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TIB MOLBIOL

LightMix® Kit for the detection of *CYP 2D6* alleles **3, *4 and *5/*5*

Cat.-No. 40-0305-32

Kit with reagents for the detection of the *CYP 2D6 *3, *4 and *5/*5* polymorphisms using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do NOT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 II / cobas z 480 Instrument see pages 6-7

1. Introduction

The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism. Cytochrome P450 2D6 (*CYP2D6*) is primarily localized in the liver and is known to metabolize 20% of commonly prescribed drugs, in particular, cardiovascular and psychotropic agents (debrisoquine, sparteine, propafenone, amitryptiline etc.).

The gene is highly polymorphic with more then 80 different alleles described to date [1]. About 5-10% of the Caucasian population is affected by nonfunctional alleles, which result in poor and intermediate metabolizer phenotypes. The alleles *CYP2D6*3*, *CYP2D6*4* and *CYP2D6*5* represent more than 90% of the inactivating alleles (2).

Allele CYP2D6*3 is a frameshift mutation generated by a 1-bp deletion (2637delA) in exon 5.
Allele CYP2D6*4 has an incorrect splicing due to transition 1934G-A at the junction intron 3 to exon 4.
Allele *CYP2D6*5* is characterized by a deletion of the entire CYP2D6 gene.

[1] http://www.cypalleles.ki.se/cyp2d6.htm

[2] CYP2D6 worldwide genetic variation shows high frequency of altered activity variants and no continental structure. J.Sistonen, A.Sajantila, O.Lao, J.Corander, G.Barbujani and S.Fuselli. Pharmacogenetics and Genomics 2007, 17:93–101

The LightMix® Kit *CYP 2D6* provides a fast, easy and accurate system to identify alleles **3, *4* as well as a homozygous deletion of the gene *(*5/*5)* using a nucleic acid extract obtained from peripheral blood.

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 II (96 well and 384 well formats) Instruments using the Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

2. Description

After amplification with specific primers the genotypes are identified through the specific melting points (Tm) recorded during the melting curve analysis. For the analysis for *CYP 2D6 *3* a 317 bp fragment from exon 5 is amplified and analyzed with a SimpleProbe® oligomer (channel 530) depicting a Tm of 61.3°C for the wild type allele and 55.8°C for the deletion 2637 delA allele. For analysis of *CYP 2D6 *4* a 336 bp fragment spanning the intron 3 - exon 4 junction is generated and analyzed with hybridization probes labeled with LightCycler® Red 640 (detected in channel 640) exhibiting a Tm of 56.5°C for the wild type allele and 65.5°C for the variant 1934A.

The deletion of the entire CYP2D6 gene (CYP 2D6*5/*5) does not produce any signal in channels 530 or 640. To demonstrate the presence of amplifiable DNA in the biological sample a 234 bp fragment of the human chemokine receptor type 5 (hCR5) is co-amplified with specific primers. The hCR5 amplification is detected with hybridization probes labeled with LightCycler® Red 690 (detected in channel 705) exhibiting a specific melting peak at a Tm of about 49°C.

For use in LightCycler® 480 II Instruments use filter combination 483-533 instead of channel 530 and filter combination 483-640 instead of channel 640 for detection.

3. Set Contents

3 Vials with **red** cap containing lyophilized primers and probes for 32 PCR reactions *CYP 2D6*

1 Vial with colorless cap containing wild type control DNA, 10^5 target equivalents per reaction
1 Vial with colorless cap containing variant control DNA (**3* and **4*), 10^5 target equivalents per rxn
1 Vial with colorless cap with mixed control DNA (**1/*3* and **1/*4*), 10^5 target equivalents per rxn

4. Additional Reagents and items required

	Roche Diagnostics
ColorCompensation HybProbe order n°40-0318-00	Cat. -No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001

5. Product Characteristics

PCR results are obtained within 50 minutes (45 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (45 cycles and melting curve) with the LightCycler® 480 Instruments.

Sensitivity
These reagents detect 5 ng of *CYP 2D* genomic DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments.

Measuring range
The linear measuring range of the assay is 5 ng to 100 ng of *CYP 2D6* genomic DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25°C). See expiry on the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).

6. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a MSDS.

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

7. Experimental Protocol

The following procedure was developed for use with the LightCycler® 1.x / 2.0 / 480 II Instruments. Start programming before preparing the solutions. See the Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. 'High Pure PCR Template Preparation Kit' for genomic DNA samples).

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.

7.1. Preparation of parameter-specific reagents (32 reactions):

One reagent vial with a red cap contains primers and probes to run 32 rxns for *CYP 2D6.*

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**Use 2 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure t o light.

7.2. Preparation of the control DNA

Add 80 µl PCR-grade water to each vial (1.6 x 10^6 target molecules) with a colorless cap. Mix the target DNA by pipetting the solution up and down 10 times.

Note: Control DNA can be dissolved up to 160 µl to achieve 32 control reactions.

►**Use 5 µl** control DNA for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage free ze at -20°C. Avoid repeated freezing thawing cycles. For the heterozygote control DNA provided with the kit please note that the relative amounts of wild type DNA and mutant DNA may change during time.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

7.3. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart Master	
Single reaction	**Component**
9.4 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
2.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **7.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

Table 1

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary (LightCycler® 1.x / 2.0 Instruments) or to a multiwell plate (LightCycler® 480 II Instrument).

Add 5 µl of sample or control DNA to each capillary or well for a final reaction volume of 20 µl.

Start run.

8. LightCycler® 1.x / 2.0 Instruments

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [℃]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [℃/s]	20	20	20	20	20	20	0.2	20
Acquisition Mode	None	None	Single	None	None	None	Continuous	None

Table 2

8.2. Data Analysis

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640, channel F3 instead of channel 705 and channel F1 instead of channel 530 for detection. We recommend upgrading LightCycler® 1.x instruments to software version 4.1.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the Color Compensation Kit HybProbe 530/640/690'. Perform data analysis, as described in the LightCycler® Instrument operator's manual.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *CYP 2D6 *3* data in channel 530, *CYP 2D6 *4* data in channel 640 and *CYP 2D6 *5/*5* deletion (hCR5 amplification control) data in channel 705. "Tm Calling" Analysis mode (LightCycler® 2.0 Instrument) or Melting Curves mode (LightCycler® 1.x Instrument). The negative control (NTC) must show no signal.

8.3. Sample Data – Typical Results

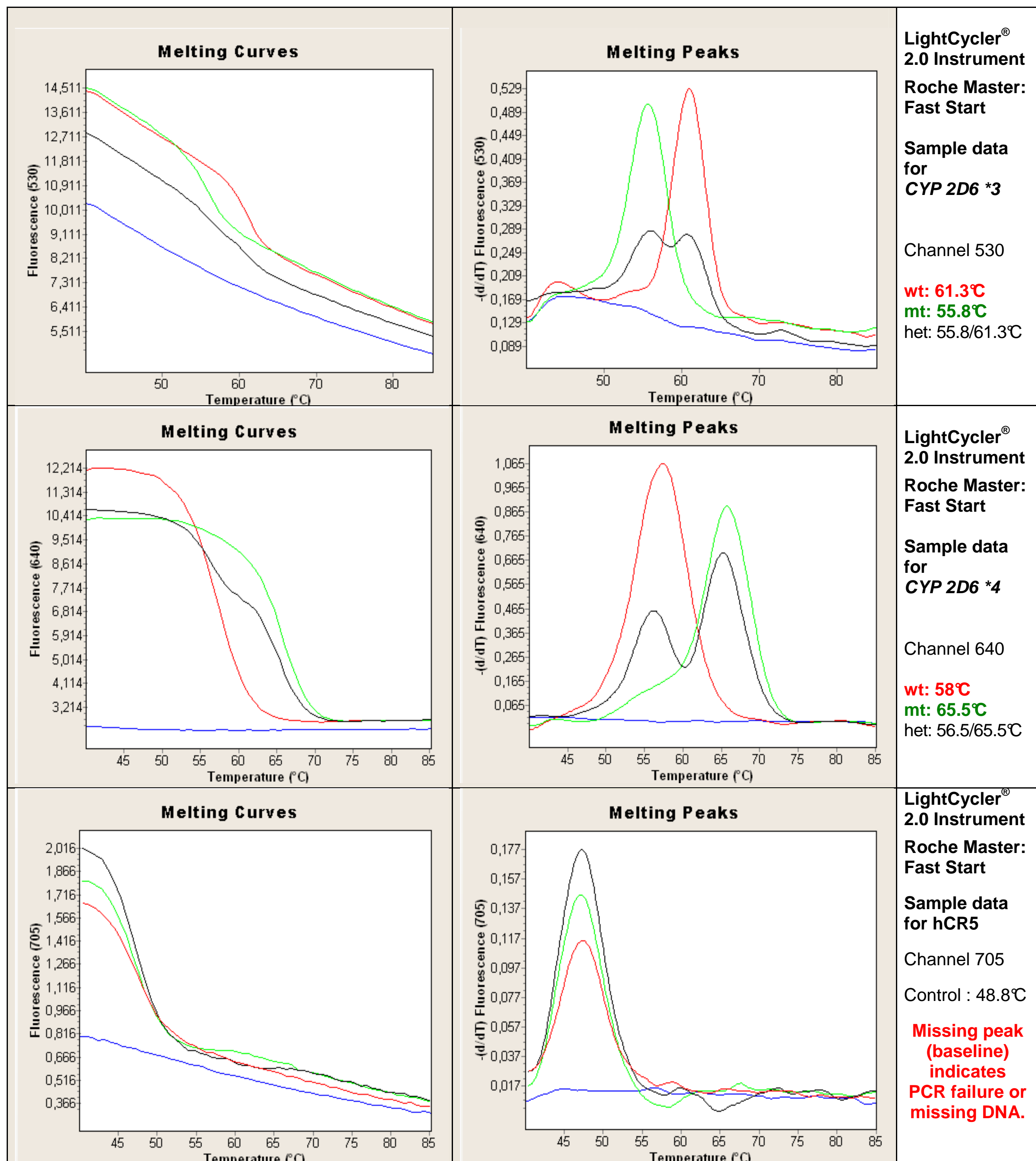

Fig.1. Sample data from LightCycler® 2.0 Instrument for the CYP 2D6 *3*4*5/5 detection system.

Upper panels: Channel 530. Left panel melting curves, right panel melting peaks for *CYP 2D6 *3.*
Wildtype (wt) corresponds to *CYP 2D6 *3* A/A2637, heterozygote (het) corresponds with *CYP 2D6 *3* -/A2637 and mutant (mt) corresponds to *CYP 2D6 *3* -/-2637.

Middle panels: Channel 640. Left panel melting curves, right panel melting peaks for *CYP 2D6 *4*
Wildtype (wt) corresponds to *CYP 2D6 *4* G1934, heterozygote (het) corresponds with *CYP 2D6 *4 G1934A* and mutant (mt) corresponds to *CYP 2D6 *4 1934A.*

Lower panels: Channel 705. Left panel melting curves, right panel melting peaks for *hCR5* (amplification control).

Please refer to chapter **10. Interpretation of Data** for the combined interpretation of results.

9. LightCycler® 480 II / cobas z 480 Instruments

9.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660
cobas z 480 Instrument: 465-510, 498-645, 498-660

Program Step:	**Denaturation**	**Cycling**			**Melting**			**Cooling**
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [℃]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [℃/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [℃/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per ℃]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**

Table 3

9.2. Data Analysis

Note: cobas z480 Instruments signal levels are about 50% compared to LightCycler® 480 II results.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the Color Compensation Kit HybProbe 530/640/690'.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *CYP 2D6 *3* data with Filter Combination 465-510, *CYP 2D6 *4* data with Filter Combination 498-640 and *CYP 2D6 *5/*5* deletion (hCR5 amplification control) data with Filter Combination 498-660, Tm Calling" Analysis mode. The negative control (NTC) must show no signal.

9.3. Sample Data – Typical Results

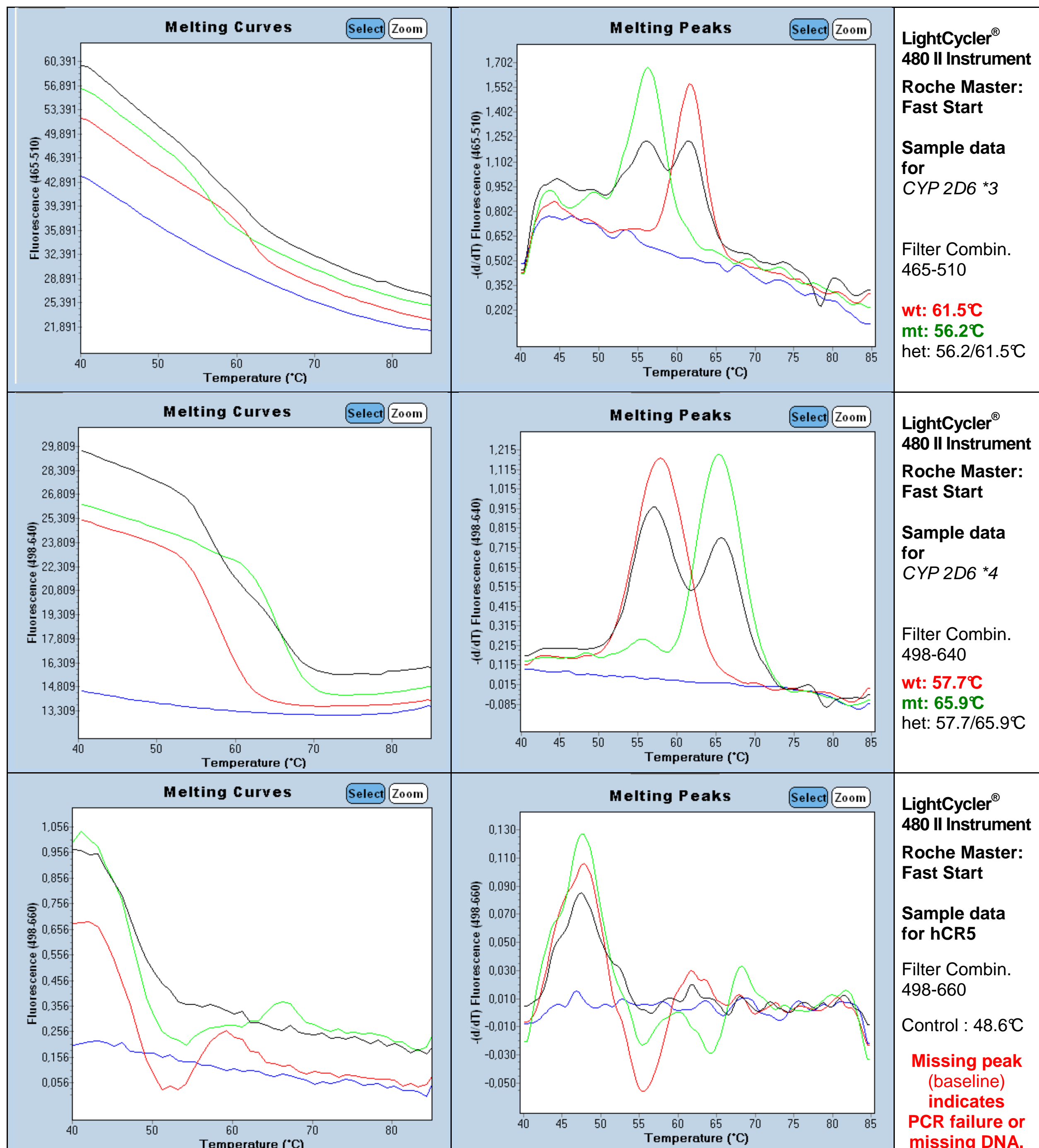

Fig.2. Sample data from LightCycler® 480 II Instrument for the *CYP 2D6 *3*4*5/5* detection system.

Upper panels: Filter Combination 465-510. Left panel melting curves, right panel melting peaks for *CYP 2D6 *3*. Wildtype (wt) corresponds to *CYP 2D6 *3* A/A2637, heterozygote (het) corresponds to *CYP 2D6 *3 -*/A2637 and mutant (mt) corresponds to *CYP 2D6 *3 -/-*2637.

Middle panels: Filter Combination 498-640. Left panel melting curves, right panel melting peaks for *CYP 2D6 *4.* Wildtype (wt) corresponds to *CYP 2D6 *4* G1934, heterozygote (het) corresponds to *CYP 2D6 *4 G1934A* and mutant (mt) corresponds to *CYP 2D6 *4 1934A.*

Lower panels: Filter Combination 498-660. Left panel melting curves, right panel melting peaks for *hCR5* (amplification control).

Please refer to chapter **10. Interpretation of Data** for the combined interpretation of results.

10. Interpretation of Data

CYP 2D6 *3 Channel 530 Filter 465-510 Melting point(s)		CYP 2D6 *4 Channel 640 Filter 498-640 Melting point(s)		hCR5 (control) Channel 705 Filter 498-660 Melting point	CYP 2D6 alleles	Metabolizer Type Phenotype
-	61.5°C	57.7°C	-	~ 49°C (not relevant)	*1/*1 (wild type) § or *1/*5 (deletion) %	Extensive (or intermediate)
56.2°C	61.5°C	57.7°C	-	~ 49°C (not relevant)	*1/*3 heterozygous	Intermediate
-	61.5°C	57.7°C	65.9°C	~ 49°C (not relevant)	*1/*4 heterozygous	Intermediate
56.2°C	-	57.7°C	-	~ 49°C (not relevant)	*3/*3 or *3/*5 %	Poor
-	61.5°C	-	65.9°C	~ 49°C (not relevant)	*4/*4 or *4/*5 %	Poor
56.2°C	61.5°C	57.7°C	65.9°C	~ 49°C (not relevant)	*3/*4 heterozygous	Poor
-	-	-	-	**~ 49°C**	*5/*5 (deletion) $	Poor
-	-	-	-	**NONE**	PCR failure #	**Repeat test**
ΔTm 5.3°C		ΔTm 8.2°C		-		

Table 4. Typical analysis results (Roche Master: Fast Start)

§ Other haplotypes not showing the mutations analyzed by this kit are, for simplicity, classified as *1.
As more than 90% of all gene defects in Caucasian individuals are *3 or *4 there will be a fraction of less than 10% samples misclassified as wild type *1 because other gene variants are not analyzed.

% Any homozygous results might be a combination of the same allele and a deletion of the other gene copy (*5*).

$ In case that no melting signals are visible in channel 510 nor channel 640, but the control gene has been amplified as shown by the melting point in channel 705, the *CYP 2D6* gene is deleted (**5/*5*).

If no signals are visible in channel 510, 640 and 705, PCR was inhibited or target DNA was missing or too low.

Notes: The values of the respective melting temperatures (T_M) may vary ±2.5°C between different experiments. The ΔT between the melting peaks for heterozygote genotypes may vary ±1.5°C. Samples with deviating melting curves should be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de).

11. Version History

Notes in red mark events require to change procedures

V110218 Release version
V110615 Minor editorial corrections
V130822 Z480 included, Expiry extended, Roche Color Compensation discontinued
V140211 Change of Tm for hCR5 (control) to ~49°C and minor editorial changes

Roche SAP order n° 06296742001

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These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
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