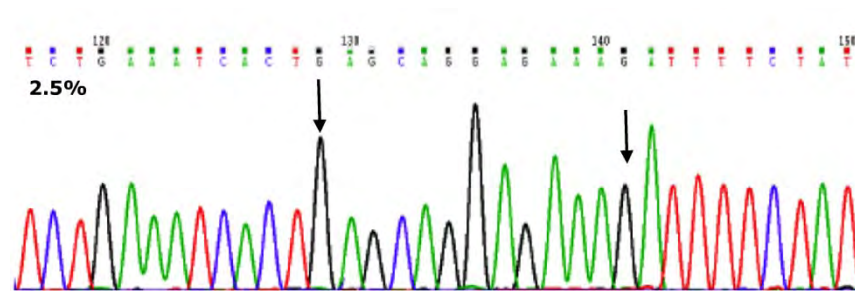


QMC PCR limit of mutation detection/HRM  
Spiking experiment using diploid cell lines  
PIK3CA/ Exon 9



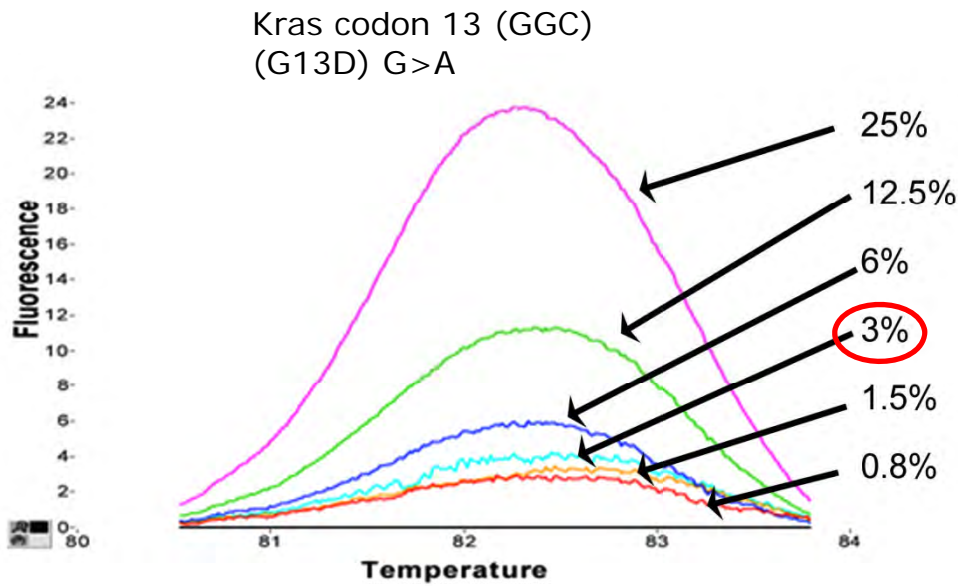


QMC PCR limit of mutation detection/ Direct sequencing  
Spiking experiment using diploid cell lines

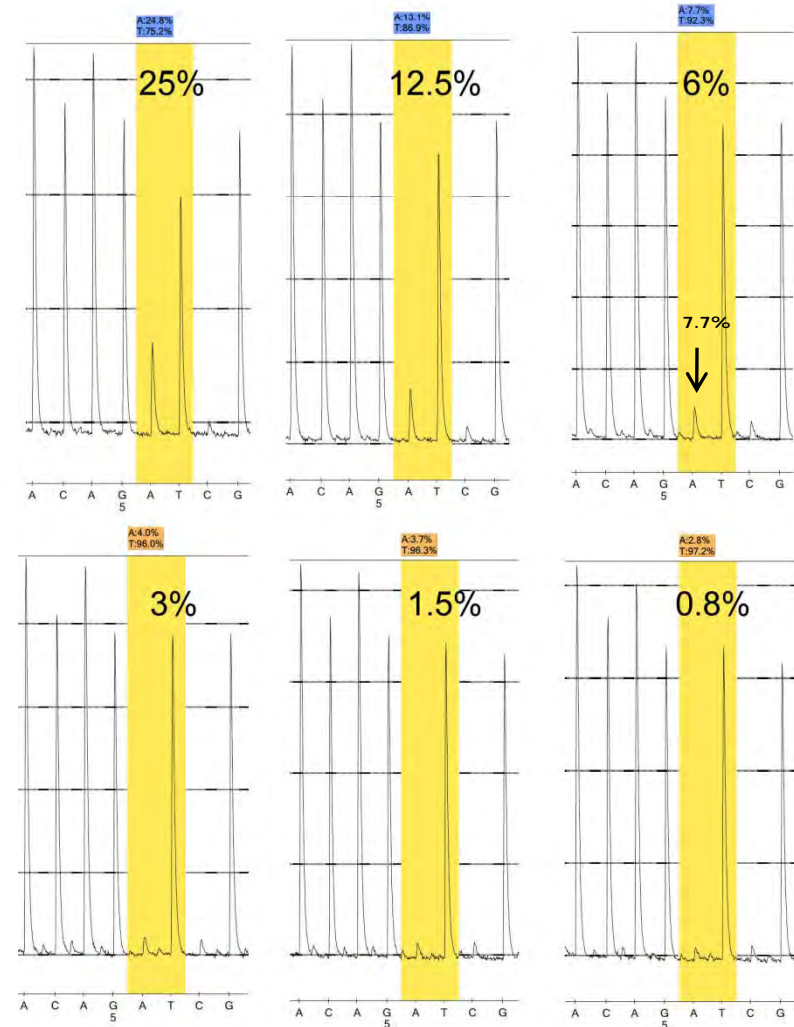
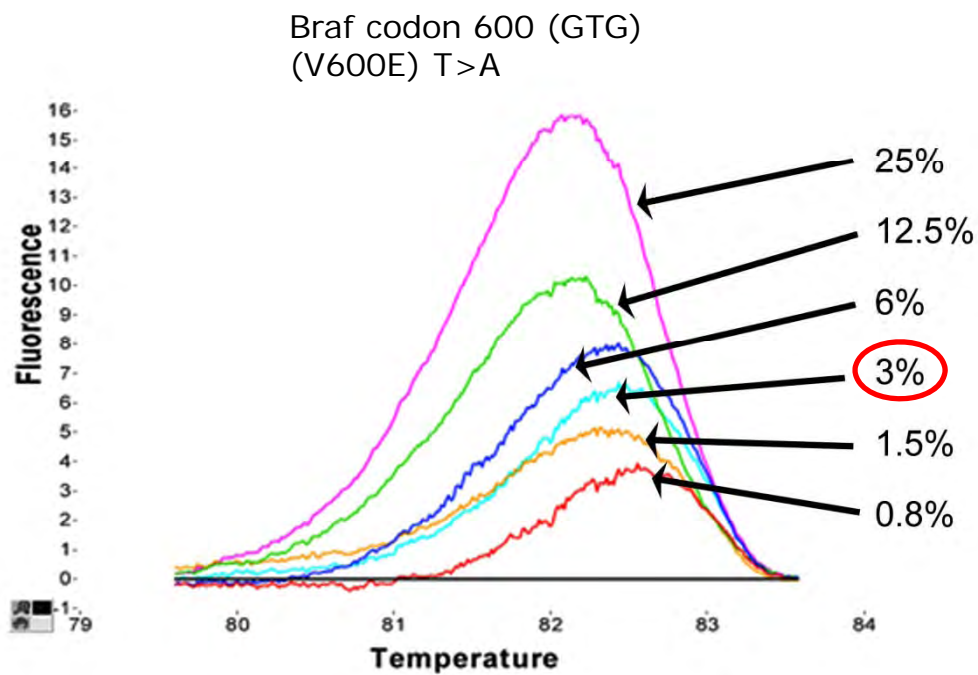




QMC PCR limit of mutation detection  
(HRM vs Pyrosequencing)  
Spiking experiment using diploid cell lines



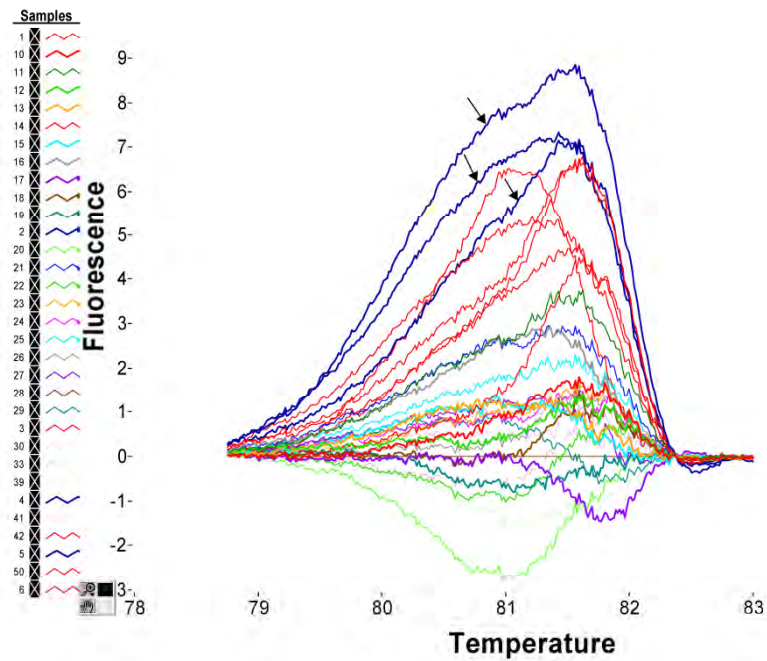
QMC PCR limit of mutation detection  
(HRM vs Pyrosequencing)  
Spiking experiment using diploid cell lines



## Elimination of Positive artefacts

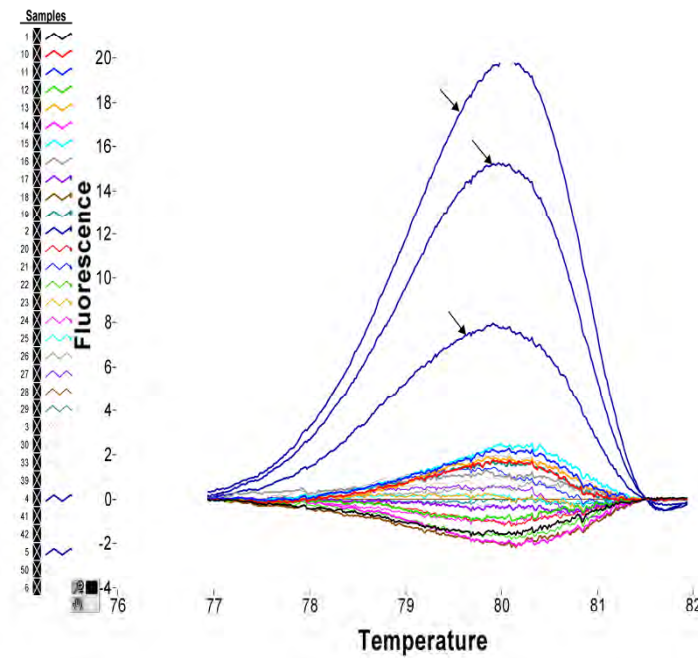
### Standard PCR

Neat DNA from FFPE tissues



### QMC PCR

Multiplex products from FFPE tissues





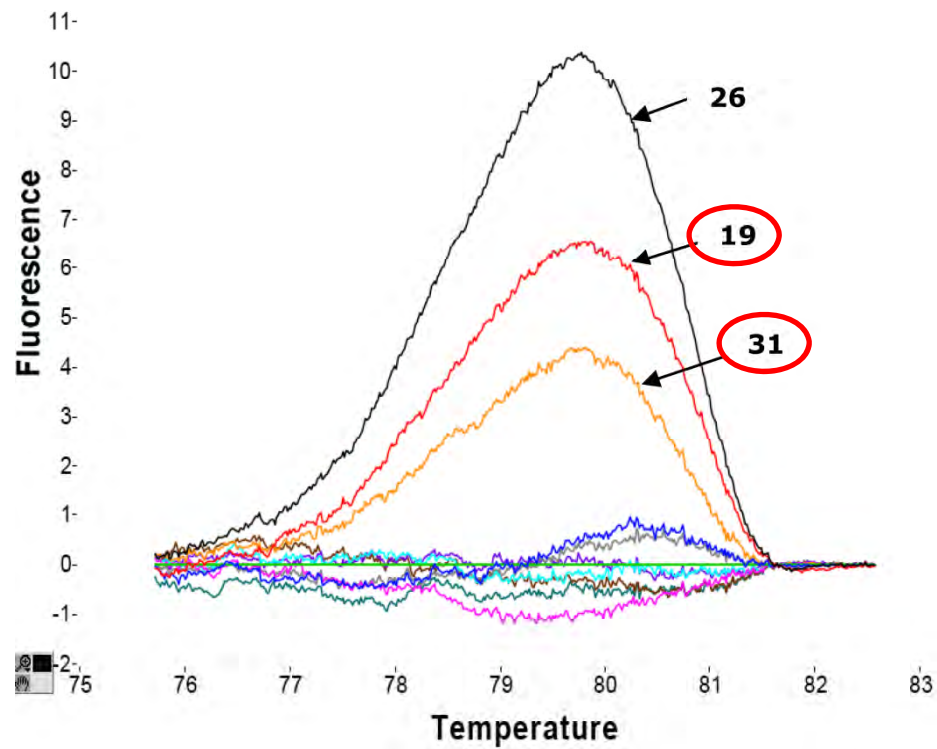
Mutation detection. Quick-multiplex-consensus-PCR and high-resolution melting analysis

			Sequencing analysis of hotspots		
			Mutant	Wild-type	
In cell lines	<i>KRAS &amp; BRAF</i> only	Mutant	23	0	(PPV 100%)
		Wild-type	0	122	(NPV 100%)
	All hotspots	Mutant	40	0	(PPV 100%)
		Wild-type	(250—not validated by sequencing)		
In FFPE tumour samples	<i>KRAS &amp; PIK3CA</i> only	Mutant	16	5	(PPV 76%)
		Wild-type	0	65	(NPV 100%)
	All hotspots	Mutant	36	7	(PPV 84%)
		Wild-type	(387—not validated by sequencing)		

FFPE, formalin-fixed paraffin-embedded; NPV, negative predictive value; PPV, positive predictive value.



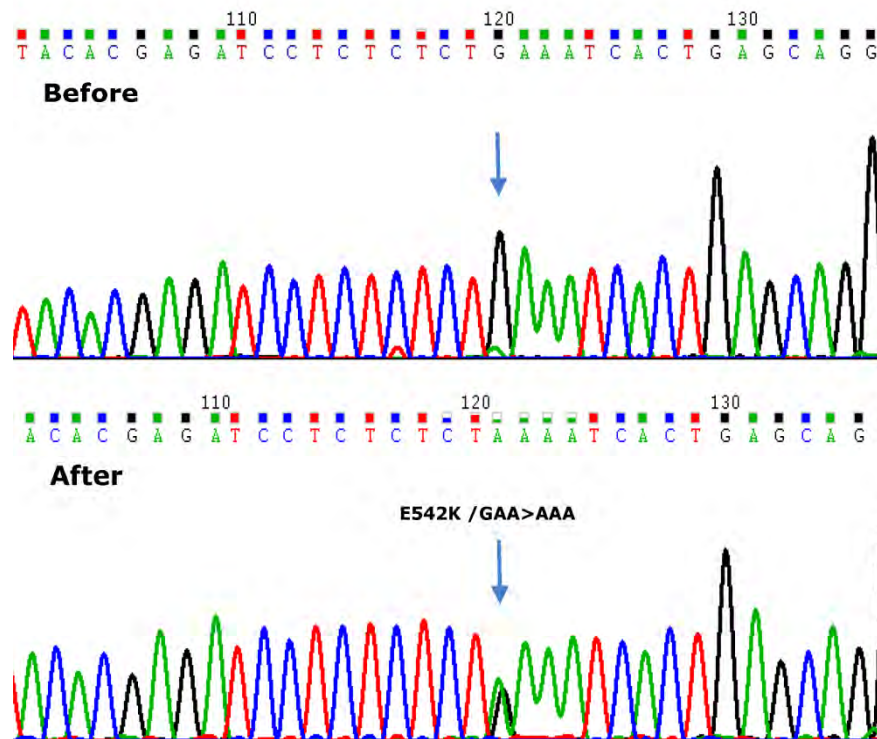
## Sequencing false negatives



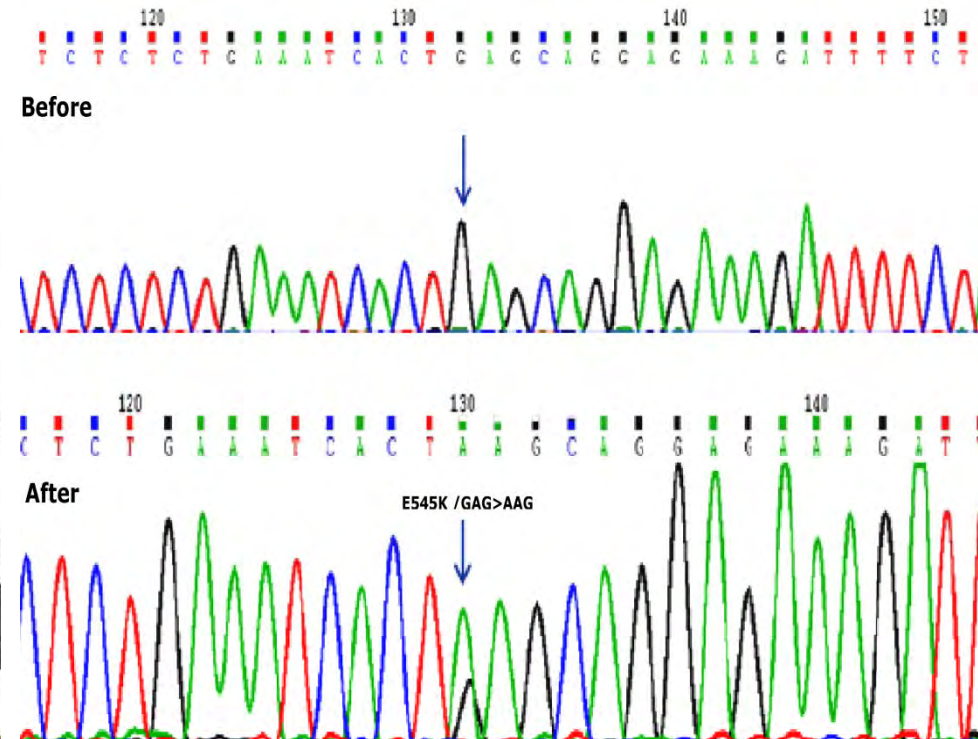


## Sequencing false negatives Mutation Enrichment by Fast Cold PCR

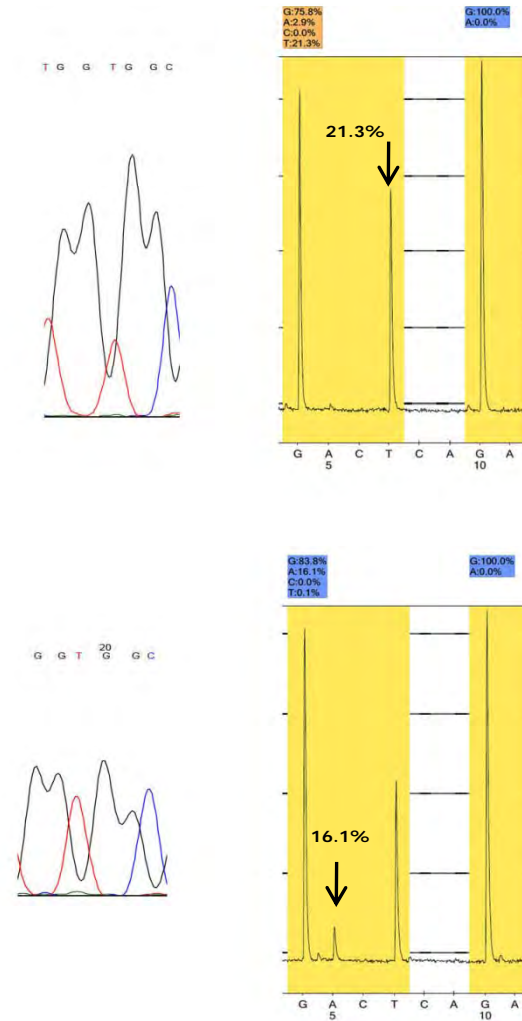
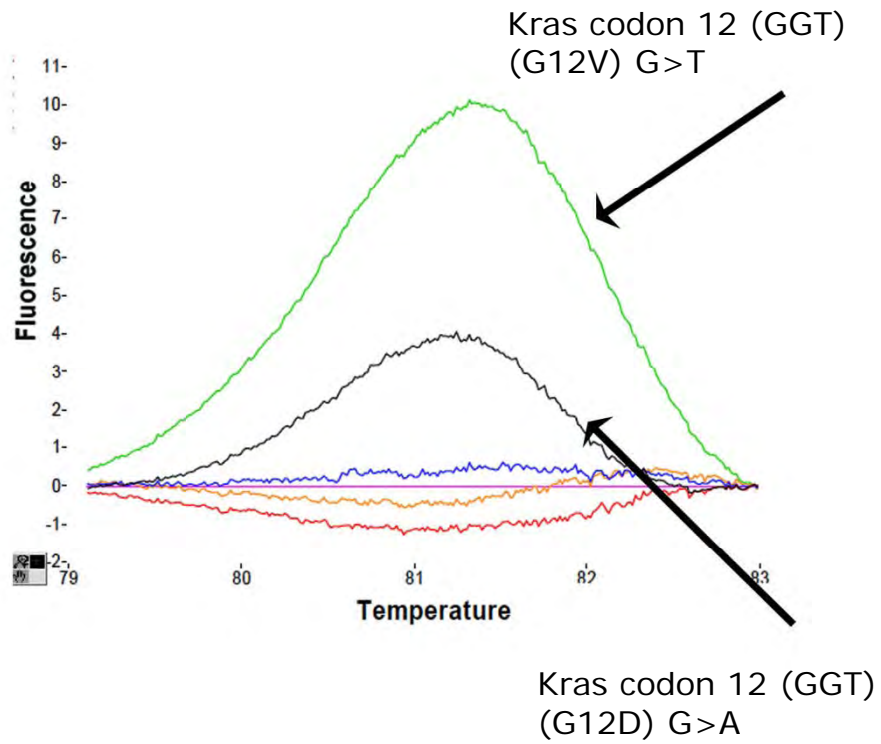
Sample 19



Sample 31



Sequencing false negatives  
Mutation Detection by Pyrosequencing



In summary, we have described a new technique based on multiplexed nested PCR and HRM analysis.

The technique is rapid, robust and highly sensitive and, most importantly, it is easy to implement (requiring a single set of cycling conditions) and the data are easy to interpret.

The technique allows multiple targets to be tested and it is ideal for tumour mutation profiling.

Given that it does not need large sophisticated pieces of equipment, it can be established in almost any setting.



Mutation detection: a comparison of real-time PCR with QMC-PCR / High Resolution Melting

Series of 444 cases from the Dutch Cairo 2 trial (CKTO 2005-02) conducted by the Dutch Colorectal Cancer Group (DCCG). (N Engl J Med 2009, 360:563-572)

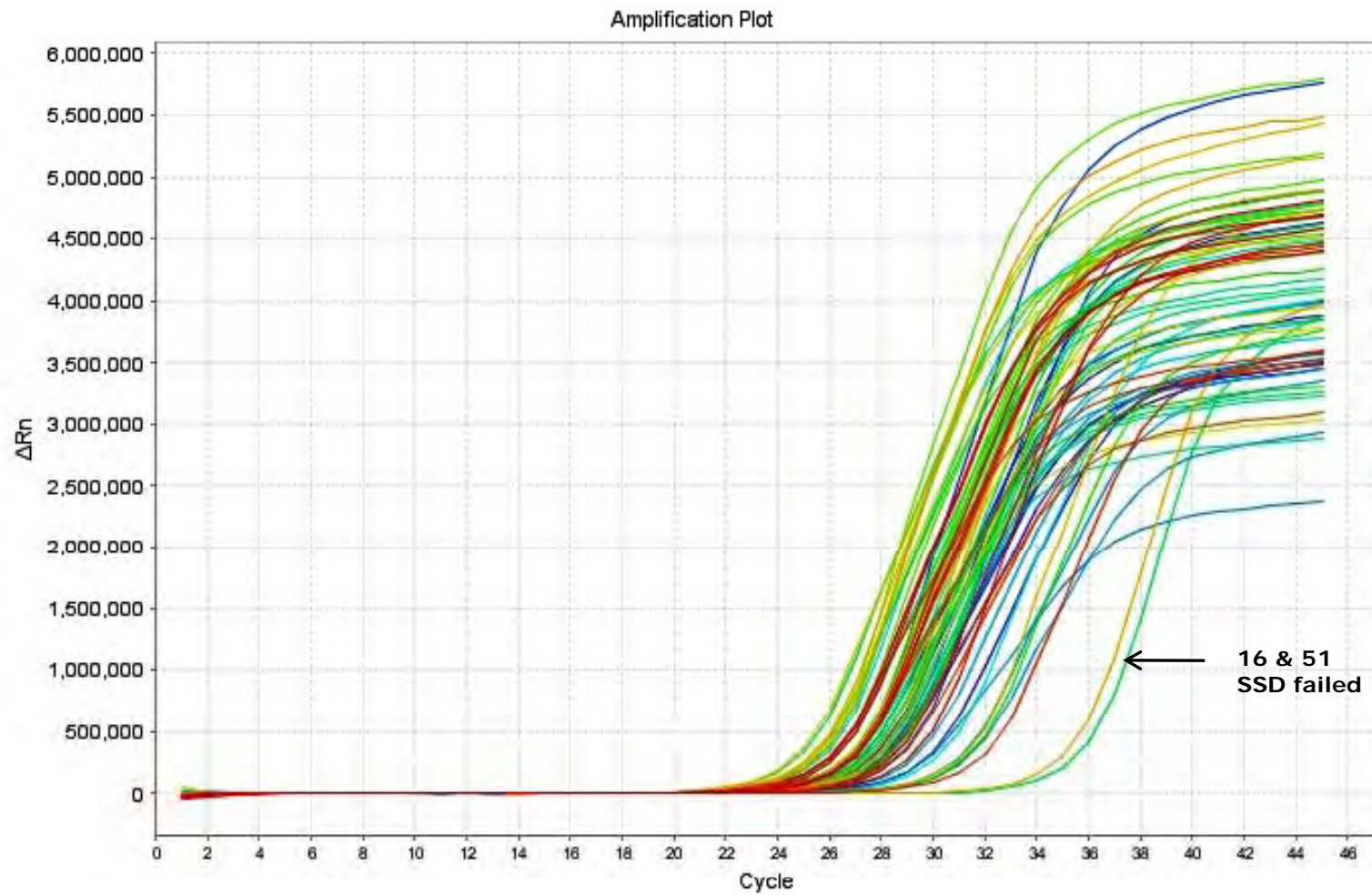
Tested for KRAS codon 12/13 mutation by the DxS real-time PCR kit

It detects the 7 most common mutations in KRAS codon 12/13.

Mutation	Base Change	Cosmic ID
Gly12Ala	(GGT>GCT)	522
Gly12Asp	(GGT>GAT)	521
Gly12Arg	(GGT>CGT)	518
Gly12Cys	(GGT>IGT)	516
Gly12Ser	(GGT>AGT)	517
Gly12Val	(GGT>GTT)	520
Gly13Asp	(GGC>GAC)	532

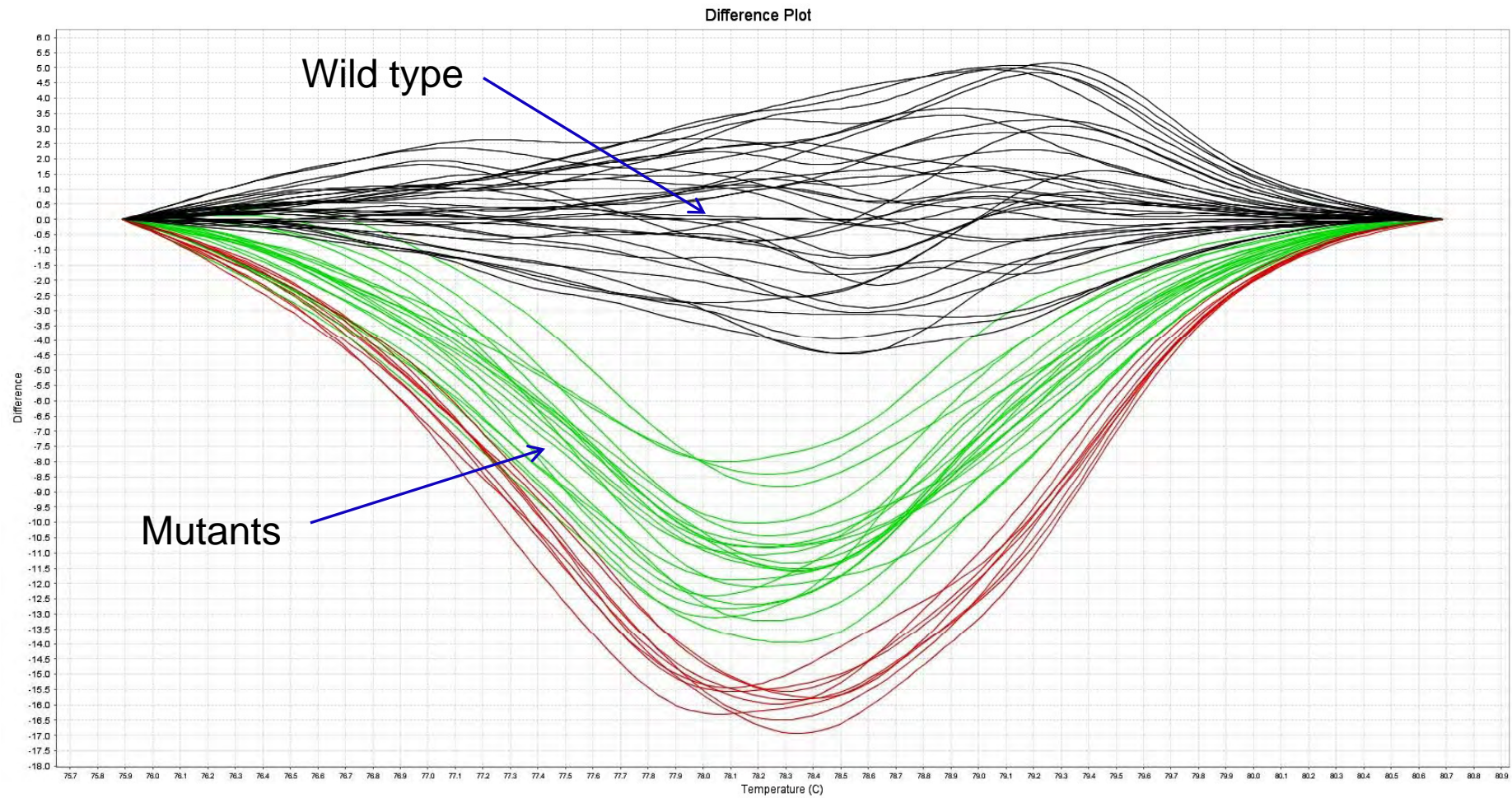
Detection of approximately 1% mutant in a background of wild-type genomic DNA is possible.

Fast 7500 real-time PCR system  
PDM Reaction



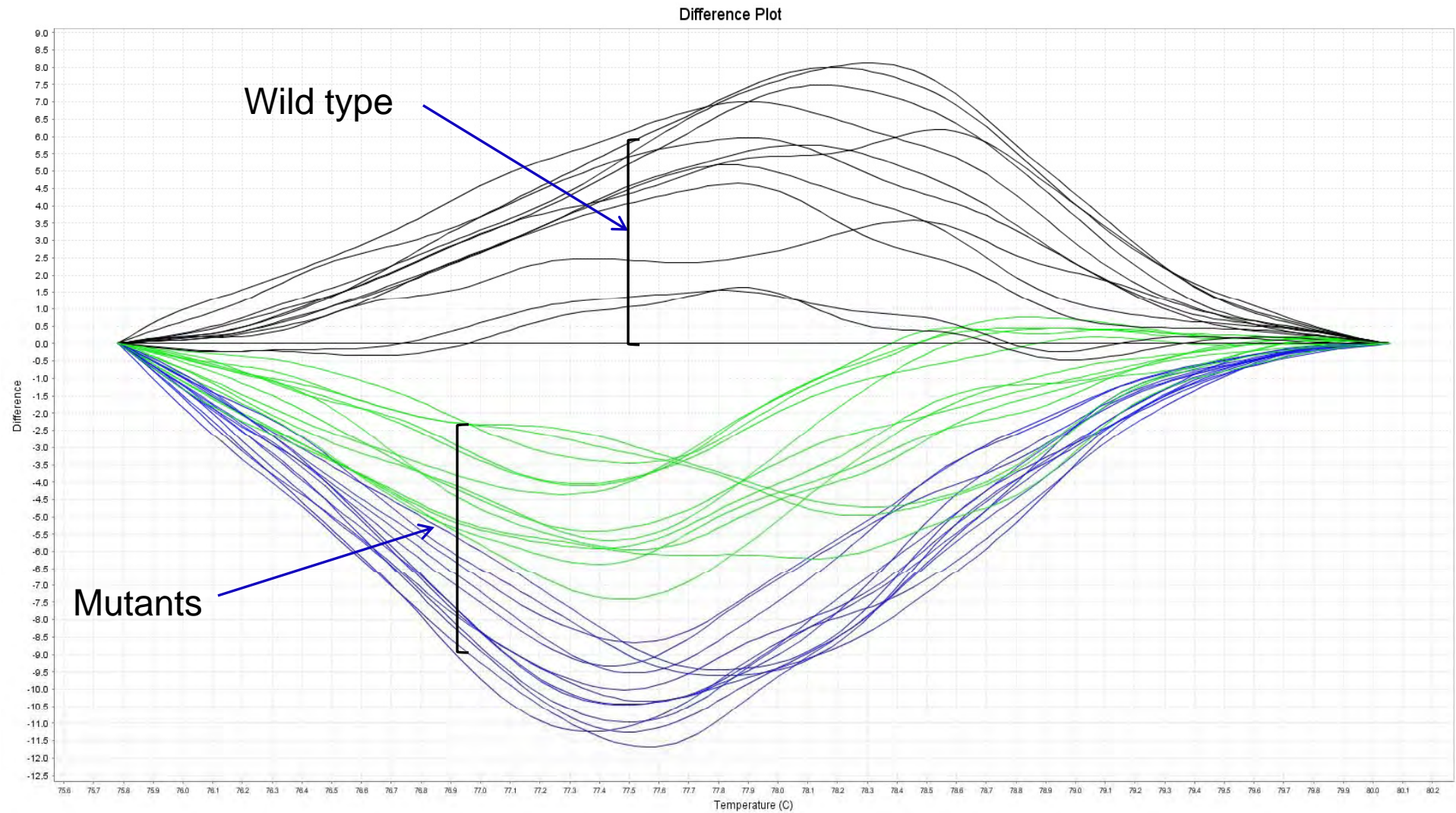


Fast 7500 real-time PCR system  
SSD reaction  
Kras 12/13





Fast 7500 real-time PCR system  
SSD reaction  
Kras 12/13





Comparison of mutation analysis for codon 12/13 of KRAS between the DxS test and the QMC-PCR/HRM1 protocol

		DxS		
		Mutant	Wild Type	Total
QMC-PCR/HRM	Mutant	168	1 <sup>GLY12GLU</sup>	169
	Wild Type	0	270	270
	Total	168	271	439

Sensitivity 100%

Specificity 99.6%



In summary, we have compared the utility of HRM and the DxS test for detecting KRAS mutations in a large series of CRC. We have found that HRM can perform on a par with the DxS test. Each assay has strengths and weaknesses but both are sufficiently accurate to use in the clinical setting.

## Acknowledgement

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Hospitals

The Dutch Cancer Society

Thank you

# Questions