

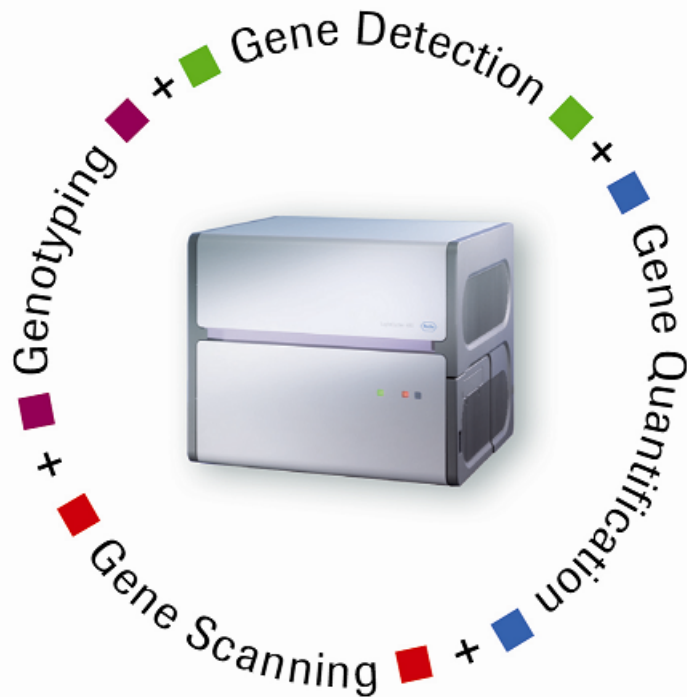
[www.roche-applied-science.com](http://www.roche-applied-science.com)

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# Genotyping Concept for the LightCycler 480<sup>®</sup> System



# Applications for the LightCycler<sup>®</sup> 480 System



- Gene Detection: detecting e.g. bacteria in sample material
- Gene Expression: analyzing expression level of gene of interest
- Genotyping: detecting **known** variants
- Gene Scanning: finding **new** variants

# Genotyping by Real-time PCR

## *Current Technologies and Developments*

### Examples for genotyping of known mutations

- using HybProbe- or Simple Probe Probes (melting curves)
- using Hydrolysis Probes (end point detection)

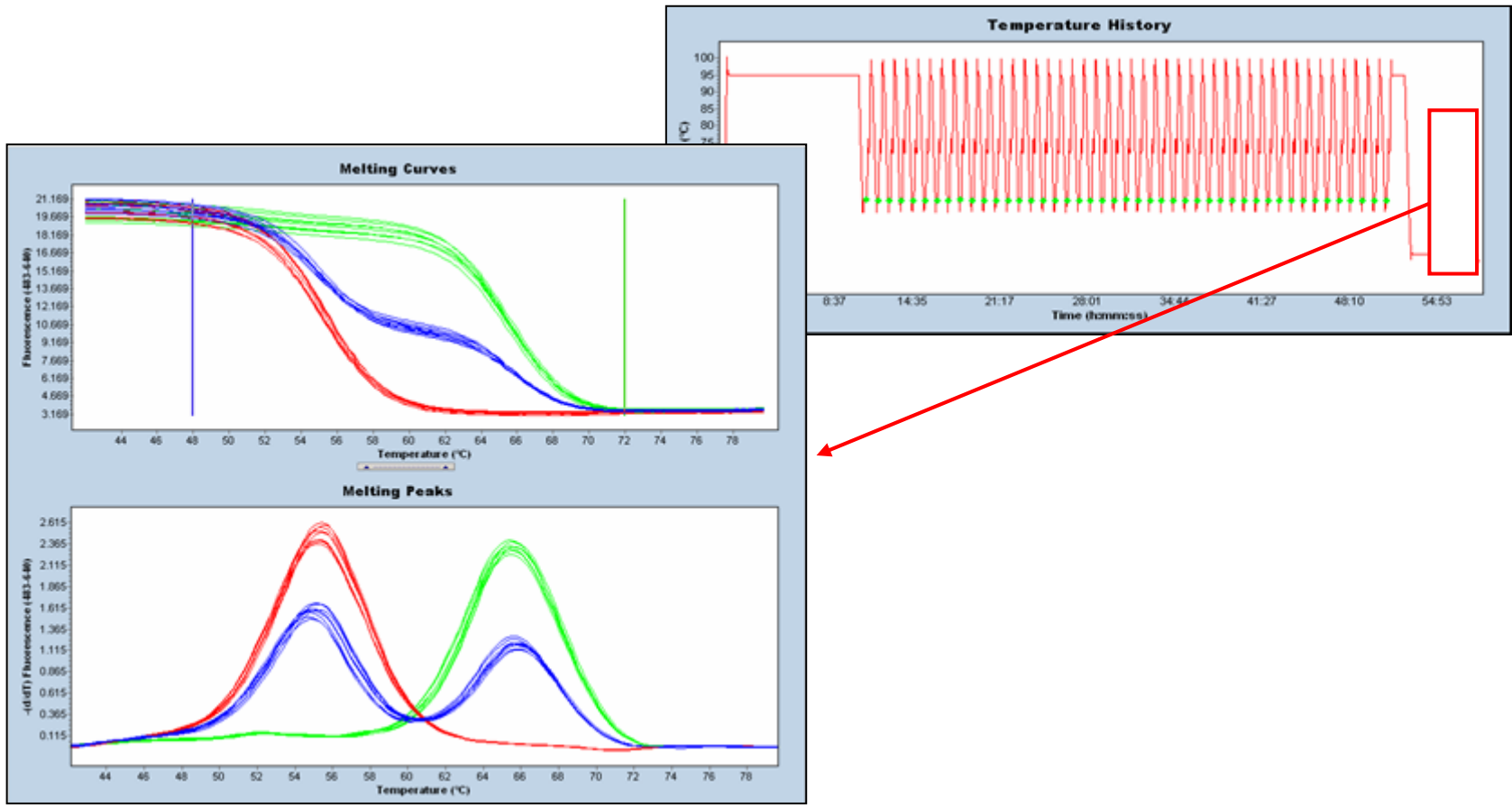
### How to find new mutations (e.g., SNPs, deletions, insertions) in specific regions of candidate genes?

- High Resolution (Amplicon) Melting using a saturating DNA-binding dye and specific algorithms for data analysis.

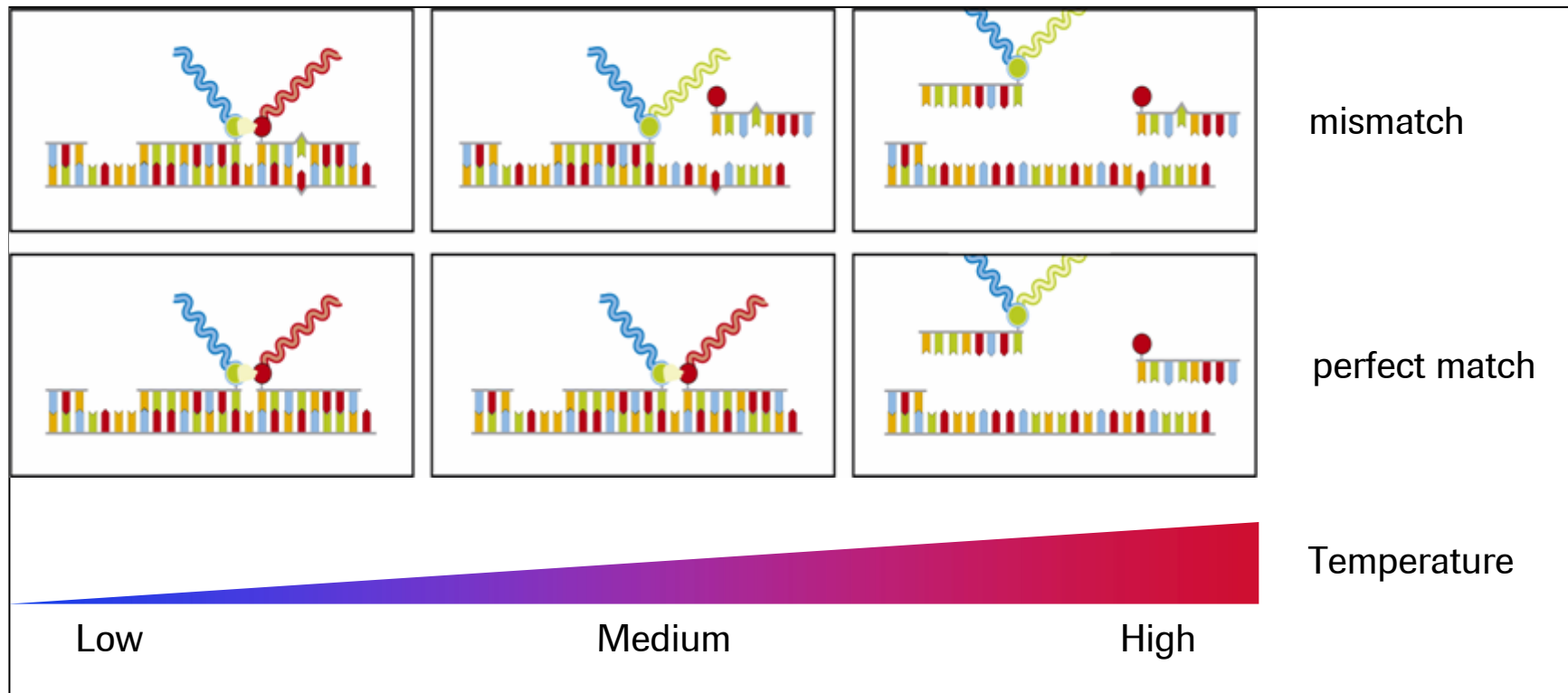
# Content

- **Principles and advantages of melting-curve based genotyping with fluorescence-labeled probes**
- Discrimination by allele specific PCR, end-point genotyping on the LightCycler<sup>®</sup> 480 System
- High-Resolution Melting for mutation scanning

# Genotyping Technique using Melting Curves



# Genotyping with HybProbe Probes



# LightCycler® 480 Genotyping Software



- performs genotyping analysis on HybProbe or SimpleProbe based experiments containing a melting curve program subsequent to PCR.
- LightCycler® 480 Genotyping Software groups samples with similar melting profiles together and identifies each group as a genotype.
- To determine genotypes, the software analyzes the shapes of all the melting curves. It compares each individual melting curve profile to a standard, and then makes a “call”.

# LightCycler<sup>®</sup> 480 Genotyping Software

## Results Screen



Sum.

Experiment

Subset Editor

Sample Editor

Analysis

Report

Analyses: Genotyping for External Melt Std

Information: Program: Melting, Color Compensation: Off, Standard: Demo - Genotyping - Melt Standards

Subset: External Melt Std

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

●  wildtype

●  4

●  Weak Positive

●  mutant

●  5

●  Unknown

●  heterozygous

●  6

●  Negative

Samples			Results		
Include	Color	Pos Name	Group	Score	Res
<input checked="" type="checkbox"/>		D1 Sample 1	wildtype	0.99	0.99
<input checked="" type="checkbox"/>		D2 Repl. of Sample 1	wildtype	0.99	0.99
<input checked="" type="checkbox"/>		D3 Repl. of Sample 1	wildtype	1.00	0.99
<input checked="" type="checkbox"/>		D4 Sample 2	wildtype	0.99	0.99
<input checked="" type="checkbox"/>		D5 Repl. of Sample 2	wildtype	0.99	0.99
<input checked="" type="checkbox"/>		D6 Repl. of Sample 2	wildtype	0.99	0.99

**Melting Curves**

**Melting Peaks**

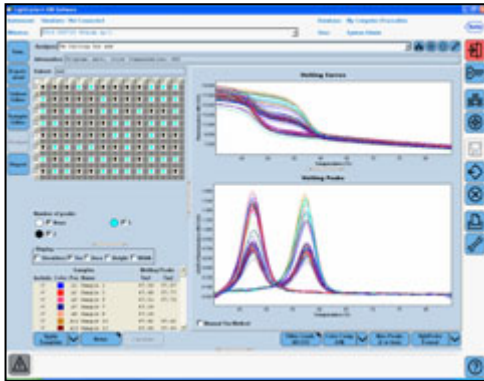
New Call:    Show Standards

Filter Comb. 483-640 | Color Comp (Off) | Standards (External)

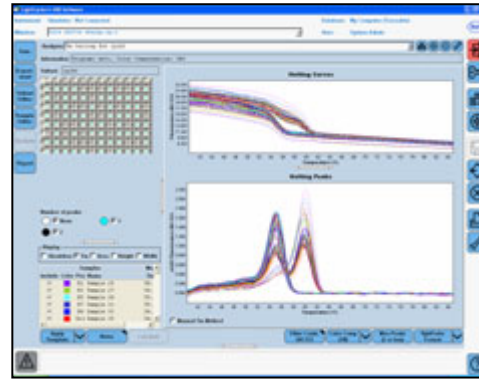


# LightCycler<sup>®</sup> 480 Performance

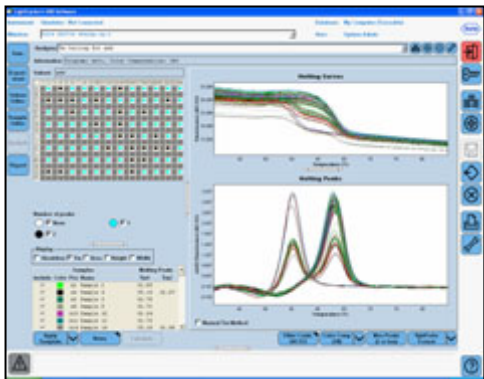
## *High-Throughput SNP Analysis*



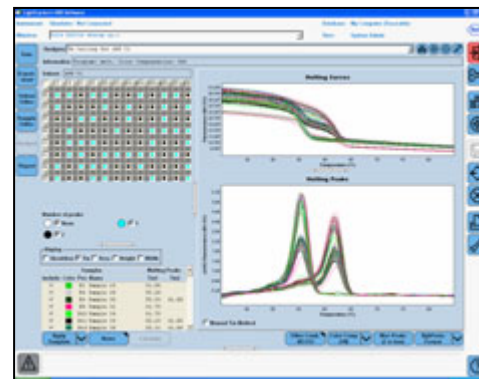
Set A1-O23  
**MDR1 C/T**



Set B1-P23  
**LPLH3 C/A**



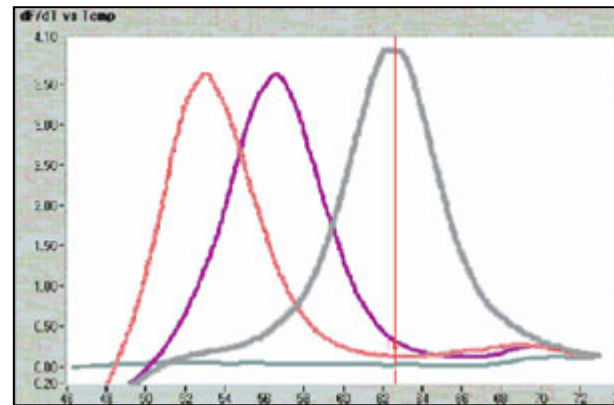
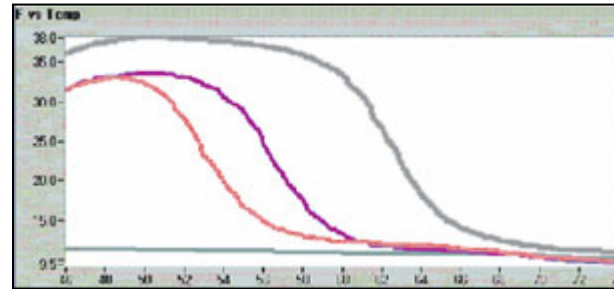
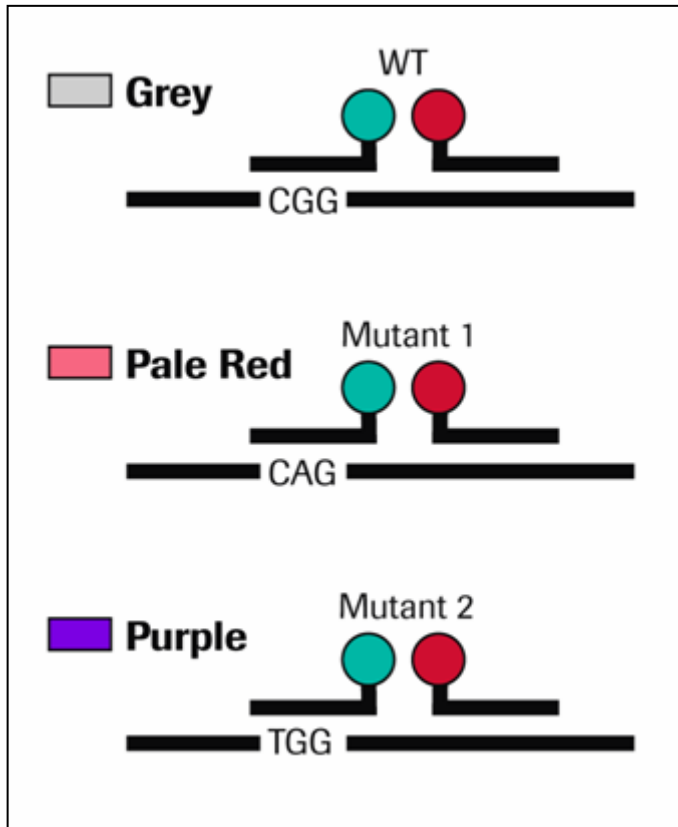
Set A2-O24  
**ADD1 C/A**



Set B2-P24  
**ADR1-C1 C/T**

# LightCycler<sup>®</sup> Genotyping: Example

## *SNP with Three Different Alleles*



Template:  
Plasmid DNAs

Target:  
Apolipoprotein B

Single Color:  
LC RED 640

# LightCycler<sup>®</sup> 480 System

## *Benefits for Genotyping*

- **Melting curve principle:**
  - post-PCR, biophysical measurement, more robust than enzymatic assays.
  - curve shapes and peaks more informative than PCR end-points.
- **Optimized for hybridization probes:**
  - cover several nearby SNPs in the same reaction
  - resolve nearby SNPs, and identify unknown new allelic variants.
- **Broad range of specific filters for excitation and detection:**
  - easily set-up multiplex assays by combining colors and Tms
- **Highly reproducible results on an instrument designed for automated workflows.**

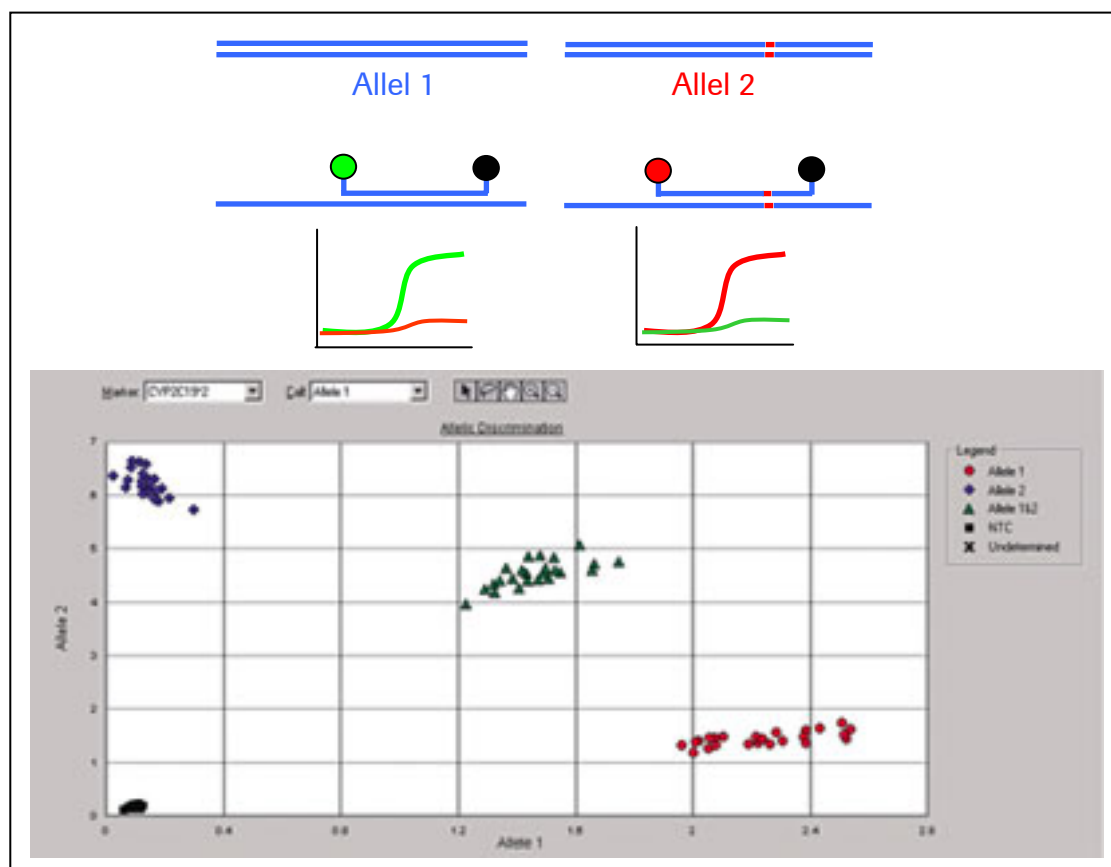
# Content

- Principles and advantages of melting-curve based genotyping with fluorescence-labeled probes
- **Discrimination by allele specific PCR, end-point genotyping on the LightCycler<sup>®</sup> 480 System**
- High-Resolution Melting for mutation scanning

# Allelic Discrimination

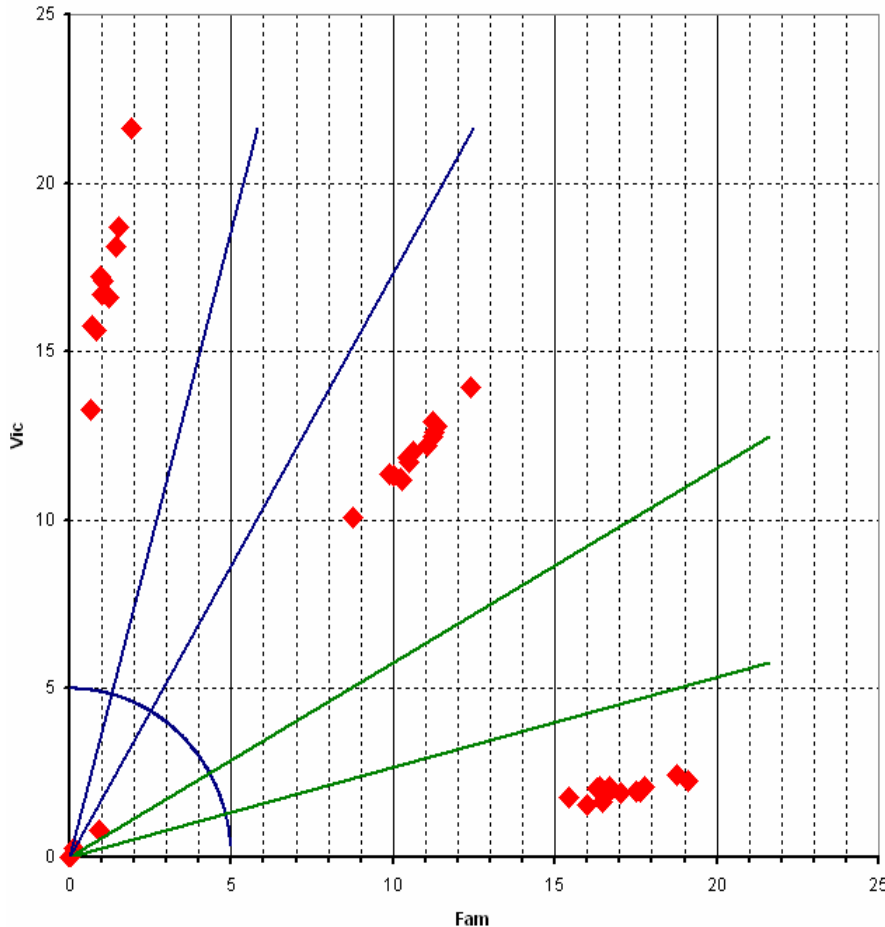
- Allelic discrimination is Genotyping by the use of Hydrolysis Probes for each homozygous type (wildtype and mutant)
- Multiplex approach with e.g., Fam- and Hex-labeled Hydrolysis Probes
- Mismatches between probe and target reduce efficiency of probe hybridization. The enzyme is more likely to replace mismatched probe without cleaving it (lower or no fluorescent signal)

# Allele Specific PCR



# LightCycler<sup>®</sup> 480 End-Point Genotyping

## *Analysis of Hydrolysis Probe Assays*



- **Excel worksheet (“TaxcelTool”)** available for analysis of **LightCycler<sup>®</sup> 480 data (on request)**
- **Bridging the gap between launch of SW 1.5 with module for Hydrolysis Probe Genotyping**

# Create LightCycler<sup>®</sup> 480 Data for “TaxcelTool” Analysis

The screenshot displays the software interface for configuring a PCR program. The 'Setup' section shows 'Detection Format' as 'Multi Color Hydrolysis Probe'. The 'Programs' table lists two programs: 'Program' (1 cycle, 'Melting Curves' analysis mode) and 'cooling' (1 cycle, 'None' analysis mode). The 'Temperature Targets' table is configured as follows:

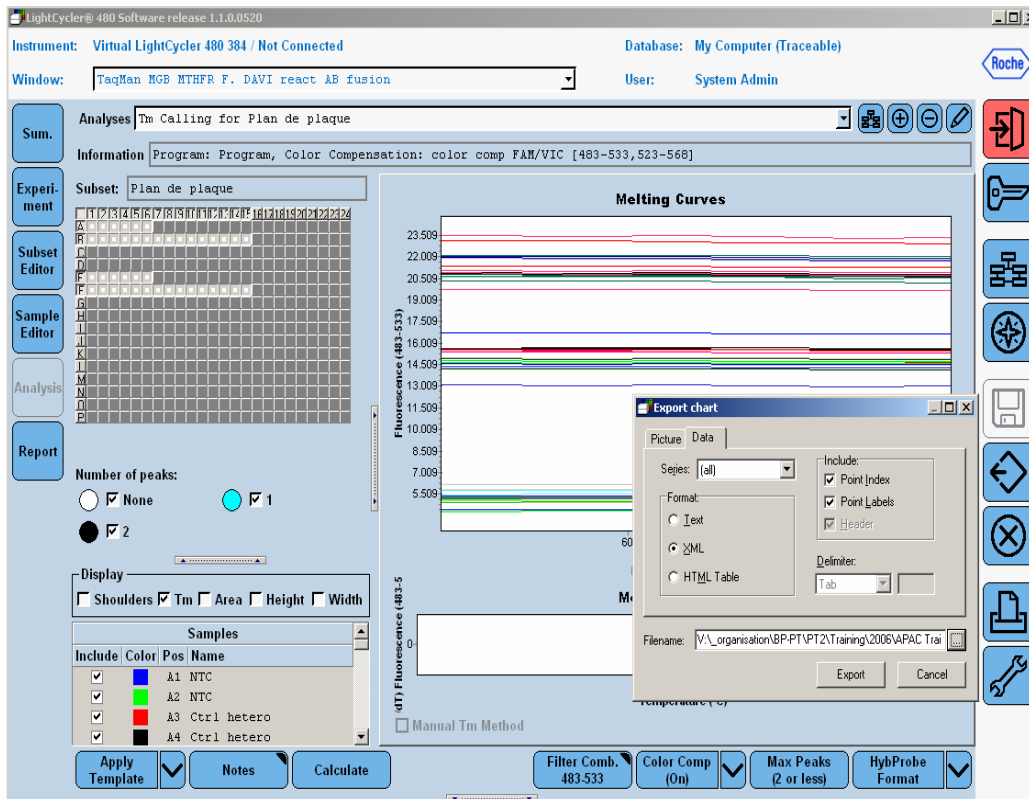
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
60	None	00:00:01	4.8				
61	Continuous			5			

The 'Overview' graph shows the temperature profile over time, with a ramp up to 60°C, a 1-second hold, a continuous ramp to 61°C, and a final ramp down.

After PCR with Hydrolysis probes (run on LC480) perform an endpoint measurement by programming a short "Melting Curve" from 60-61°C.



# Analyze and Export LightCycler<sup>®</sup> 480 Data for “TaxcelTool” Analysis



**Open analysis "Tm Calling":**

- **Settings for FAM data:**

**Filter Comb. 483-533**  
**Color Comp. (On)**

- **Settings for VIC/Hex data:**

**Filter Comb. 523-568**  
**Color Comp. (On)**

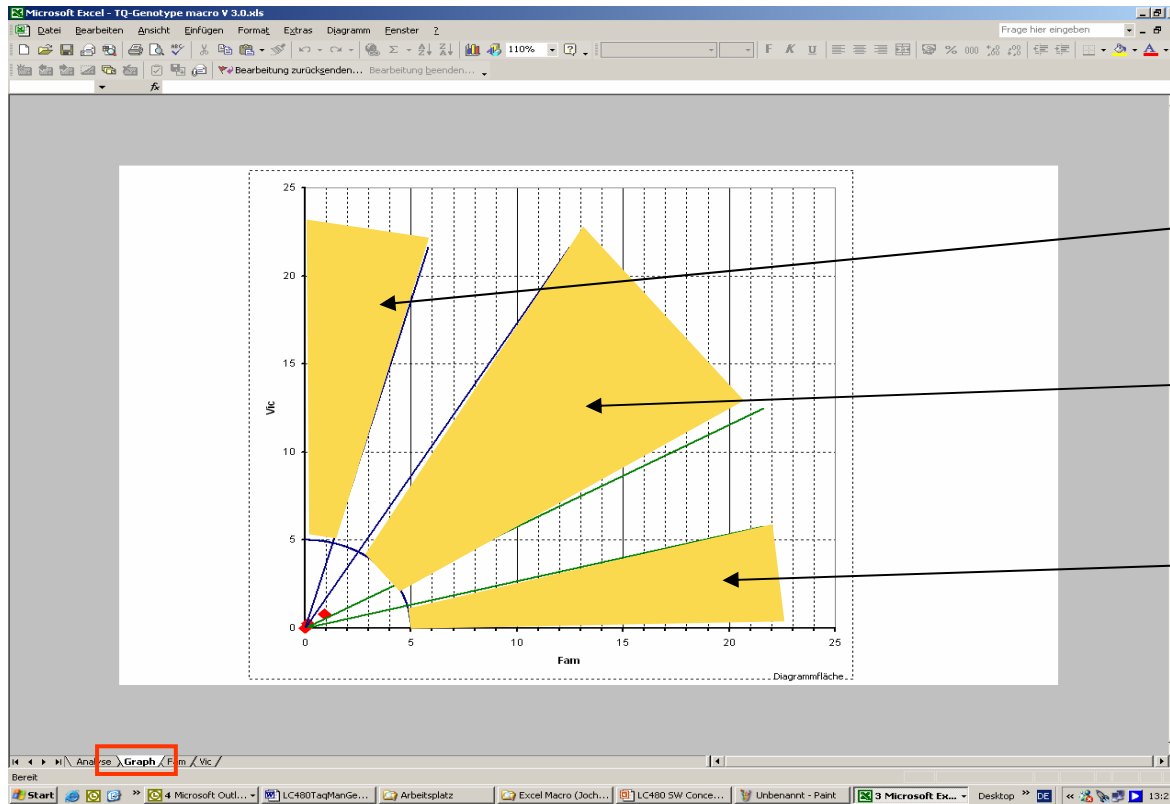
**Export raw data (right mouse click into Melting Curves graph), save as XML format**

# Open LightCycler<sup>®</sup> 480 Data in Excel for “TaxcelTool” Analysis

1	/chart						
2	/series#id	/series@title	/series@type	/series/points@count	/series/points/point@index	/series/points/point@X	/series/points/point@Y
3	1 A1: NTC	Line	6	0	59.5	4.445696685	
4	1 A1: NTC	Line	6	1	59.93	4.456921625	
5	1 A1: NTC	Line	6	2	60.09	4.46521963	
6	1 A1: NTC	Line	6	3	60.35	4.46550546	
7	1 A1: NTC	Line	6	4	60.61	4.459057577	
8	1 A1: NTC	Line	6	5	60.86	4.476251049	
9	2 A2: NTC	Line	6	0	59.5	5.183088071	
10	2 A2: NTC	Line	6	1	59.93	5.188867509	
11	2 A2: NTC	Line	6	2	60.09	5.203294035	
12	2 A2: NTC	Line	6	3	60.35	5.20005429	
13	2 A2: NTC	Line	6	4	60.61	5.216321608	
14	2 A2: NTC	Line	6	5	60.86	5.212149281	
15	3 A3: Ctrl hetero	Line	6	0	59.5	14.80496221	
16	3 A3: Ctrl hetero	Line	6	1	59.93	14.72645295	
17	3 A3: Ctrl hetero	Line	6	2	60.09	14.79052434	
18	3 A3: Ctrl hetero	Line	6	3	60.35	14.74491115	
19	3 A3: Ctrl hetero	Line	6	4	60.61	14.71229756	
20	3 A3: Ctrl hetero	Line	6	5	60.86	14.65034176	
21	4 A4: Ctrl hetero	Line	6	0	59.5	15.60785183	
22	4 A4: Ctrl hetero	Line	6	1	59.93	15.59485551	
23	4 A4: Ctrl hetero	Line	6	2	60.09	15.64536224	
24	4 A4: Ctrl hetero	Line	6	3	60.35	15.58118668	
25	4 A4: Ctrl hetero	Line	6	4	60.61	15.55275382	
26	4 A4: Ctrl hetero	Line	6	5	60.86	15.53475405	
27	5 A5: Ctrl Homo	Line	6	0	59.5	21.04933865	
28	5 A5: Ctrl Homo	Line	6	1	59.93	21.03878361	
29	5 A5: Ctrl Homo	Line	6	2	60.09	21.07704713	
30	5 A5: Ctrl Homo	Line	6	3	60.35	20.99402775	
31	5 A5: Ctrl Homo	Line	6	4	60.61	20.96075513	
32	5 A5: Ctrl Homo	Line	6	5	60.86	20.92264095	
33	6 A6: Ctrl Homo	Line	6	0	59.5	20.65787456	
34	6 A6: Ctrl Homo	Line	6	1	59.93	20.62698406	
35	6 A6: Ctrl Homo	Line	6	2	60.09	20.65346928	
36	6 A6: Ctrl Homo	Line	6	3	60.35	20.60220708	
37	6 A6: Ctrl Homo	Line	6	4	60.61	20.52184827	
38	6 A6: Ctrl Homo	Line	6	5	60.86	20.53432507	
39	7 B1: 1	Line	6	0	59.5	5.365309605	
40	7 B1: 1	Line	6	1	59.93	5.354681713	
41	7 B1: 1	Line	6	2	60.09	5.38742005	

**On Excel:  
Open FAM.xml and VIC.xml**

# “TaxcelTool” for the Analysis of LightCycler<sup>®</sup> 480 Data (1)



homozygote VIC

heterozygote

homozygote FAM

Import FAM and VIC excel sheets into “TaxcelTool“. Click on Graph to visualize groups

# “TaxcelTool” for the Analysis of LightCycler® 480 Data (2)

**Genotyping Auto Call**

Parameter	Value	Slope	max Fam	max Vic	y / x-max
Angle FAM: Determines Area for Homozygote VIC	15	0.26794919	1.00E-12	21.6075156	21.60752
Angle VIC: Determines Area for Homozygote FAM	75	3.73205081	1.00E-12	21.6075156	21.60752
Minimum Expression: Samples with an Expression Vector below this will be called Negative	5	0.57735027	1.00E-12	21.6075156	21.60752
Angle of Unknown zone: Determines the size of the area between VIC, Heterozygote, and FAM areas. Samples in this area will be called Unknown	15	1.73205081	1.00E-12	21.6075156	21.60752

Sample Name	Call	X: Fam	Y: Vic	X Fam	Y Vic	Vector	angle
A1: NTC	Negative	4.461442	3.50588022	4.461442	3.5058802	0.150166	0.251349
A2: NTC	Negative	5.20062913	4.03311399	5.200629	4.033114	0.893953	0.778583
A3: Ctl hetero	Heterozygote	14.7302487	16.1264849	14.73025	15.126485	10.42697	11.67195
A4: Ctl hetero	Heterozygote	15.6861273	16.8932483	15.68613	16.893248	11.27485	12.62872
A5: Ctl homo	Homozygote FAM	21.0070869	5.32915425	21.0071	5.3291543	16.69682	2.074623
A6: Ctl homo	Homozygote FAM	20.5994514	5.31600626	20.59945	5.3160063	16.28818	2.061475
B1: 1	Homozygote VIC	5.3722123	20.3368799	5.372212	20.33688	1.060936	17.08235
B2: 2	Homozygote VIC	5.30457894	19.9491035	5.304579	19.949103	0.993303	16.69457
B3: 3	Homozygote VIC	4.9671526	16.5095734	4.967153	16.509573	0.655877	13.25504
B4: 4	Homozygote VIC	5.20911011	24.862047	5.20911	24.862047	1.897834	21.60752
B5: 5	Homozygote VIC	5.74288801	21.3609305	5.742888	21.36093	1.431612	18.1064
B6: 6	Heterozygote	14.1816509	14.6175484	14.18155	14.617548	9.890275	11.36302
B7: 7	Heterozygote	13.0521521	13.3509337	13.05215	13.350934	8.740876	10.0864
B8: 8	Heterozygote	16.5145461	16.7467348	16.51455	16.746735	11.20327	12.4822
B9: 9	Heterozygote	16.7051372	17.2088415	16.70514	17.208841	12.39986	13.95431
B10: 10	Heterozygote	14.776116	14.9621571	14.77612	14.962157	10.46484	11.70763
B11: 11	Homozygote FAM	21.3450507	5.150626	21.34505	5.150626	17.03377	1.896095
B12: 12	Homozygote FAM	20.8533643	5.0939484	20.85336	5.093948	16.54241	1.814463
B13: 13	Homozygote FAM	23.4199909	5.51075197	23.41999	5.510752	19.10871	2.256221
B14: 14	Homozygote FAM	20.2902039	4.81440811	20.2902	4.8144081	15.97893	1.559877
B15: 15	Homozygote FAM	21.834738	5.2208446	21.83474	5.2208446	17.52346	1.966313
E1: NTC	Negative	4.4514185	3.40189889	4.451418	3.4018987	0.140142	0.147367
E2: NTC	Negative	4.31127609	3.25453142	4.311276	3.2545314	0	0

Click on Analysis to visualize the listed genotypes and to adjust angle settings

# Content

- Principles and advantages of melting-curve based genotyping with fluorescence-labeled probes
- Discrimination by allele specific PCR, end-point genotyping on the LightCycler<sup>®</sup> 480 System
- **High-Resolution Melting for mutation scanning**

# High-Resolution Melting Analysis

## *Introduction*

- Melting curve analysis (MCA) is well established as a method to characterize amplicons, e.g., with SYBR Green I, HybProbe (FRET) or SimpleProbe probes.
- HRM is an extension of melting curve analysis
  - allowing to extract even more information (also unknown variants) out of melting curves at lower cost and with less effort
  - requiring special fluorophores, a high-performance instrument (block homogeneity, suitable filters, optical sensitivity and resolution) and special analysis algorithms.

# Possible Applications

- Mutation Discovery and SNP Detection
- DNA methylation analysis
- DNA Mapping
- Species identification
- ...

# High-Resolution Melting

## *Innovations and Prerequisites*

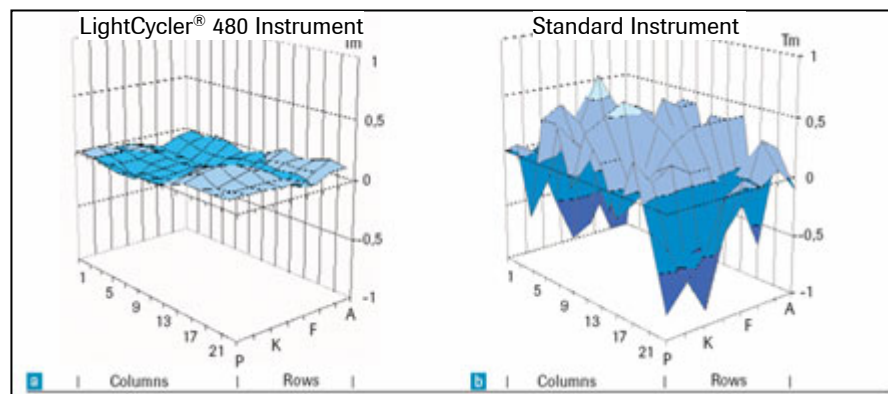
- **Precise Instrument** to allow genotyping and/or mutation scanning of whole PCR products.
  - homogenous temperature profile and temperature control
  - high sensitivity optical system (light source and detection system)
- **Novel intercalating dye** to identify heteroduplex DNA
  - saturating, non-inhibitory dsDNA binding without redistribution during melting
- **Software** generating normalized and temperature shifted fluorescence difference plot instead of derivative melting curves revealing higher resolution of subtle changes in the melting behaviour of heteroduplexes



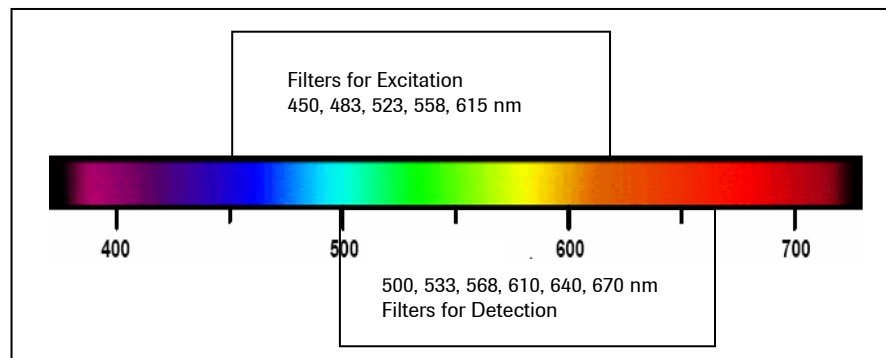
# LightCycler® 480 System Instrumentation



- **Optimized heating and cooling technology for increased speed and maximized temperature uniformity**



- **Optimized arrangement of optical components for homogeneous excitation and fluorescence detection**

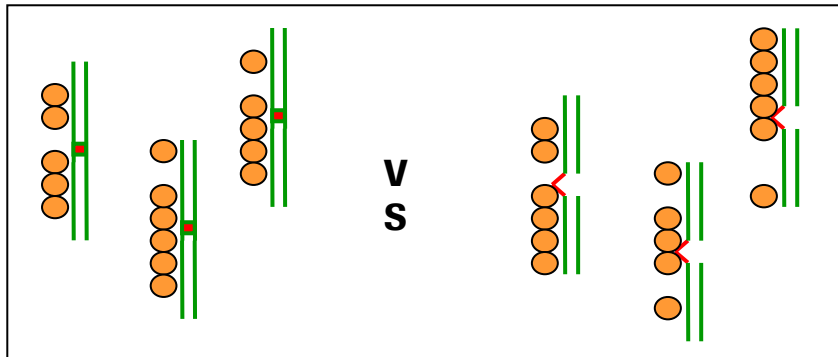


# Mutation Detection using HRM

## *Saturating Dyes*

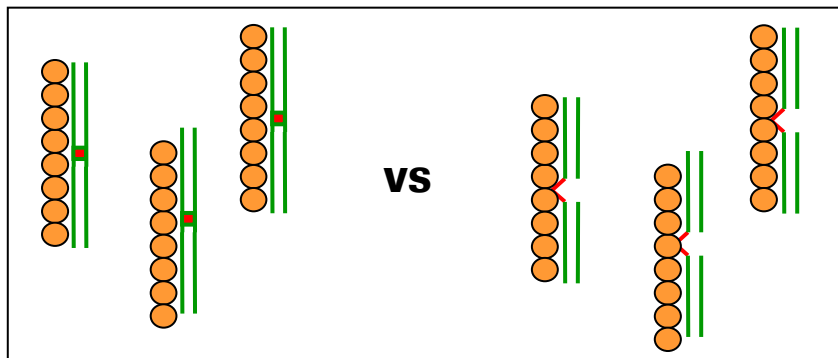
**homoduplexes**

**heteroduplexes**



Fluorescent ds-DNA specific dyes  
(e.g., SYBR Green I)

- individual curves not sharp
- overlap is the same for homo- and heteroduplexes

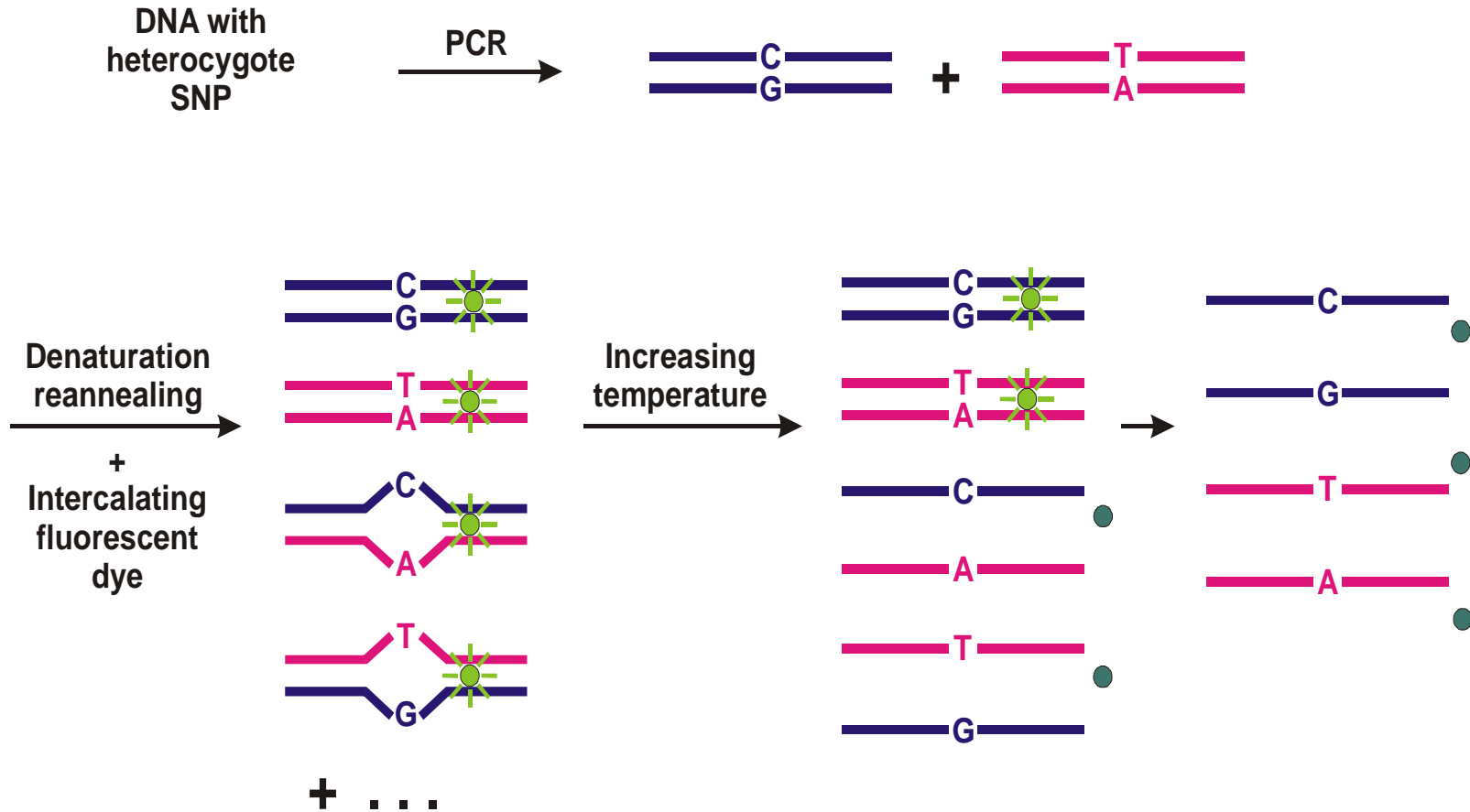


Saturating dye

- uniform, sharp signals
- only sequence but not dye makes a difference

# Amplicon Melting

## *Principle of Gene Scanning by HRM*



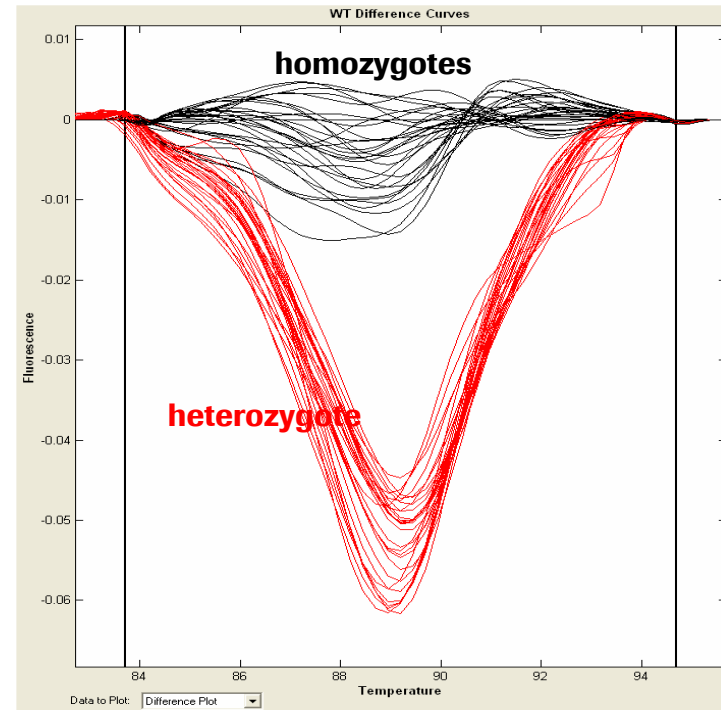
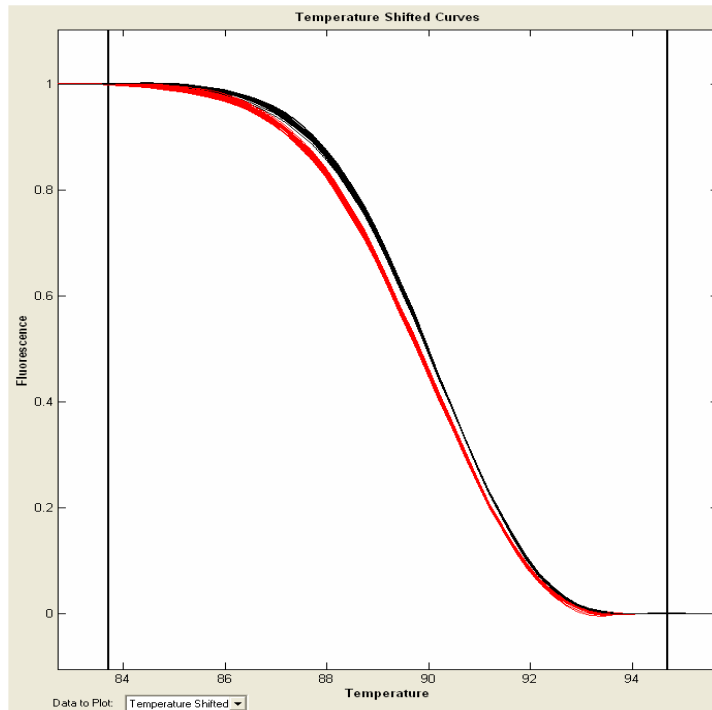
# Heterozygote/Homozygote Distinction

## *Target mdr-1*

**mdr-1 SNP (C→T)**, DNA of 60 independent blood donors

Detection dye: R27

Amplicon length: 247 bp



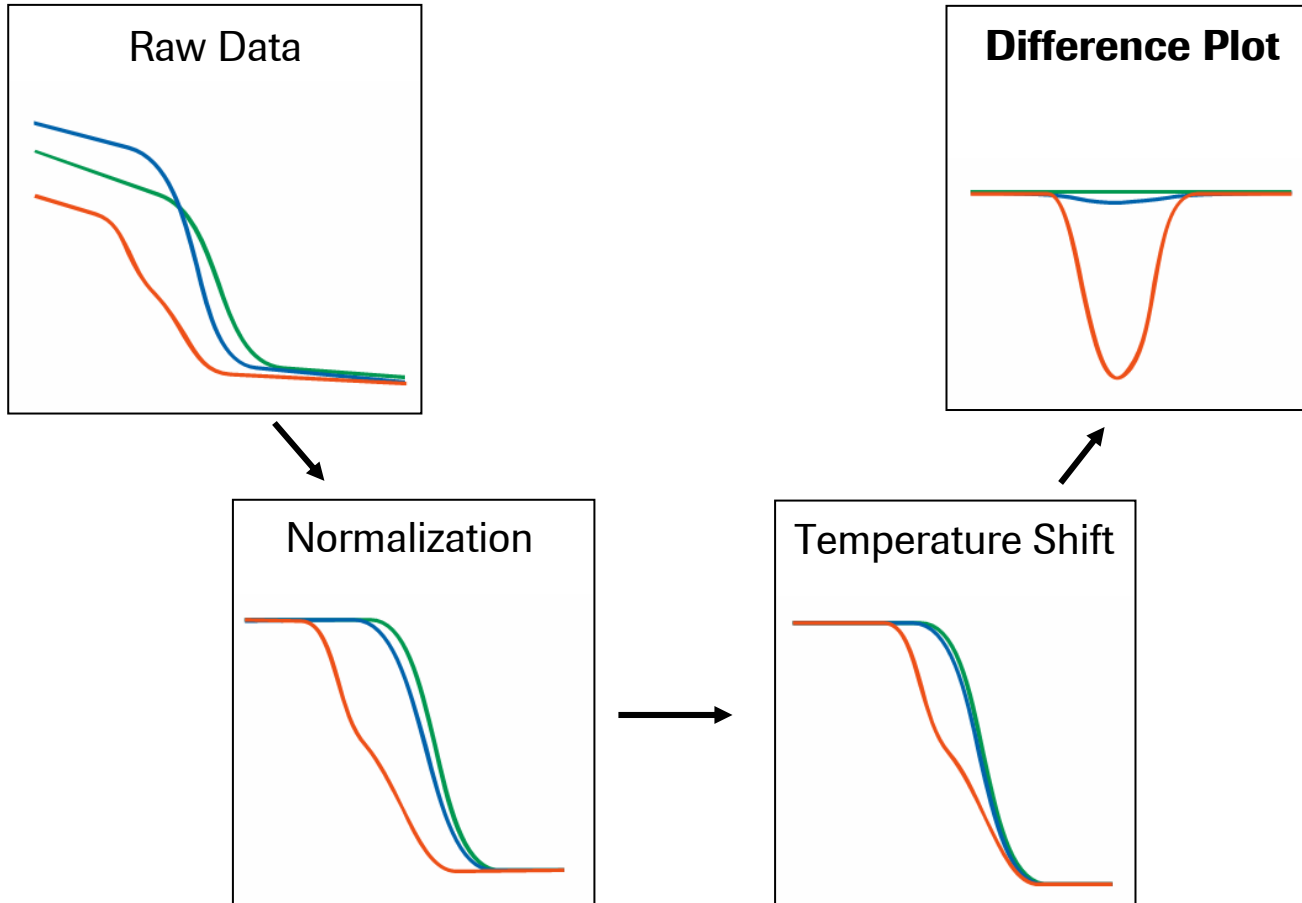
# Mutation Detection using HRM

## *Finding Heterozygotes*

- **Amplicon Melting of homozygote samples (containing homoduplexes wt or mut) give very similar curve shapes.**
- **Amplicon Melting of heterozygote samples (containing homo and heteroduplexes) give curve shapes which are highly distinct.**
- **No identification of specific sequences (genotyping);  
Only differences between two genomes are detected.**

# High-Resolution Melting Curve Analysis

## *Difference Plot*



# High-Resolution Melting

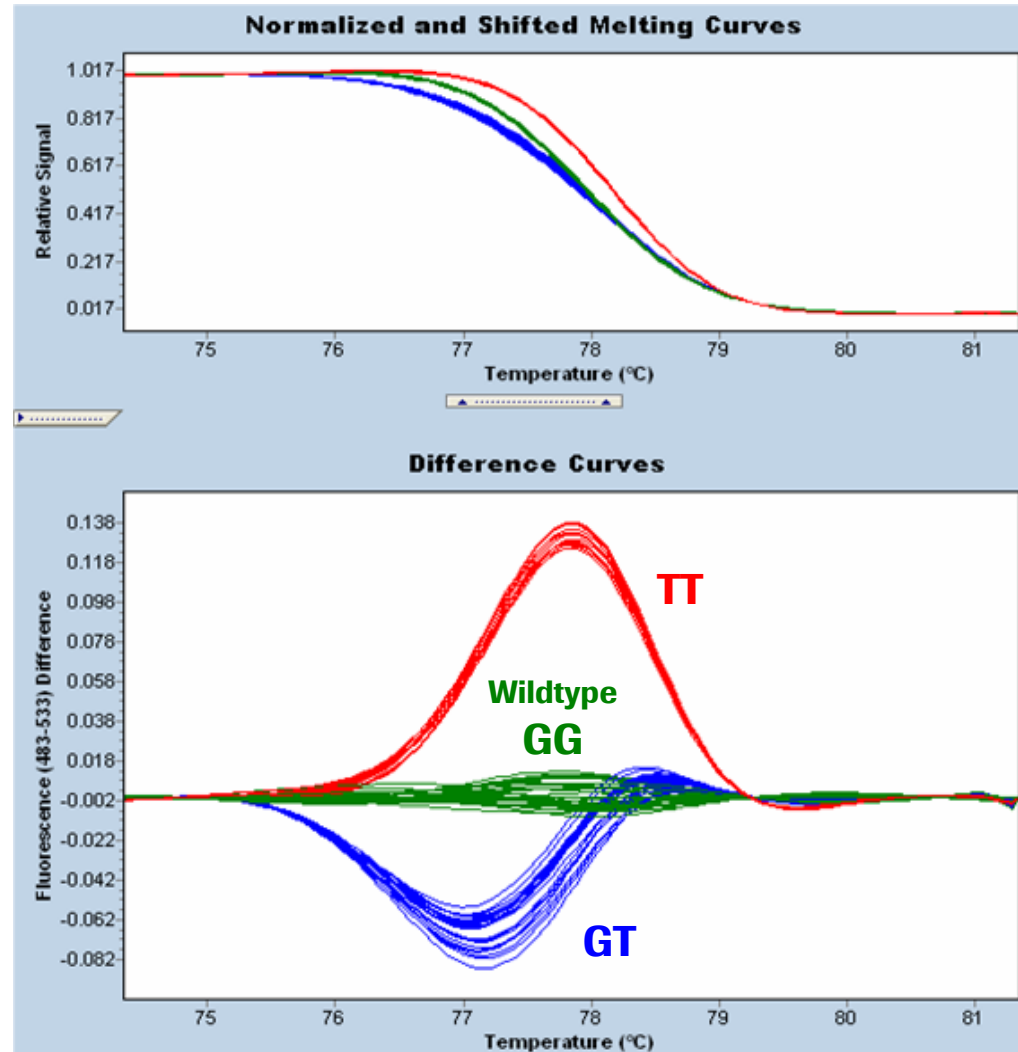
## Example 1

**Target: LPLH3**

**SNP G→T**

**Amplicon 164 bp**

**72 samples**



# Gene Scanning

## Example 2

### Target: MBL2

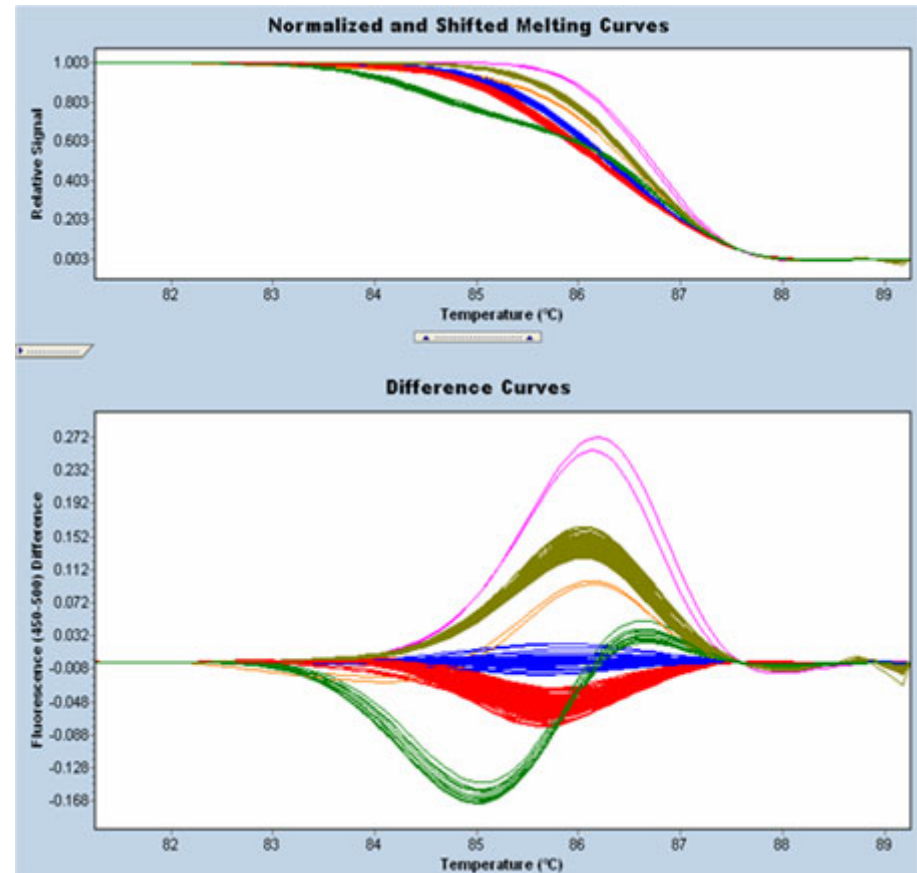
Screening for sequence variants in  
384 unknown samples

Amplicon 219 bp

4 common and 2 rare groups of  
different sequences are found

In literature, 3 polymorphic sites are  
described, the most frequent alleles are  
the 4 variants:

- A) C / G / G
- B) C / A / G
- C) C / G / A
- D) T / G / G





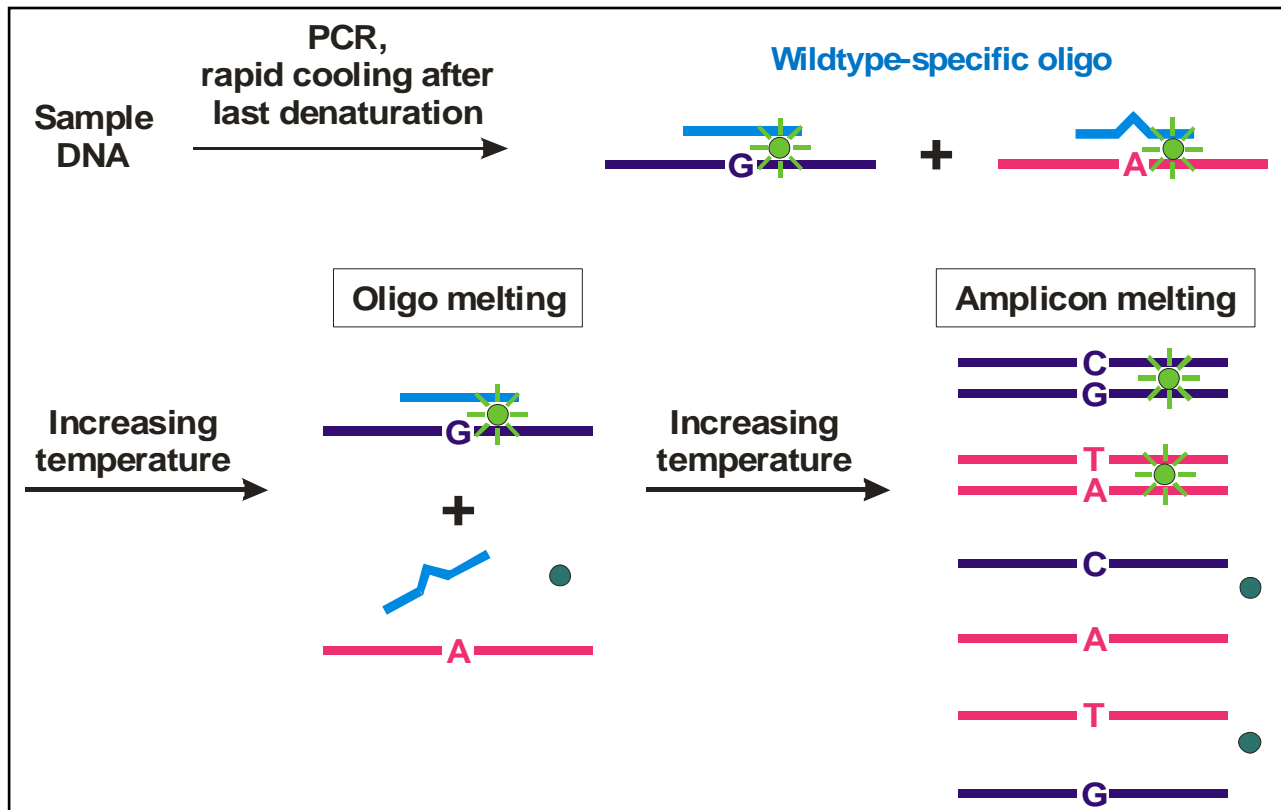
# Recommendations

- Prefer smaller amplicons for melting (up to 400bp)
- Use highly purified DNA for amplification
- Well established PCR amplification products
  - Bias free amplification (e.g., free of primer dimer, by-products)
  - Optimized conditions (optimized Roche-Master will be available)
  - Keep salt- and primer concentration as low as possible
- Check amplification curves
  - Similar curve shape
  - Similar plateau phase (same PCR product concentration)
- Accurate normalization of data

# Unlabeled Probe Melting

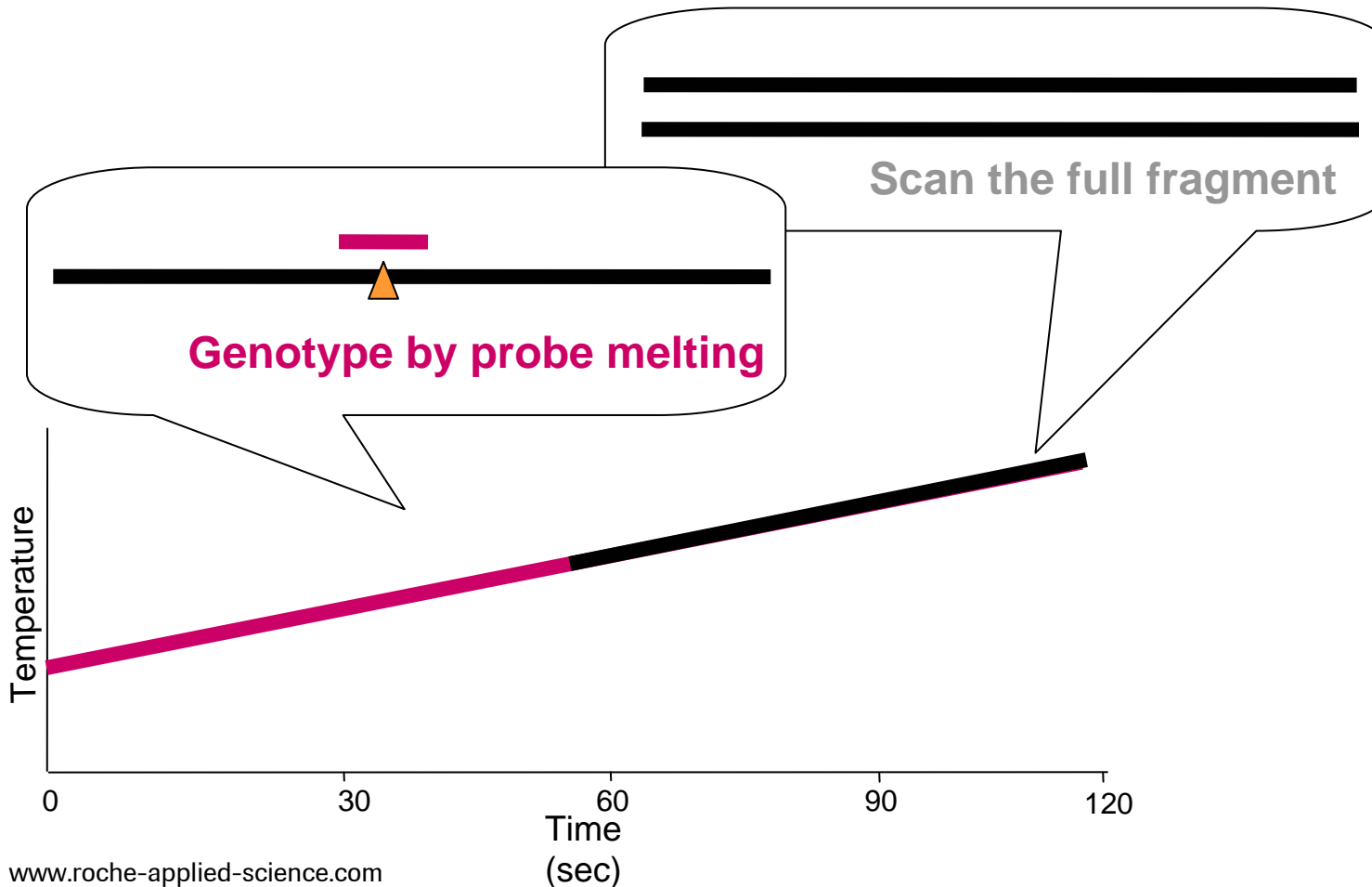
## *Principle of Genotyping by HRM*

High-Resolution Melting with intercalating dye and unlabeled oligo specific for known mutation site



# Simultaneous Genotyping and Scanning

## *Unlabeled Probe Genotyping and Amplicon Melting*



# High-Resolution Melting

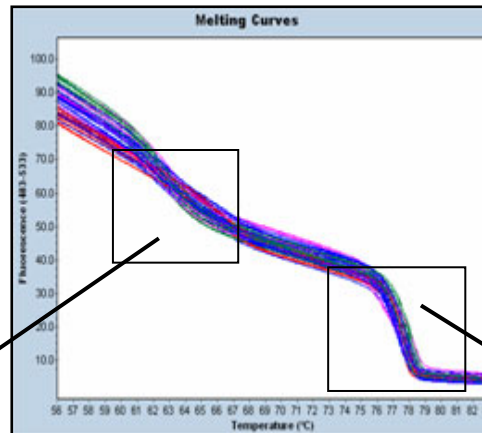
## *Combined Unlabeled Probe and Amplicon Melting*

**Target: TNF $\alpha$**

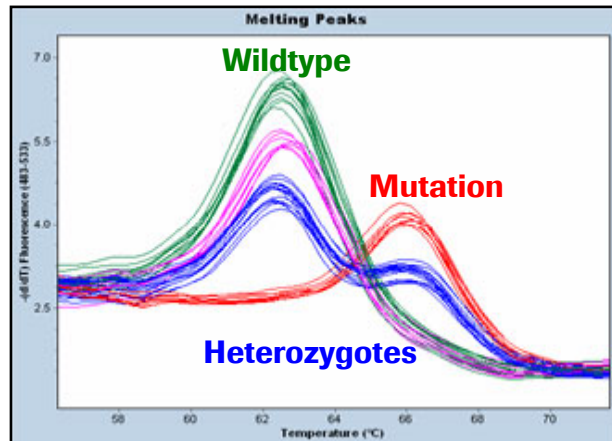
Probe for SNP C $\rightarrow$ T

Amplicon 136 bp

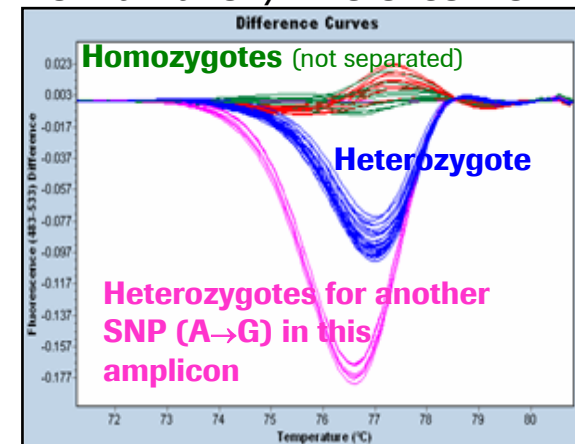
96 samples



**1st Derivative**

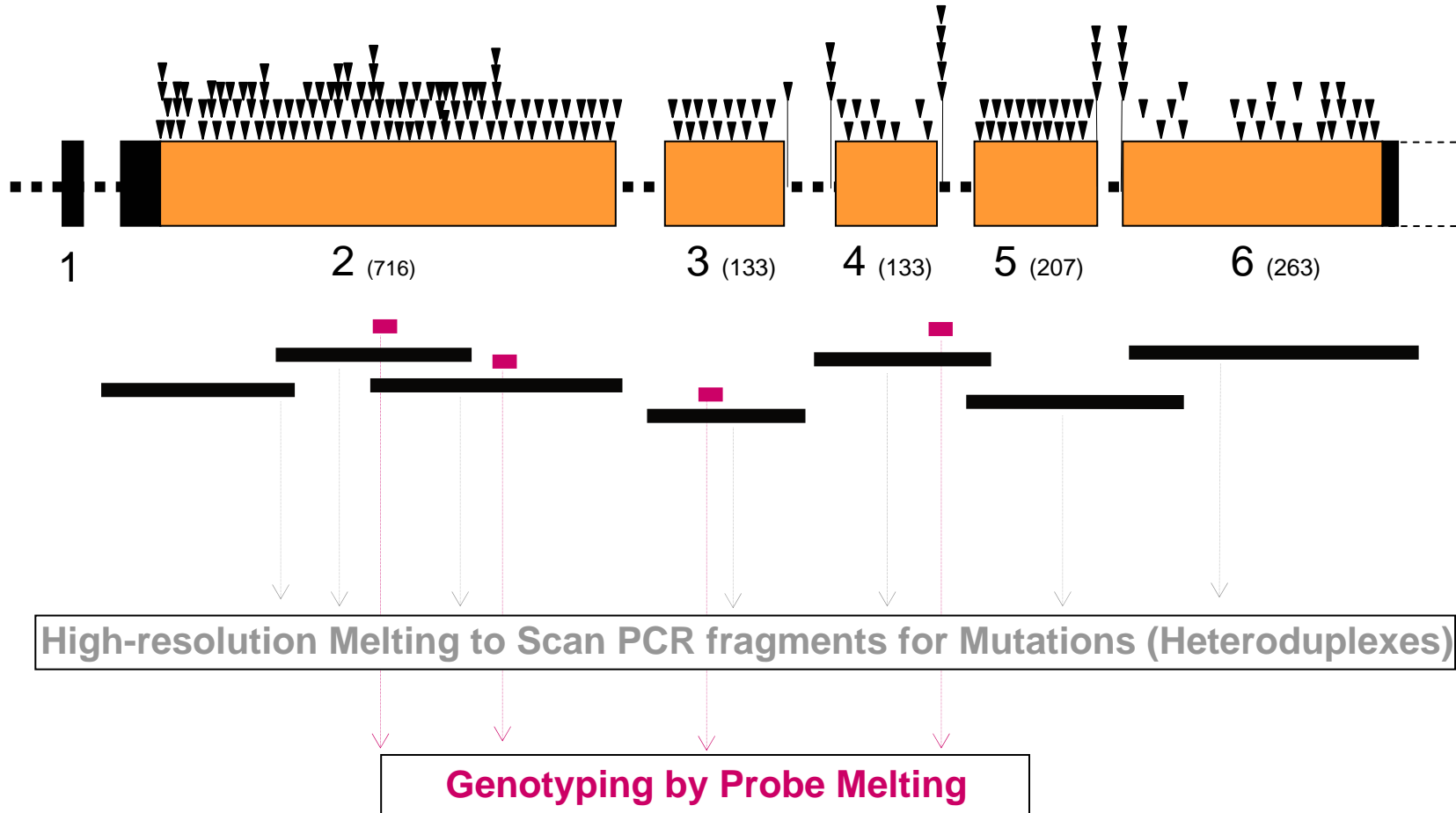


**Normalization, Difference Plot**



# SNP Detection and Analysis

## *Two Combined Approaches*



# Benefits and Requirements

- Simple and flexible technology
  - More information, faster
  - No need for probe based assays
- Robust instrumentation
  - Lower maintenance requirements compared to e.g., to dHPLC
- Well established PCR amplification products
  - Bias free amplification (e.g., free of primer dimer, by-products)
  - Optimized conditions (optimized Roche-Master will be available)

# Literature on High-Resolution Melting

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