Roche

Diagnostics

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Genotyping Concept for the LightCycler 480® System

Applications for the LightCycler® 480 System

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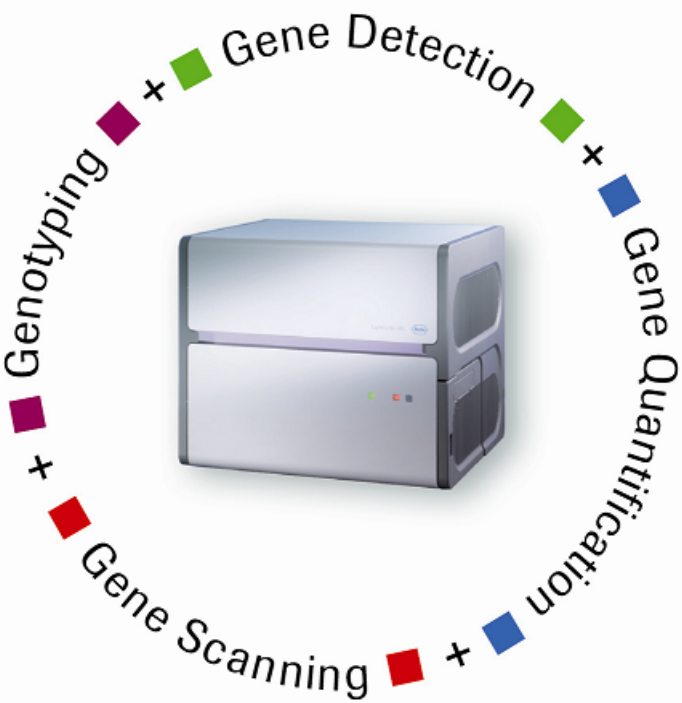
Diagnostics



- 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

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Diagnostics

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.

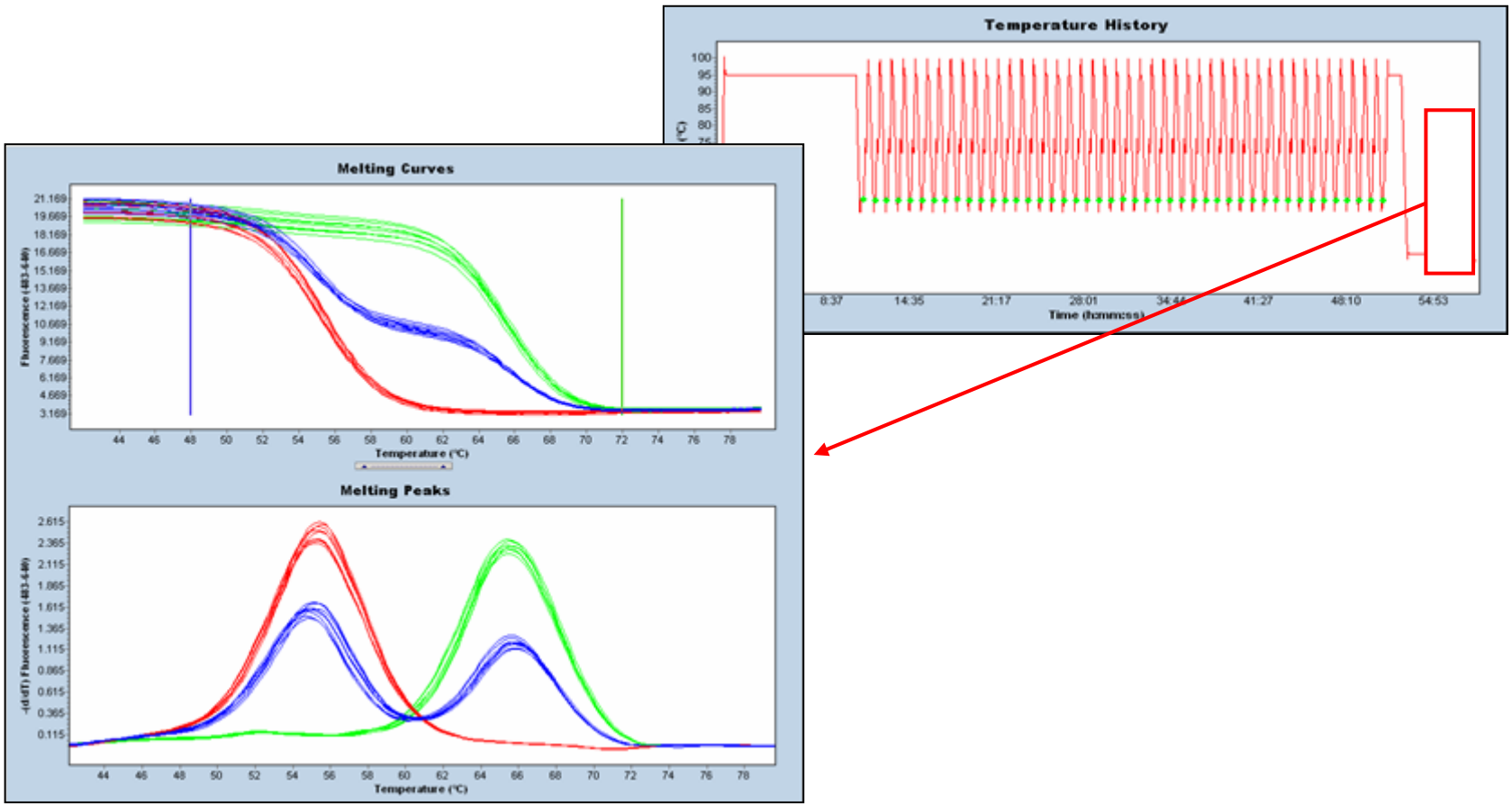
Content

- **Principles and advantages of melting-curve based genotyping with fluorescence-labeled probes**

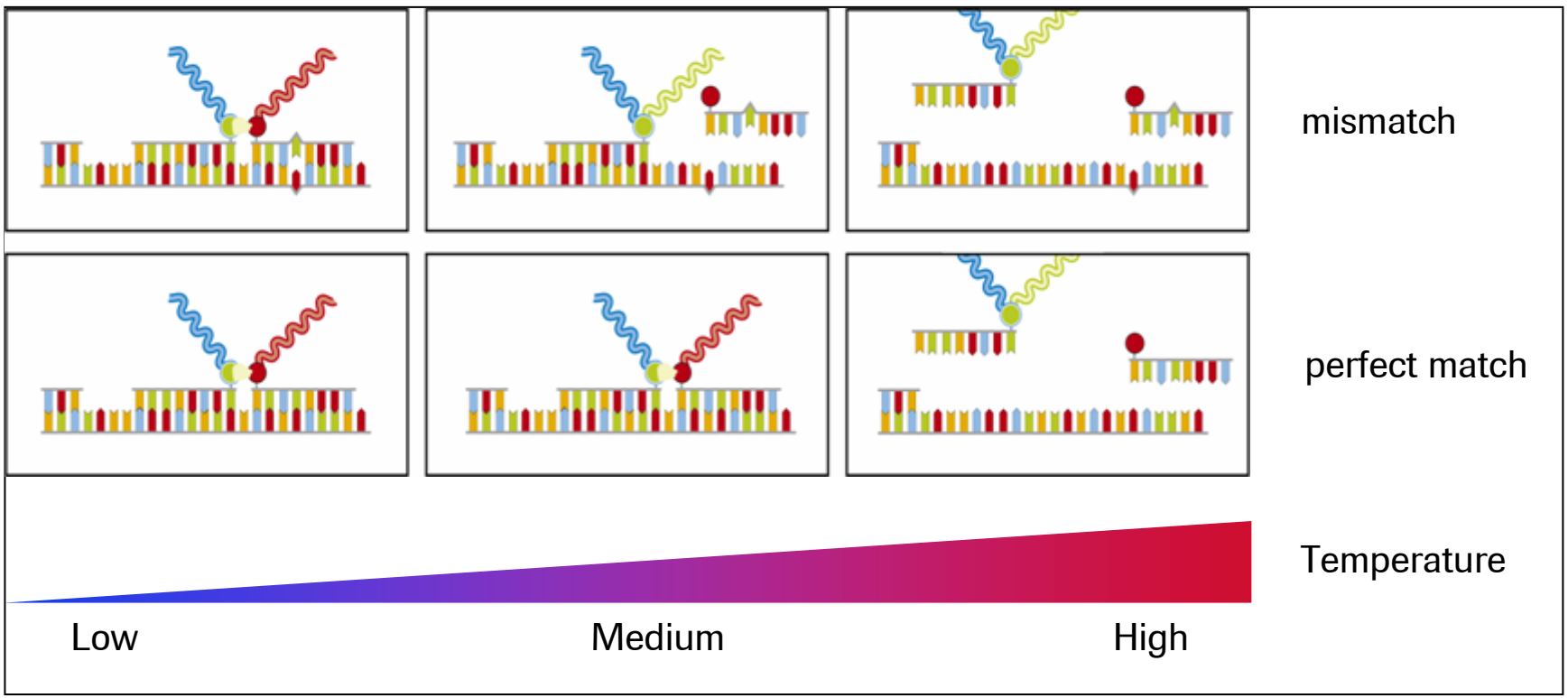
- Discrimination by allele specific PCR, end-point genotyping on the LightCycler® 480 System

- High-Resolution Melting for mutation scanning

Genotyping Technique using Melting Curves



Genotyping with HybProbe Probes



LightCycler® 480 Genotyping Software

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Diagnostics

- 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® 480 Genotyping Software

Results Screen

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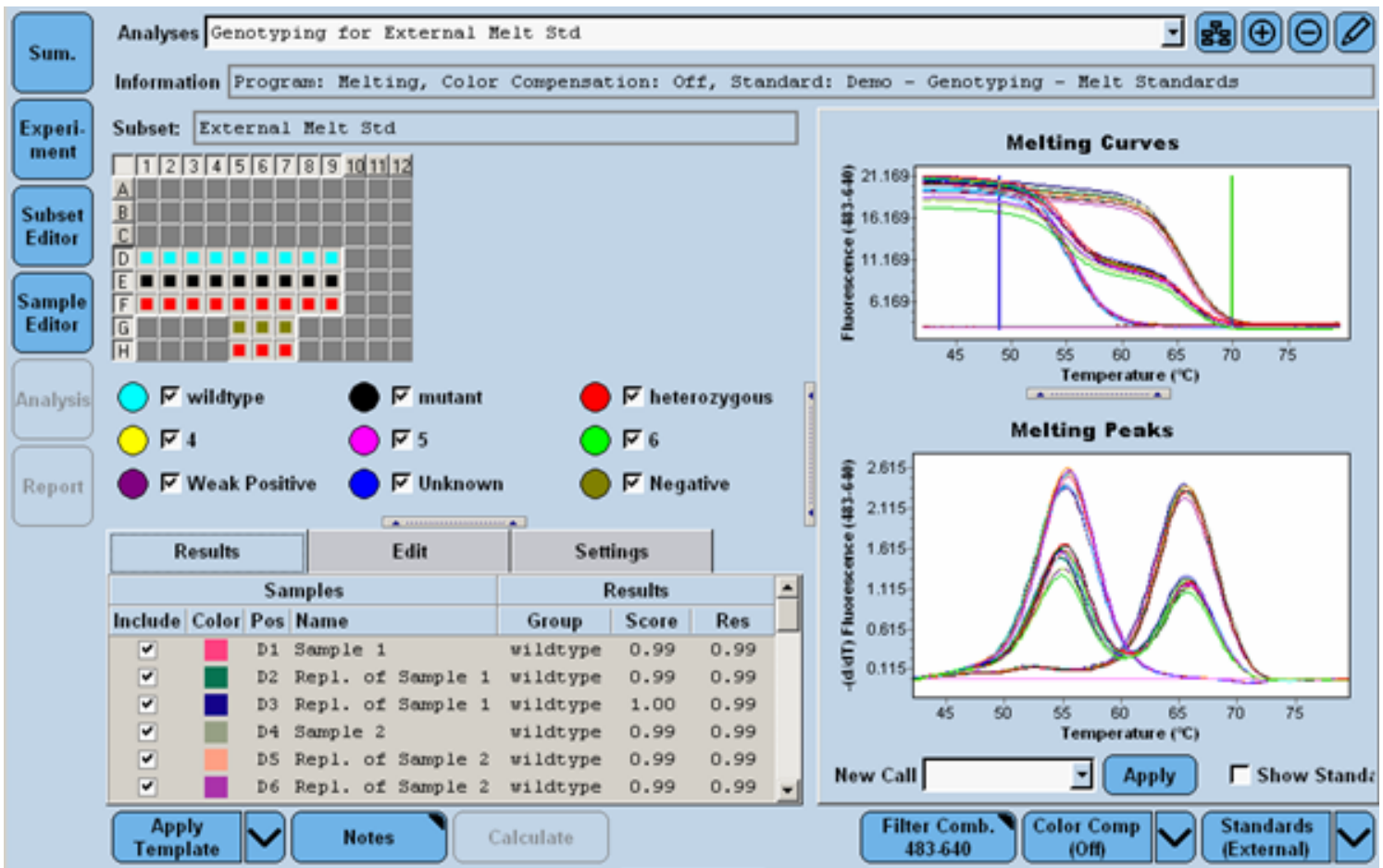
Diagnostics



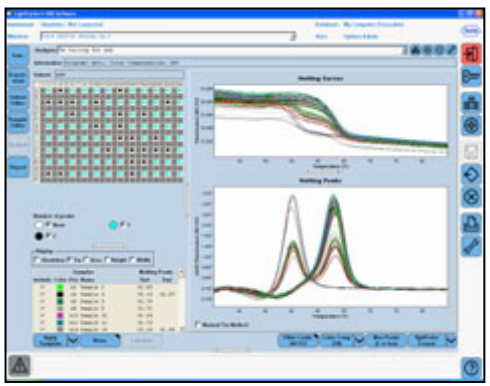
LightCycler® 480 Performance

High-Throughput SNP Analysis

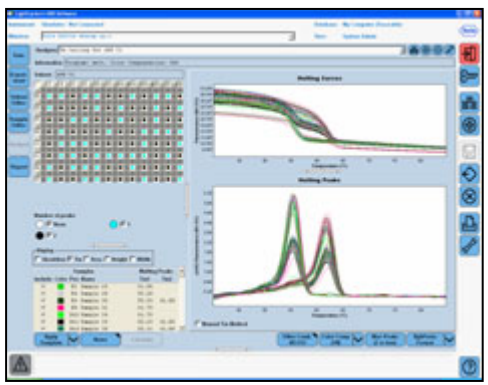
Roche Diagnostics

Set A1-O23
MDR1 C/T

Set B1-P23
LPLH3 C/A



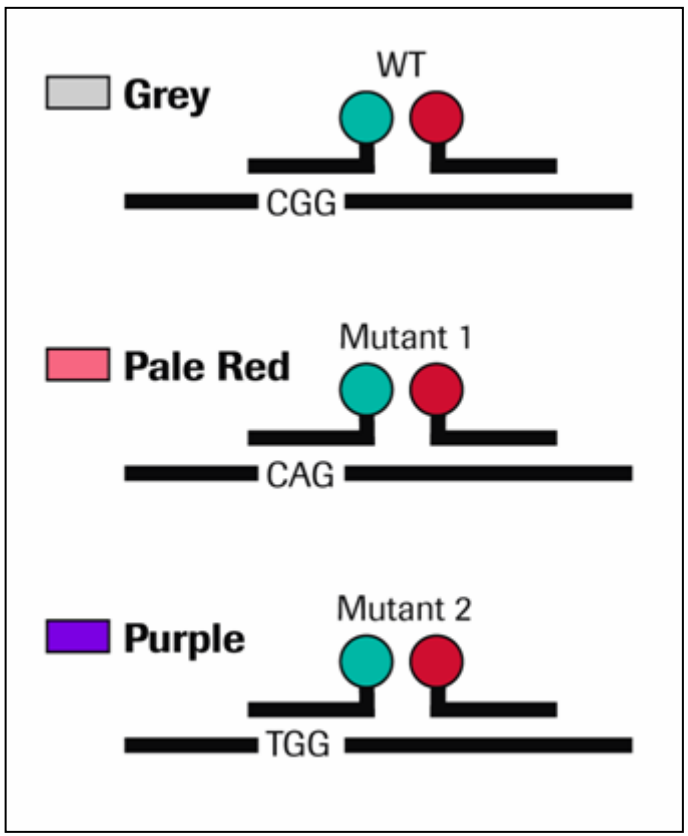
Set A2-O24
ADD1 C/A

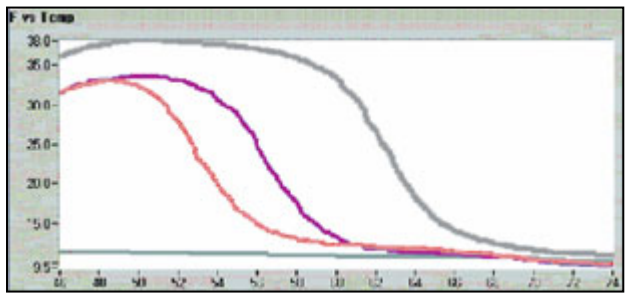


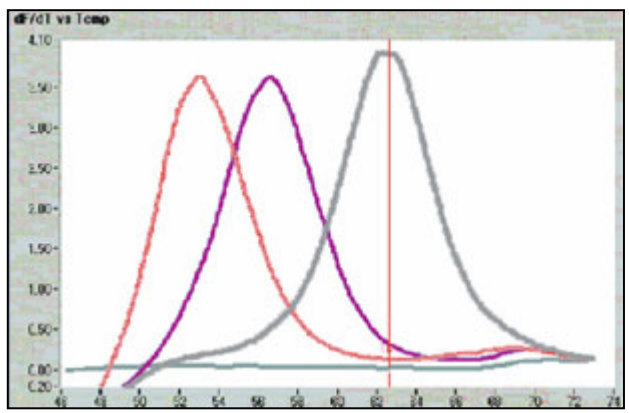
Set B2-P24
ADR1-C1 C/T

LightCycler® Genotyping: Example

SNP with Three Different Alleles







Template:
Plasmid DNAs

Target:
Apolipoprotein B

Single Color:
LC RED 640

LightCycler® 480 System
Benefits for Genotyping

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Diagnostics

- **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® 480 System**
- High-Resolution Melting for mutation scanning

Allelic Discrimination

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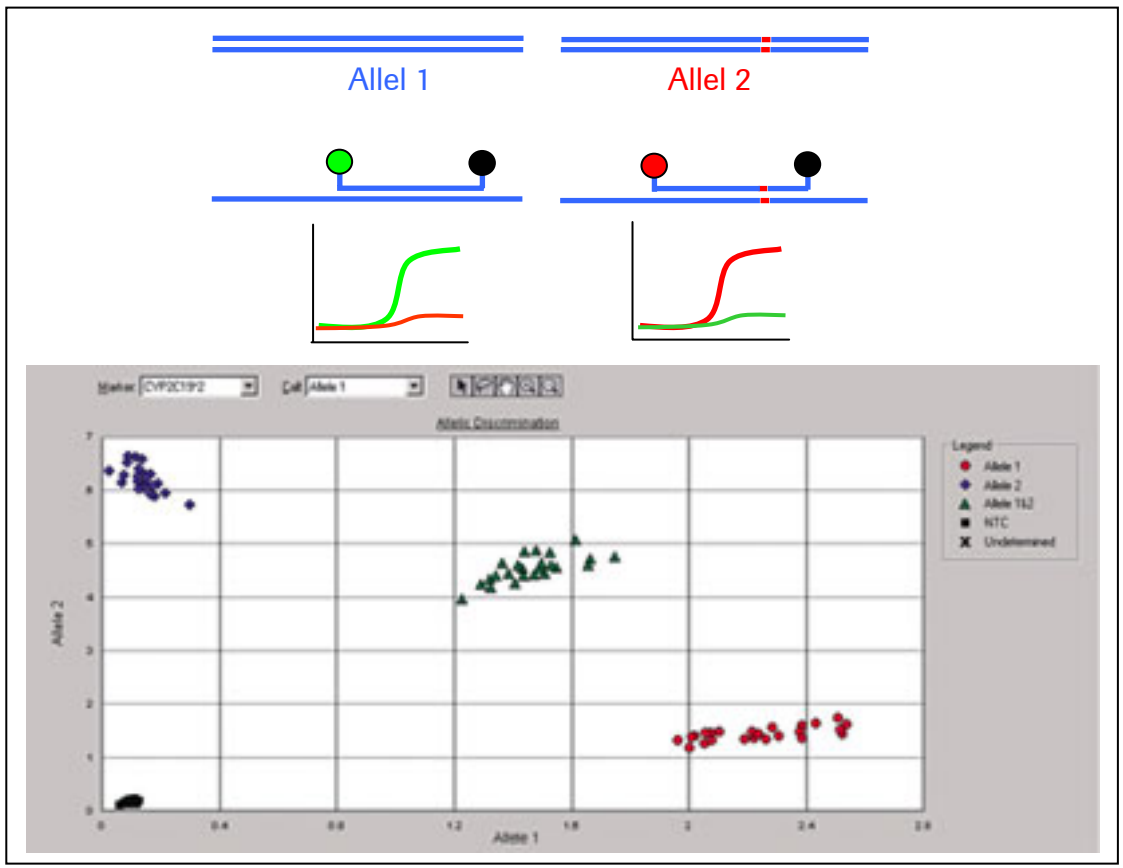
Diagnostics

- 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® 480 End-Point Genotyping

Analysis of Hydrolysis Probe Assays

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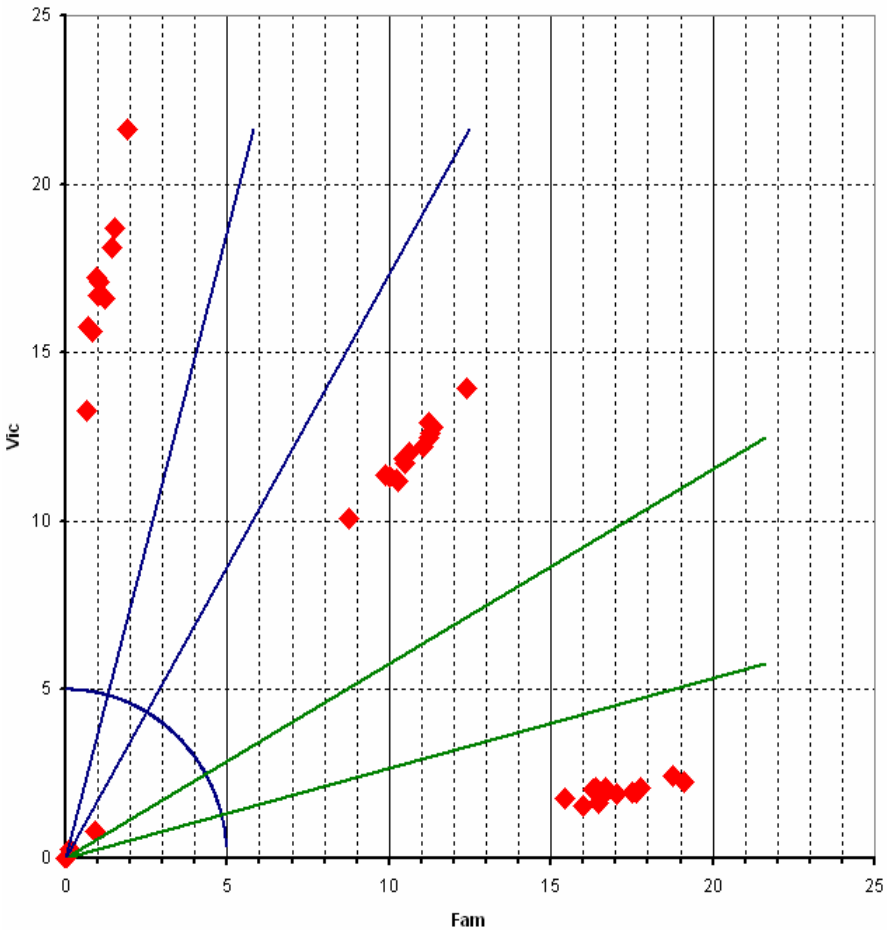
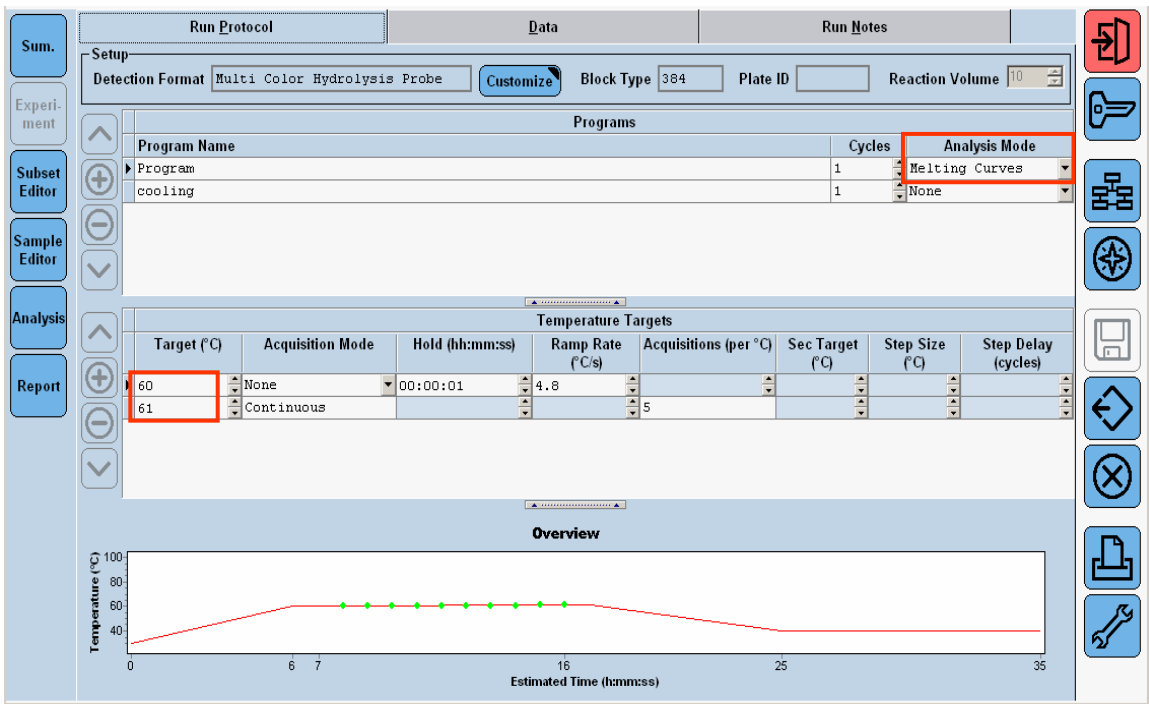
Diagnostics



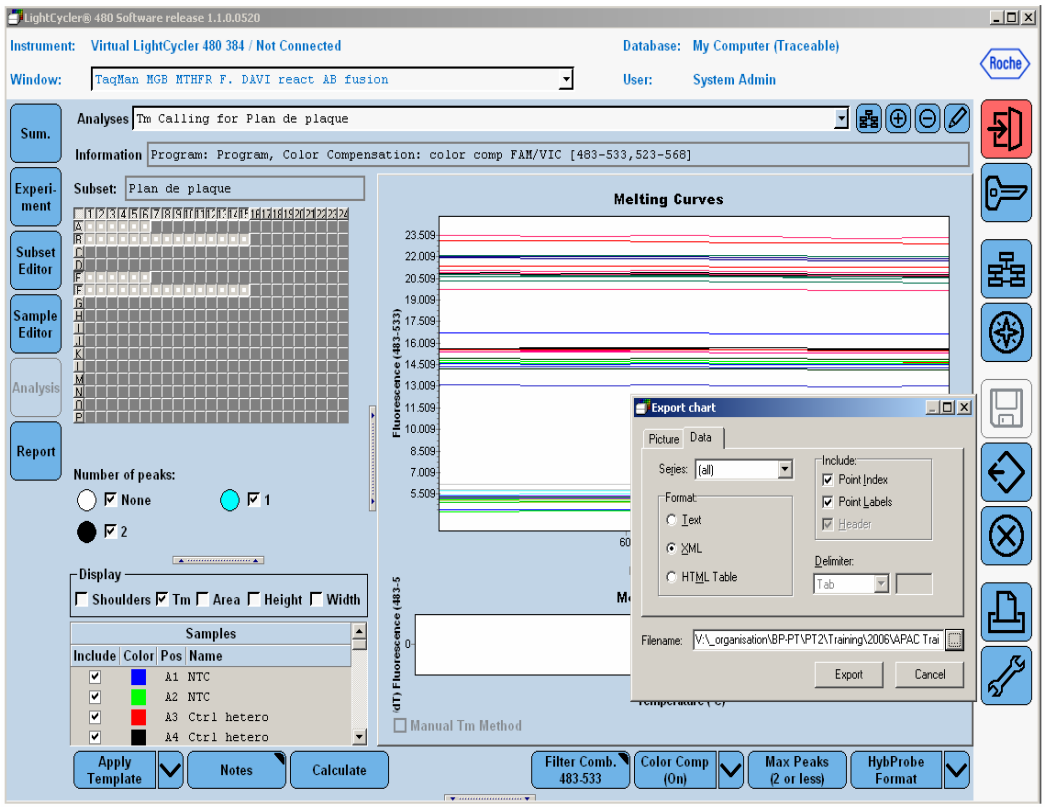
- **Excel worksheet (“TaxcelTool”) available for analysis of LightCycler® 480 data (on request)**
- **Bridging the gap between launch of SW 1.5 with module for Hydrolysis Probe Genotyping**

Create LightCycler® 480 Data for “TaxcelTool” Analysis



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® 480 Data for “TaxcelTool” Analysis



Open analysis "Tm Calling“:

- **Settings for FAM data:**

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

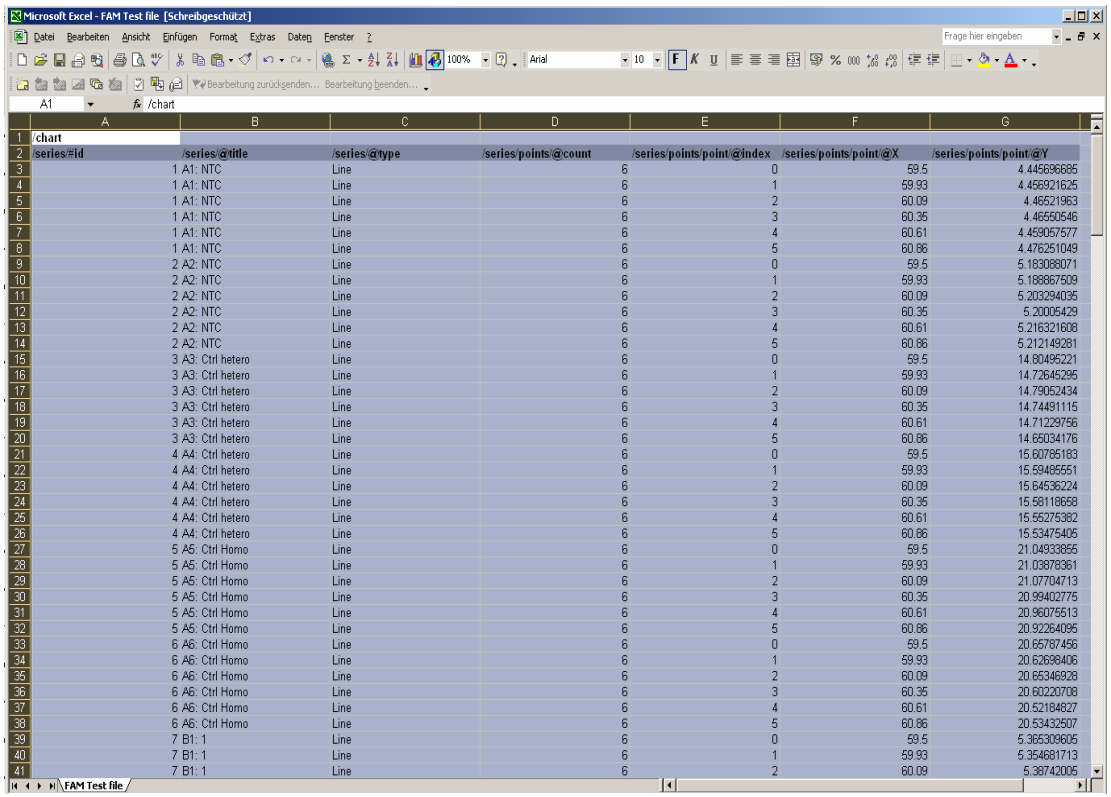
- **Settings for VIC/Hex data:**

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

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

Open LightCycler® 480 Data in Excel for “TaxcelTool” Analysis

Roche Diagnostics



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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
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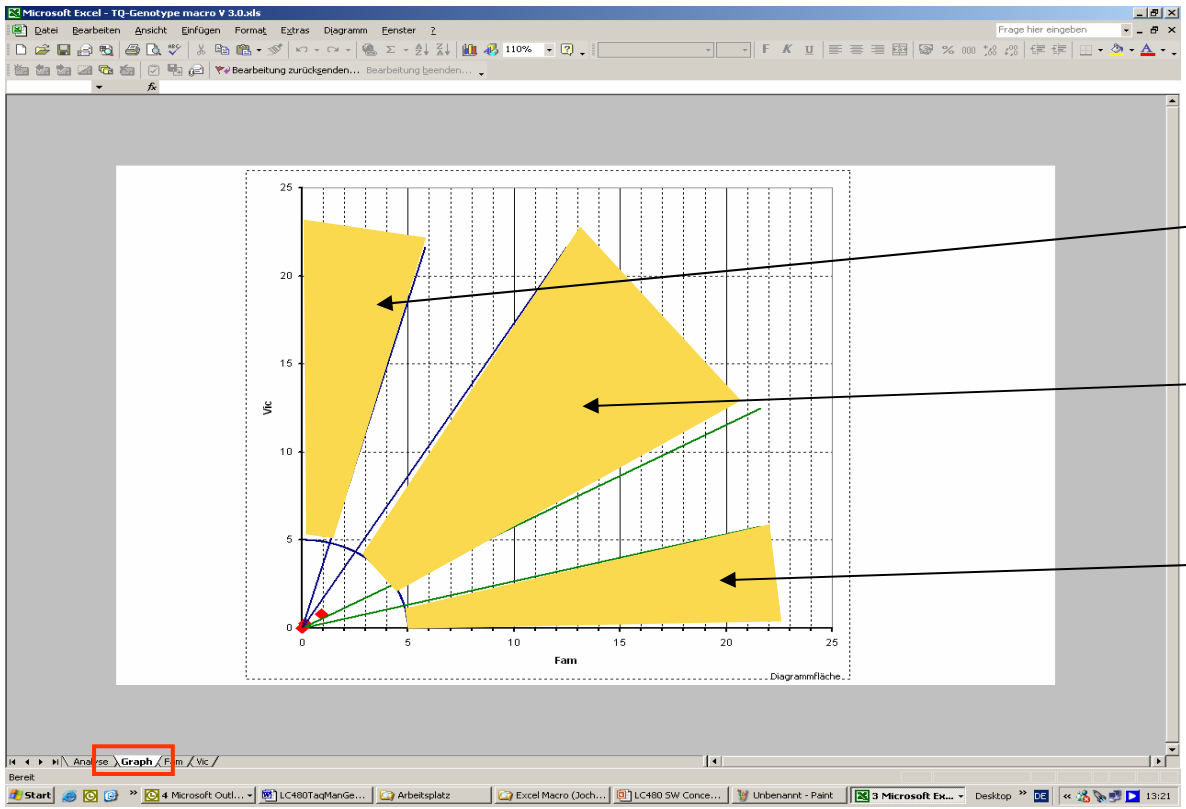
16	3	A3: Ctrl hetero	Line	6	1	59.93	14.72645295
17	3	A3: Ctrl hetero	Line	6	2	60.09	14.79052434
18	3	A3: Ctrl hetero	Line	6	3	60.35	14.74491115
19	3	A3: Ctrl hetero	Line	6	4	60.61	14.71229756
20	3	A3: Ctrl hetero	Line	6	5	60.86	14.65034176
21	4	A4: Ctrl hetero	Line	6	0	59.5	15.60785183
22	4	A4: Ctrl 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	15.58118658
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	21.04933855
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

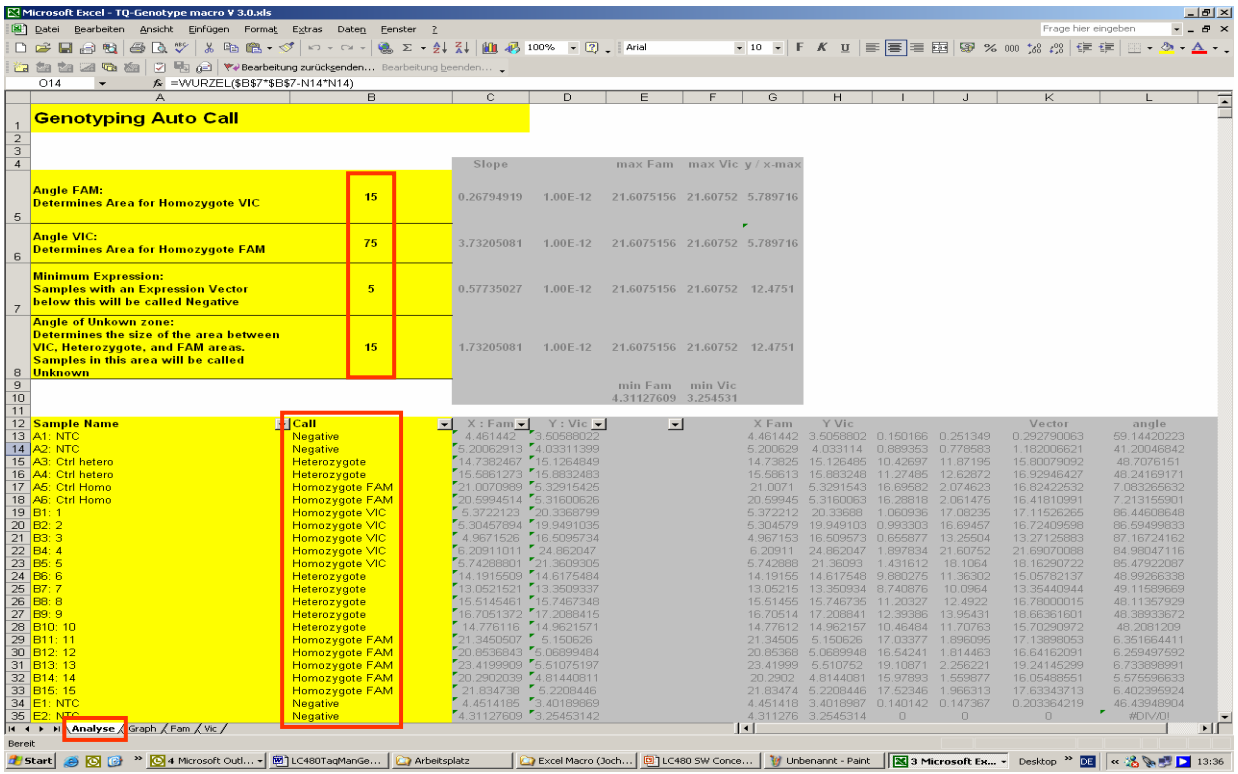
On Excel:
Open FAM.xml and VIC.xml

“TaxcelTool” for the Analysis of LightCycler® 480 Data (1)



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

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



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® 480 System
- **High-Resolution Melting for mutation scanning**

High-Resolution Melting Analysis

Introduction

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Diagnostics

- 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

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Diagnostics

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

High-Resolution Melting
Innovations and Prerequisites

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Diagnostics

- **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

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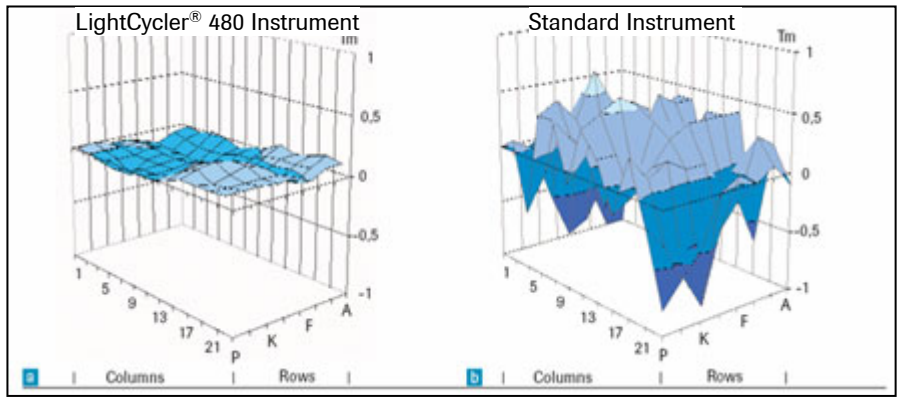
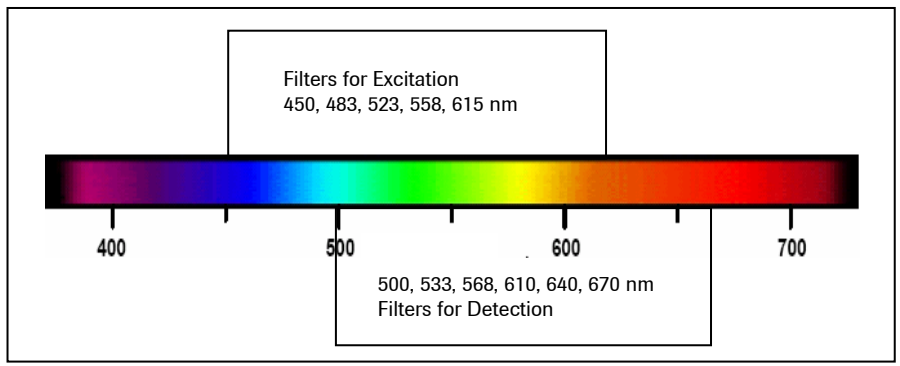
Diagnostics

- **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

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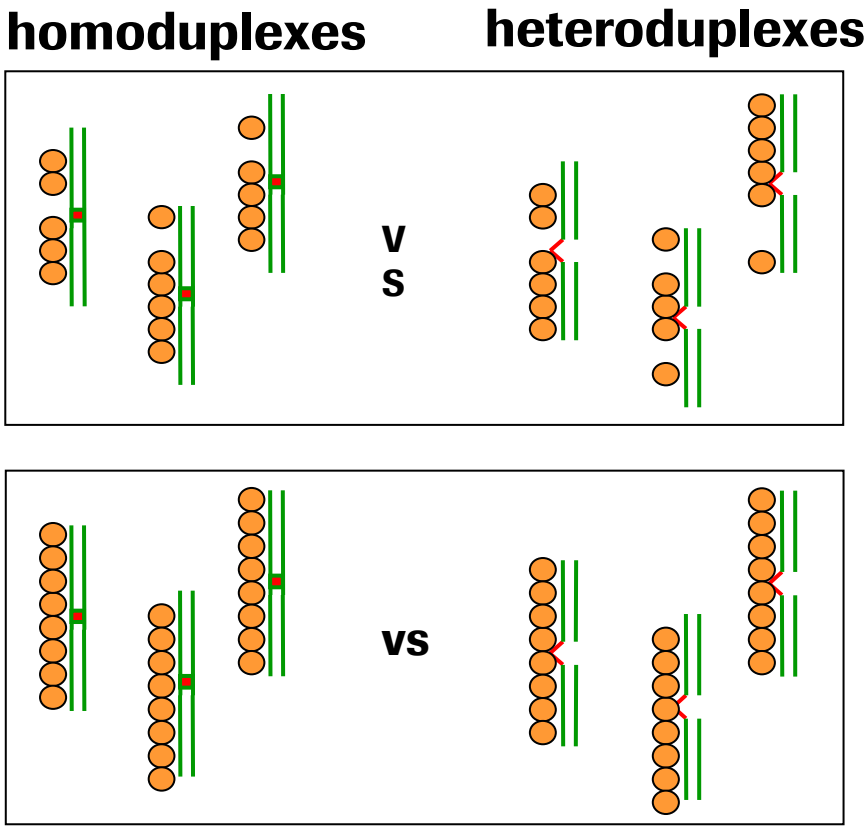
Diagnostics



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

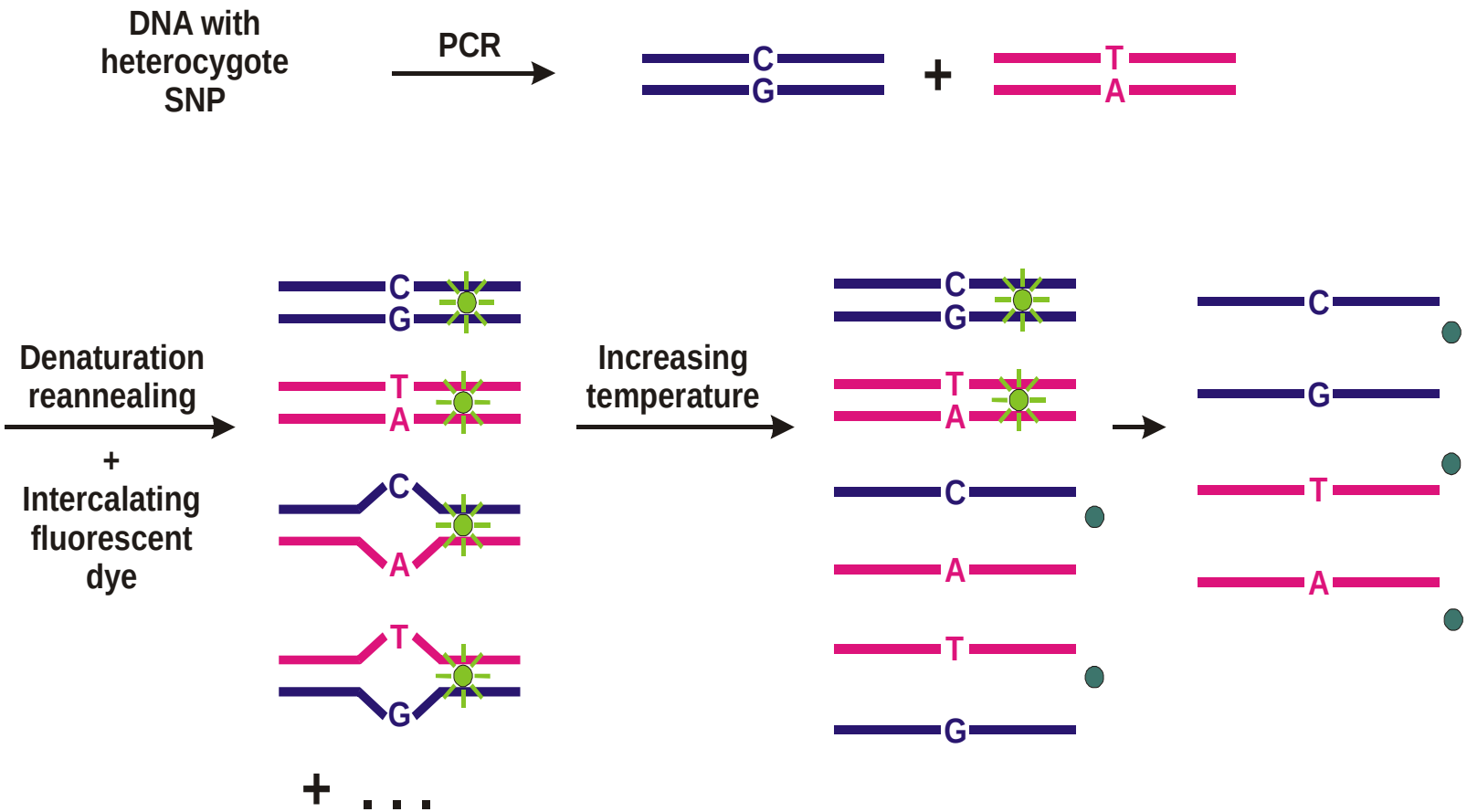
- individual curves not sharp
- overlap is the same for homo- and heteroduplexes

Saturating dye

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

Amplicon Melting

Principle of Gene Scanning by HRM



Heterozygote/Homozygote Distinction

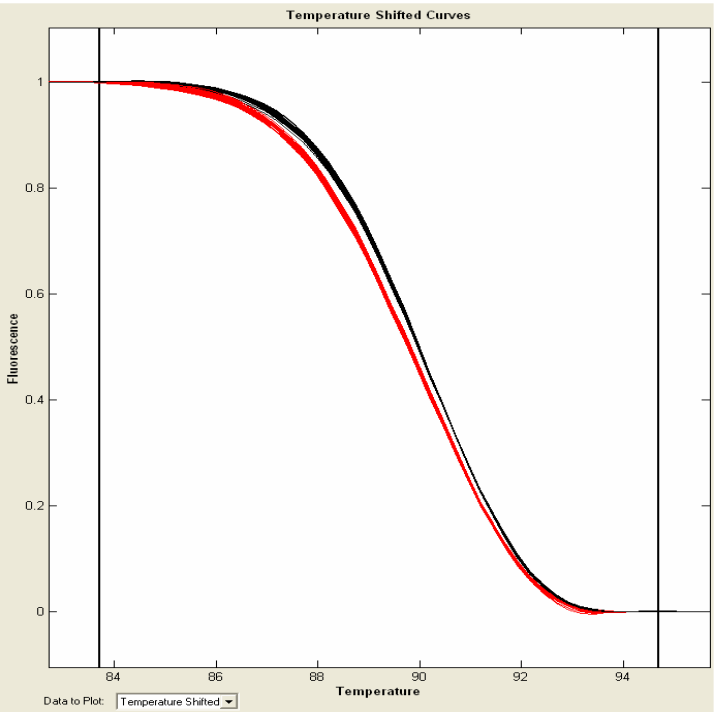
Target mdr-1

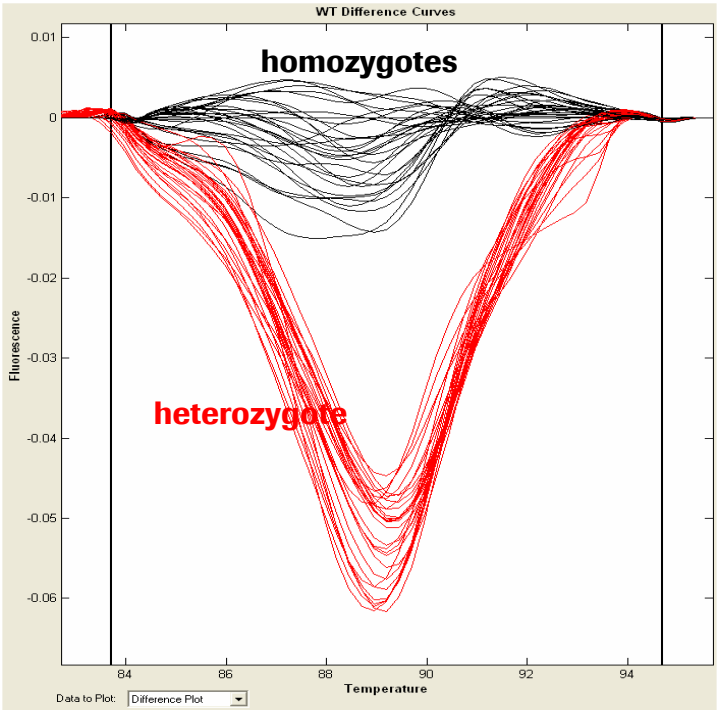
Diagnostics

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

Detection dye: R27

Amplicon length: 247 bp





Mutation Detection using HRM

Finding Heterozygotes

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Diagnostics

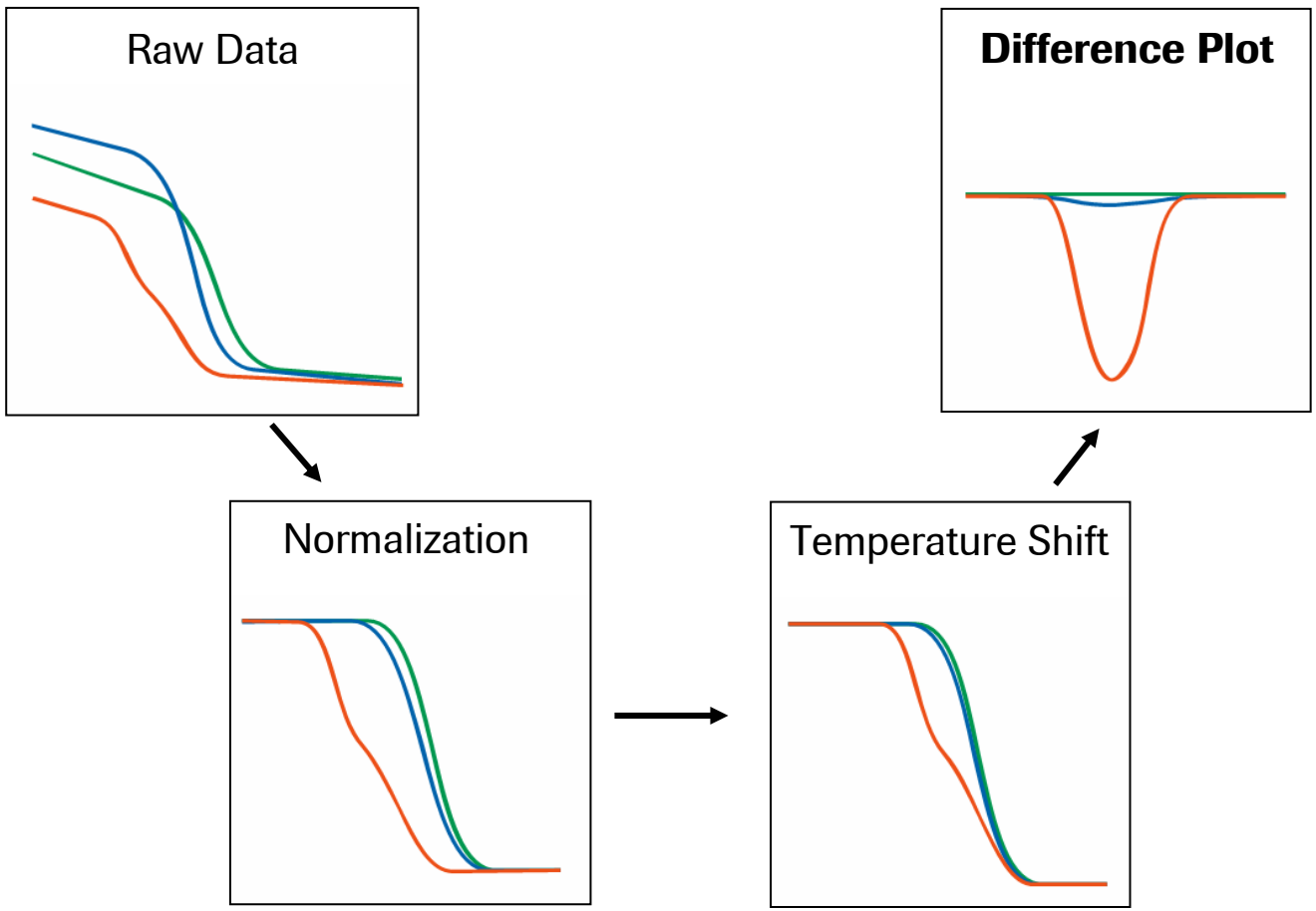
- **Amplicon Melting of homozygote samples (containing homoduplexes wt or mut) give very similar curve shapes.**

- **Amplicon Melting of heterozygote samples (containing homo and heteroduplexes) give curve shapes which are highly distinct.**

- **No identication of specific sequences (genotyping);**
  Only differences between two genomes are detected.

High-Resolution Melting Curve Analysis

Difference Plot



High-Resolution Melting

Example 1

Roche

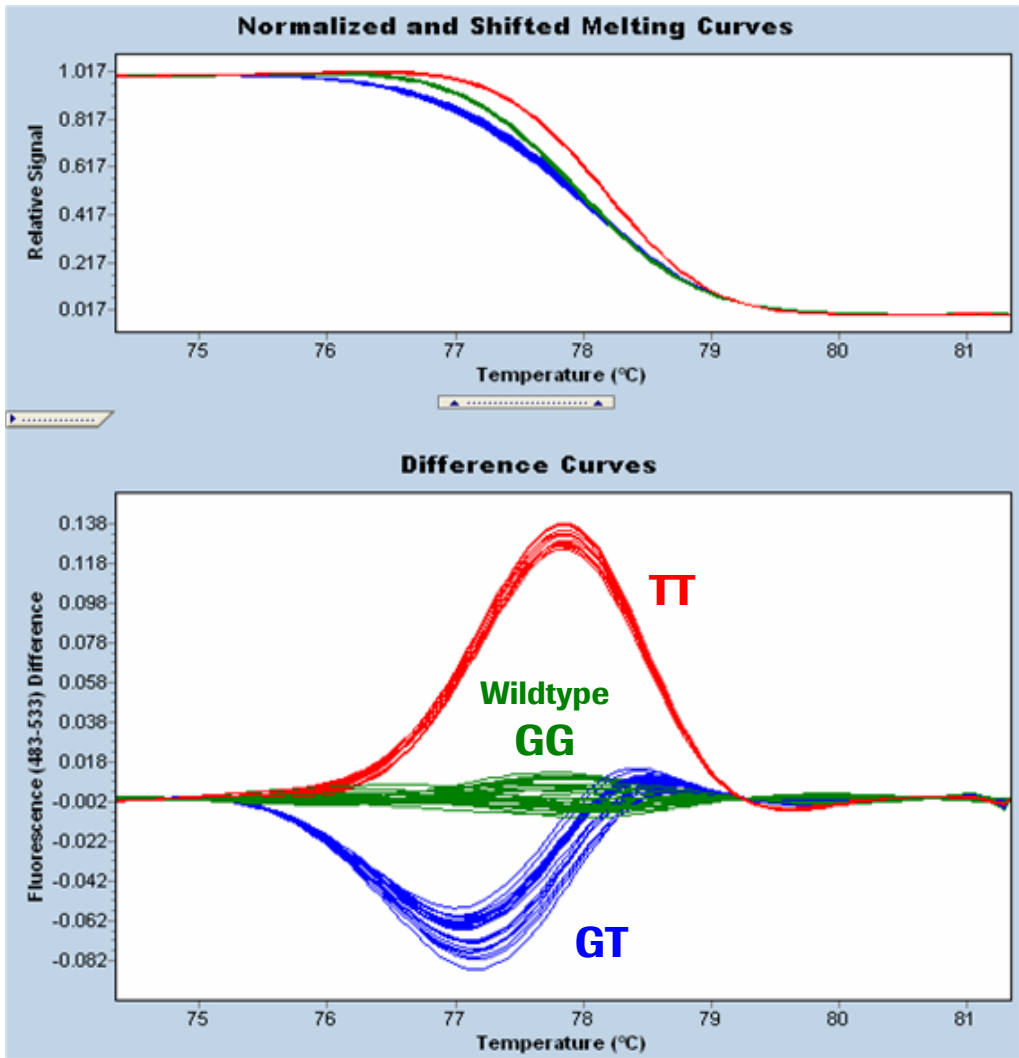
Diagnostics

Target: LPLH3

SNP G→T

Amplicon 164 bp

72 samples



Gene Scanning

Example 2

Roche Diagnostics

Target: MBL2

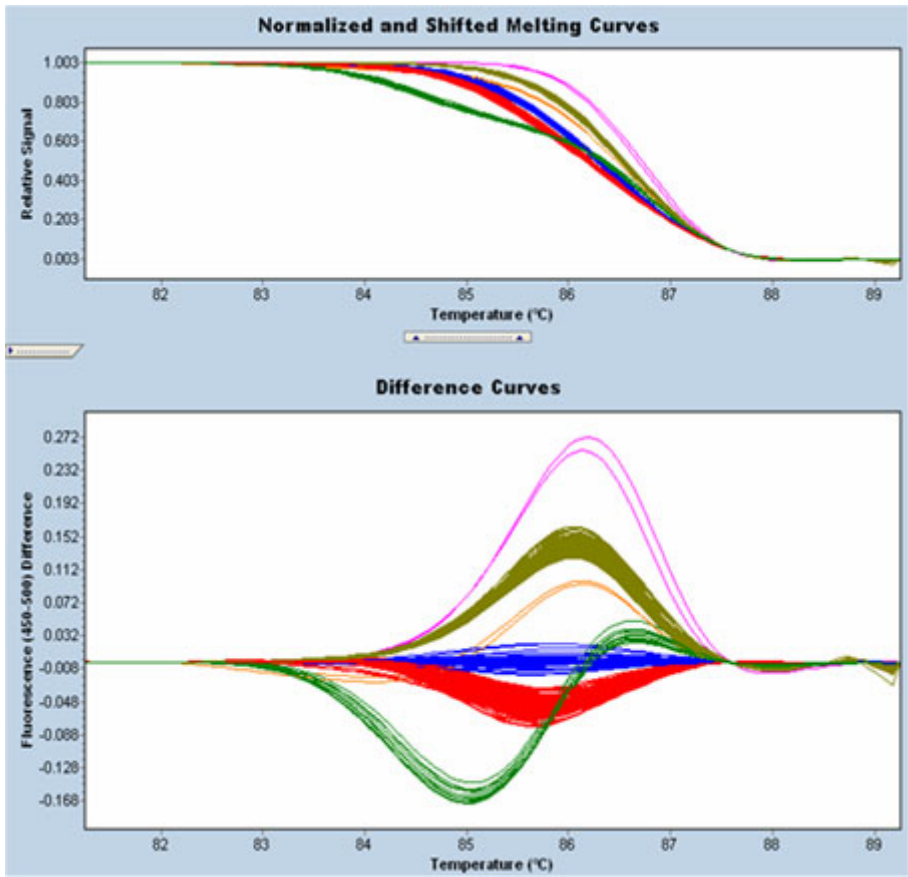
Screening for sequence variants in 384 unknown samples

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

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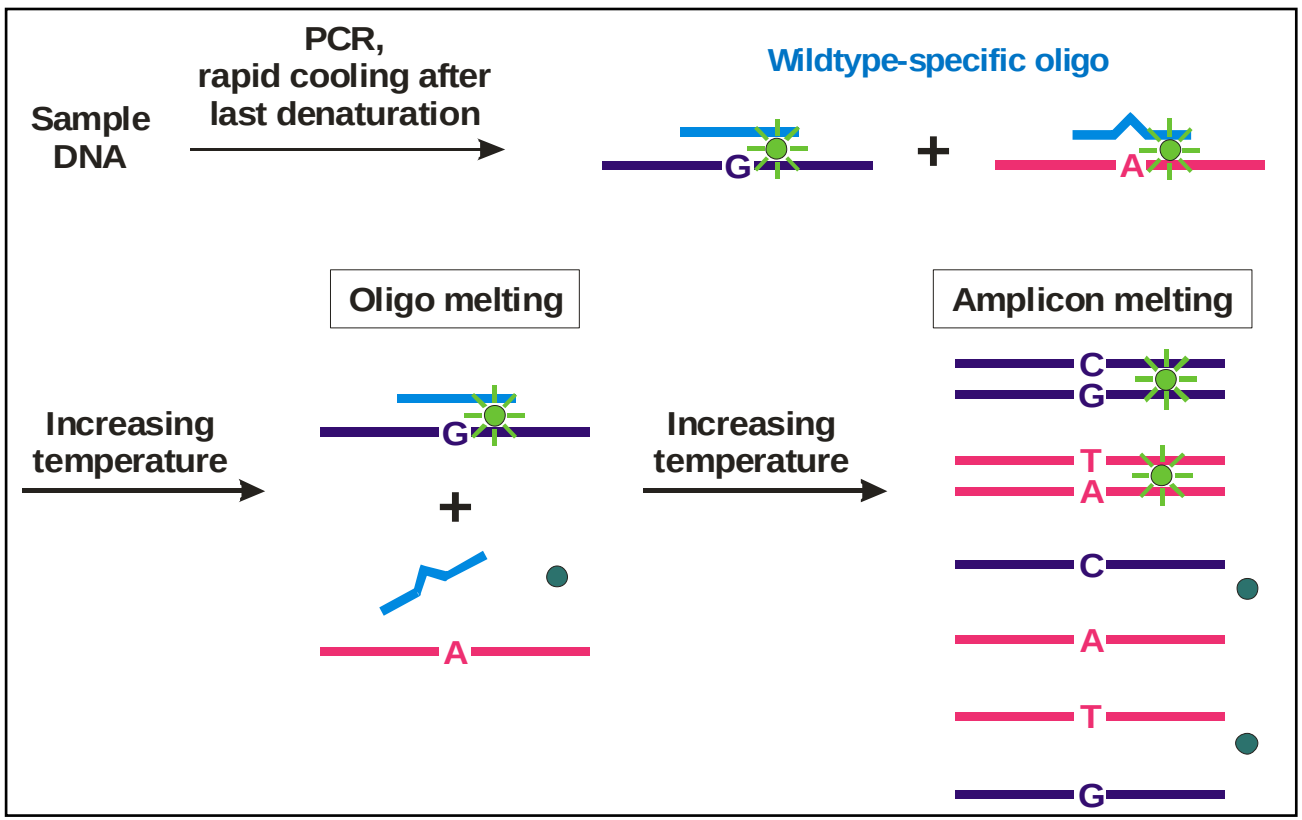
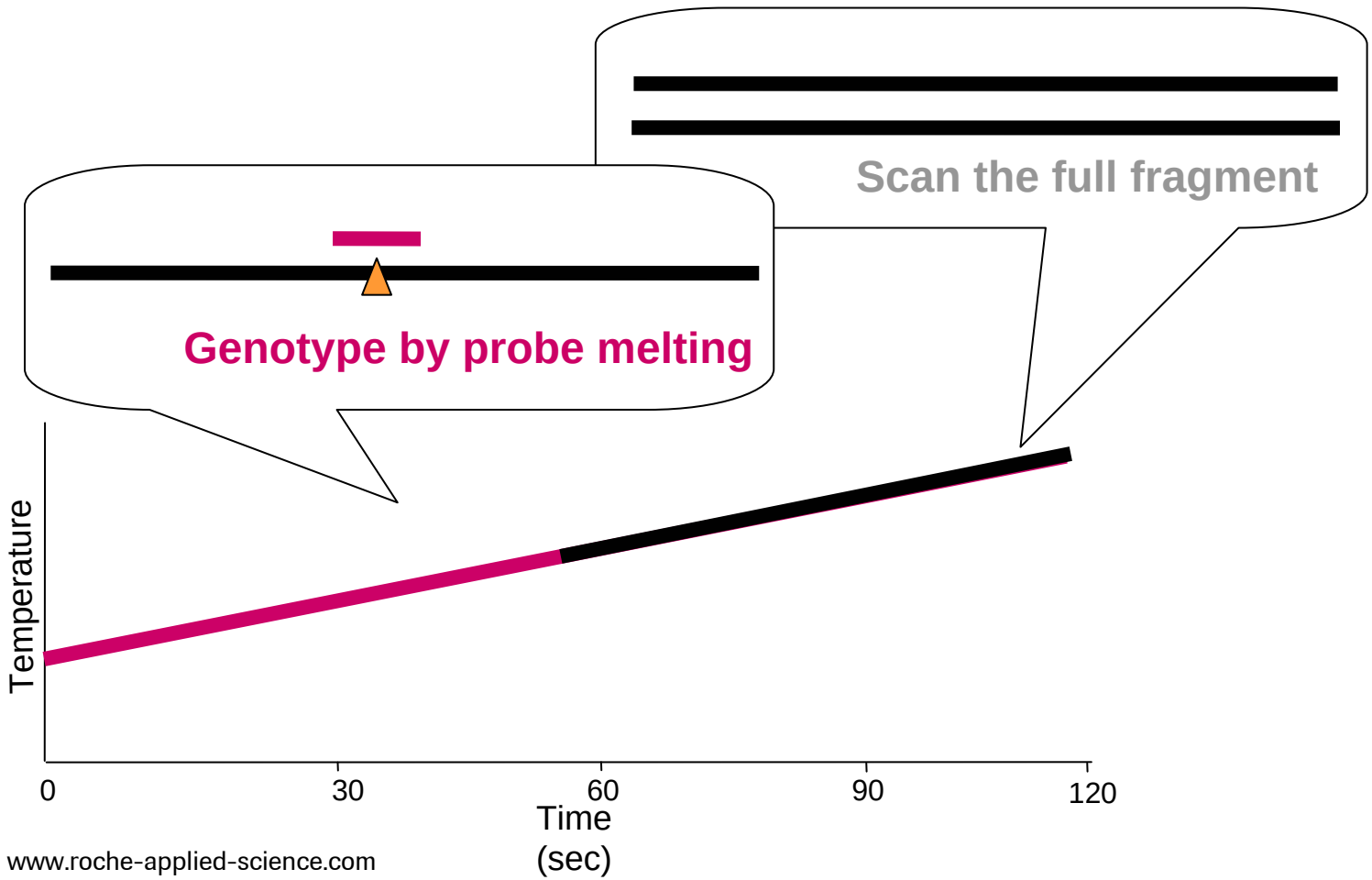
High-Resolution Melting with intercalating dye and unlabeled oligo specific for known mutation site



Simultaneous Genotyping and Scanning

Unlabeled Probe Genotyping and Amplicon Melting



High-Resolution Melting

Combined Unlabeled Probe and Amplicon Melting

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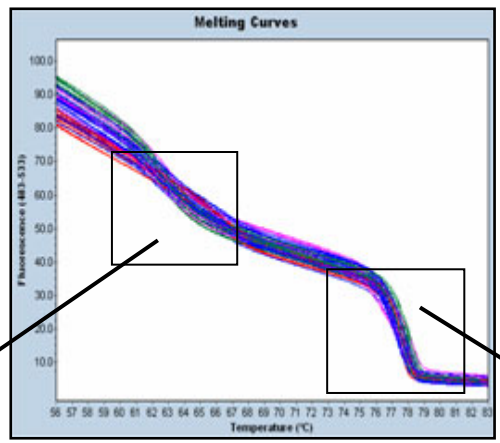
Diagnostics

Target: TNFα

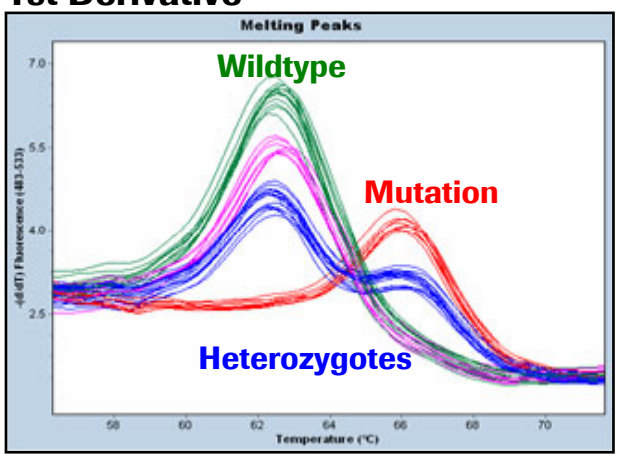
Probe for SNP C→T

Amplicon 136 bp

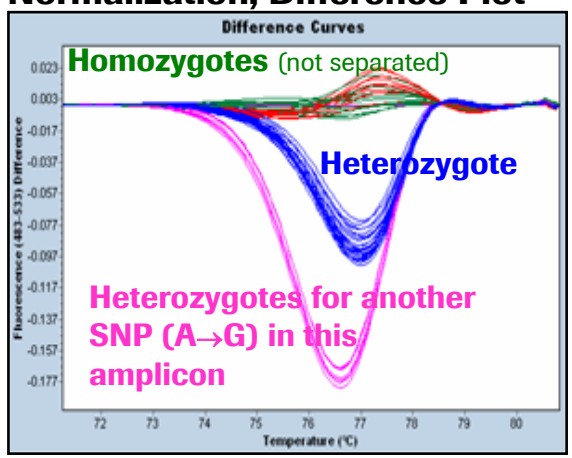
96 samples



1st Derivative



Normalization, Difference Plot



SNP Detection and Analysis

Two Combined Approaches

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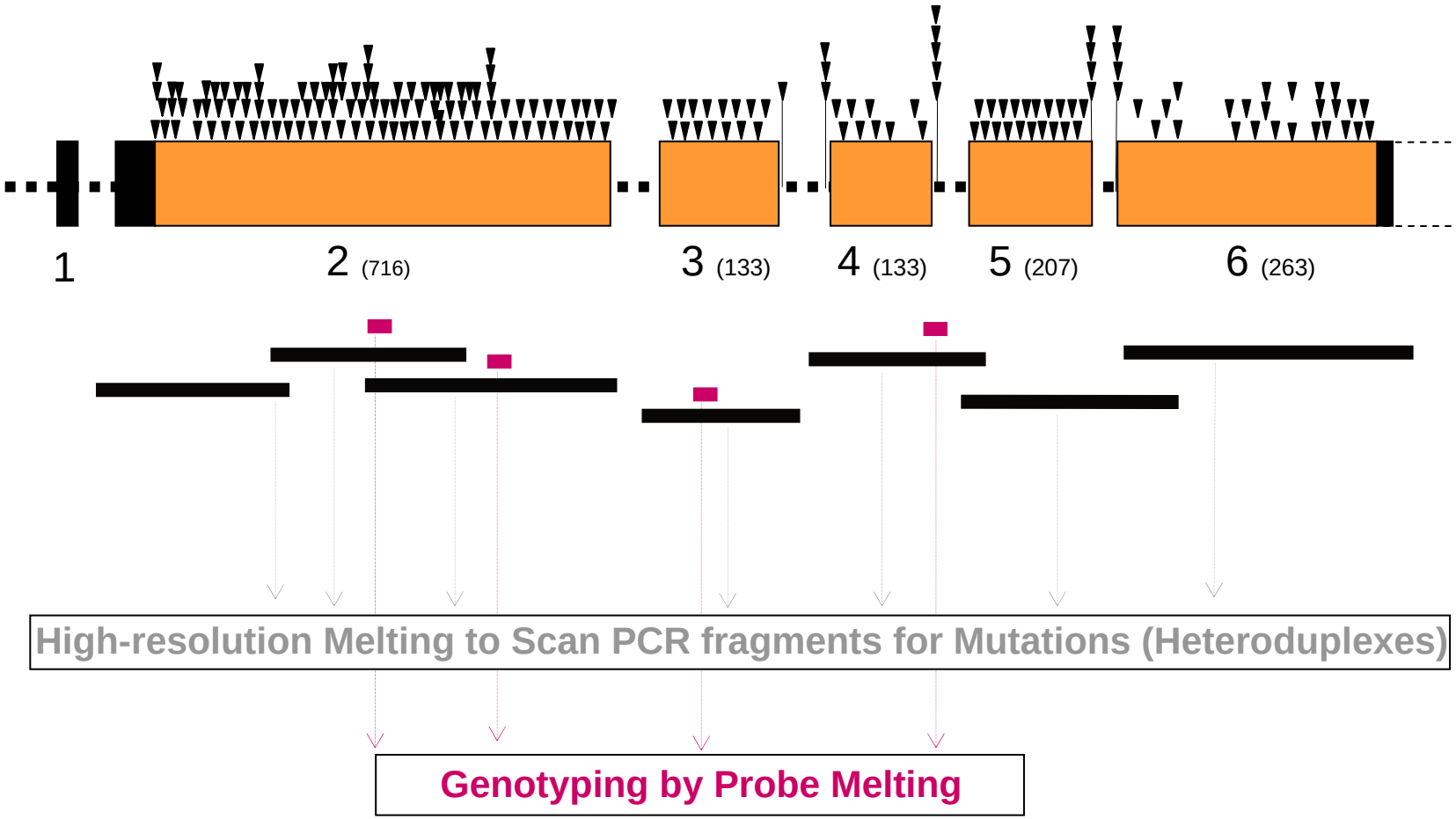

Benefits and Requirements

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Diagnostics

- 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

1. von Ahsen, N. *Two for typing: homogeneous combined single-nucleotide polymorphism scanning and genotyping*. Clin Chem 51, 1761-1762 (2005).
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