For general laboratory use. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

Roche Applied Science

LightCycler® 480 Control Kit

Version August 2005

Real-time PCR experiments for quantification and melting curve-based genotyping to prove performance of the LightCycler® 480 System

Cat. No. 04 710 924 001 Kit for 3 tests

Store the kit at −15 to −25°C

⚠ Keep vials 10, 11 and 12 away from light!

Table of Contents

PROTOCOL

1. What this Product Does

Number of tests

The kit contains reagents for 3 control runs for quantification with hydrolysis probes and 3 control runs for genotyping with HybProbe probes, with reaction volumes of 20 µl.

Kit Contents

Vial/Cap	Label	Contents / Function
1 yellow	Standard 1 10^2 copies / 5 µl	• 45 µl • target: wild type plasmid DNA
2 yellow	Standard 2 10^3 copies / 5 µl	• 405 µl • target: wild type plasmid DNA
3 yellow	Standard 3 2×10^3 copies / 5 µl	• 405 µl • target: wild type plasmid DNA
4 yellow	Standard 4 10^4 copies / 5 µl	• 45 µl • target: wild type plasmid DNA
5 yellow	Standard 5 10^5 copies / 5 µl	• 90 µl • target: wild type plasmid DNA
6 yellow	Standard 6 10^6 copies / 5 µl	• 45 µl • target: wild type plasmid DNA
7 yellow	Standard 7 Heterozygote	• 45 µl • target: heterozygous plasmid DNA
8 yellow	Standard 8 Mutation	• 45 µl • target: mutant plasmid DNA
9 blue	Primer Mix 20× conc.	• 243 µl • mix of two target-specific primers
10 red	Genotyping Probes 10× conc.	• 54 µl • HybProbe probe mix • Probe 1: Fluorescein-labeled at the 3´ end • Probe 2: LightCycler® Red 640-labeled at the 5´ end
11 green	Quantification Probe 10× conc.	• 450 µl • FAM-labeled hydrolysis probe
12 purple	Internal Control 10× conc.	• 450 µl • primer, probe and template mix, with LightCycler ® Red 610-labeled hydrolysis probe for detection of control DNA sequence
13 colorless	H_2O, PCR grade	1000 µl

Storage and Stability

- The kit is shipped on dry ice.
- Store the kit at −15 to −25°C through the expiration date printed on the label.

⚠ Keep the Genotyping Probes (vial 10), the Quantification Probe (vial 11) and the Internal Control (vial 12) away from light!

⚠ Avoid repeated freezing and thawing.

Additional Equipment and Reagents Required

- LightCycler® 480 Instrument*
- LightCycler® 480 Probes Master*
- LightCycler® 480 Multiwell Plate 384* and LightCycler® 480 Sealing Foil*
- Standard swing bucket centrifuge containing a rotor for multiwell plates with suitable adaptors
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Sterile 1.5 ml reaction tubes

** available from Roche Applied Science*

Application

The LightCycler® 480 Control Kit is designed to prove the performance of all components of the LightCycler® 480 System, including instrument, software, disposables, generic reagents and optional devices such as a pipetting robot. The kit is primarily for use with the LightCycler® 480 Probes Master* (for procedure A and B), but it can also be used with the LightCycler® 480 SYBR Green I Master (for procedure A) or the LightCycler® 480 Genotyping Master (for procedure B).

The test includes two control experiments. Experiment A is for absolute quantification of prediluted standard DNA. Experiment B is used for genotyping samples with a wild type DNA sequence as well as samples with a homozygous or heterozygous point mutation.

⚠ The performance of the kit shown in this instruction manual is guaranteed only when it is used with the LightCycler® 480 System.

Assay Time / Hands on Time

Quantification with Hydrolysis Probes	
Procedure	**Time**
Prepare PCR mixes	10 min
Pipette into plate	15 min
PCR run	40 min
Total assay time	1 h 5 min
Genotyping with HybProbe probes	
Procedure	**Time**
Prepare the PCR mix	10 min
Pipette into plate	5 min
PCR run	50 min
Total assay time	1 h 5 min

2. How To Use this Product

2.1 Before You Begin

Precautions Always wear gloves when handling the PCR mixes and plates.

Sample Material Template DNA is included in this kit.

2.2 Experimental Overview

Reagents for two different detection formats are provided with the kit:

- Procedure A: Quantification with hydrolysis probes
- Procedure B: Genotyping with HybProbe probes

The following procedures show how to use the LightCycler® 480 Control Kit together with the LightCycler® 480 Probes Master (Procedure A and B).

⚠ The procedures are optimized for a final reaction volume of 20 µl.

Procedure A: Quantification with Hydrolysis Probes

①	Set up instrument.
②	Prepare 3 reaction mixes: • PCR mix 1: for 24-fold replicates of 1000 copies of target DNA • PCR mix 2: for 24-fold replicates of 2000 copies of target DNA • PCR mix 3: for standard curve
③	Pipette into multiwell plate.
④	Run PCR on the LightCycler® 480 Instrument.
⑤	Interpret results.

Procedure B: Genotyping with HybProbe probes

①	Set up instrument.
②	Prepare reaction mix.
③	Pipette into multiwell plate.
④	Run PCR on the LightCycler® 480 Instrument.
⑤	Interpret results.

A 144 bp fragment of the Cyp2C9 gene is amplified from plasmid DNA and detected with a FAM-labeled hydrolysis probe. To test the precision of the system, replicates with only 1000 or 2000 copies of target DNA per well are distributed throughout the plate and quantified with reference to a row of standards.

PCR Program

Program the LightCycler® 480 Instrument before preparing the reaction mixes.

A LightCycler® 480 protocol for procedure A using the LightCycler® 480 Probes Master and the LightCycler® 480 Control Kit contains the following programs:

- **Pre-Incubation** to activate FastStart Taq DNA polymerase and denature the DNA
- **Amplification** of the target DNA
- **Cooling** the multiwell plate

For details on how to program the experimental protocol, see the LightCycler® 480 Operator's Manual.

Set-Up				
Detection Format	**Block Type**		**Reaction Volume**	
Multi Color Hydrolysis Probes	384		20 µl	
Filter Setting	dynamic mode, FAM (483 - 533) and Red 610 (558 - 610)			
Programs				
Program Name	**Cycles**		**Analysis Mode**	
Pre-Incubation	1		None	
Amplification	40		Quantification	
Cooling	1		None	
Temperature Targets				
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**
Pre-Incubation				
95	None	00:05:00	4.8	–
Amplification				
Segment 1: 95	None	00:00:10	4.8	–
Segment 2: 60	Single	00:00:30	2.5	–
Segment 3: 72	None	00:00:01	4.8	–
Cooling				
40	None	00:00:30	2.5	-

Preparation of the PCR Mixes

⚠ Do not touch the upper surface of the LightCycler® 480 Multiwell Plate when handling it.

❶ Thaw the following reagents, mix gently and store on ice:
- LightCycler® 480 Probes Master: vial 1
- LightCycler® 480 Control Kit: vials 1, 2, 3, 4, 5, 6, 9, 11, 12

❷ Prepare PCR mixes

⚠ Prepare all three PCR mixes (A, B, C) before dispensing to the plate.

PCR Mix A
(24-fold replicates of a standard containing 1000 copies of target DNA)
To a 1.5 ml reaction tube on ice, add the components in the order given below, mix gently, close the tube and store on ice.

Component	Vol	Final conc.
LightCycler® 480 Probes Master	270 µl	1×
Primer Mix, 20× (vial 9)	27 µl	1×
Quantification Probe, 10× (vial 11)	54 µl	1×
Internal Control, 10× (vial 12)	54 µl	1×
Standard 2 (vial 2)	135 µl	1000 copies/20 µl

PCR Mix B
(24-fold replicates of a standard containing 2000 copies of target DNA)
To a 1.5 ml reaction tube on ice, add the components in the order given below, mix gently, close the tube and store on ice.

Component	Vol	Final conc.
LightCycler® 480 Probes Master	270 µl	1×
Primer Mix, 20× (vial 9)	27 µl	1×
Quantification Probe, 10× (vial 11)	54 µl	1×
Internal Control, 10× (vial 12)	54 µl	1×
Standard 3 (vial 3)	135 µl	2000 copies/20 µl

PCR Mix C
(To generate a standard curve and negative control)
To a 1.5 ml reaction tube on ice, add the components in the order given below, mix gently, close the tube and store on ice.

Standard DNA is added to the multiwell plate after PCR Mix C is dispensed (see steps 4 and 5 below).

Component	Vol	Final conc.
LightCycler® 480 Probes Master	200 µl	1.33×
Primer Mix, 20× (vial 9)	20 µl	1.33×
Quantification Probe, 10× (vial 11)	40 µl	1.33×
Internal Control, 10× (vial 12)	40 µl	1.33×

❸ Dispense 20 µl from either PCR mix A or PCR Mix B into each of the plate wells indicated on the pipetting scheme below:
- PCR mix A (with Standard 2) into the indicated wells of columns 1, 12 and 23
- PCR mix B (with Standard 3) into the indicated wells of columns 2, 13 and 24

❹ Dispense 15 µl from PCR mix C into each of the wells in columns 3 though 8 that will contain standards. (Standards will be added in step 5.)

❺ Add 5 µl of each standard to the plate wells indicated on the pipetting scheme below:
Negative control (NC): H_2O, PCR grade (from vial 13) into three wells of column 3.
Standard curve:
Standard 1 (10^2 copies of DNA) into three wells of column 4
Standard 2 (10^3 copies of DNA) into three wells of column 5
Standard 4 (10^4 copies of DNA) into three wells of column 6
Standard 5 (10^5 copies of DNA) into three wells of column 7
Standard 6 (10^6 copies of DNA) into three wells of column 8

❻
- Seal the multiwell plate with LightCycler® 480 Sealing Foil.
- Place the multiwell plate in a standard swing-bucket centrifuge that contains a rotor for multiwell plates with suitable adaptors and balance it with a suitable counterweight (*e.g.*, another multiwell plate).
- Centrifuge at 1500 × *g* for 2 min.

❼ Transfer the multiwell plate into the LightCycler® 480 Instrument.

❽ Start the PCR program described above.

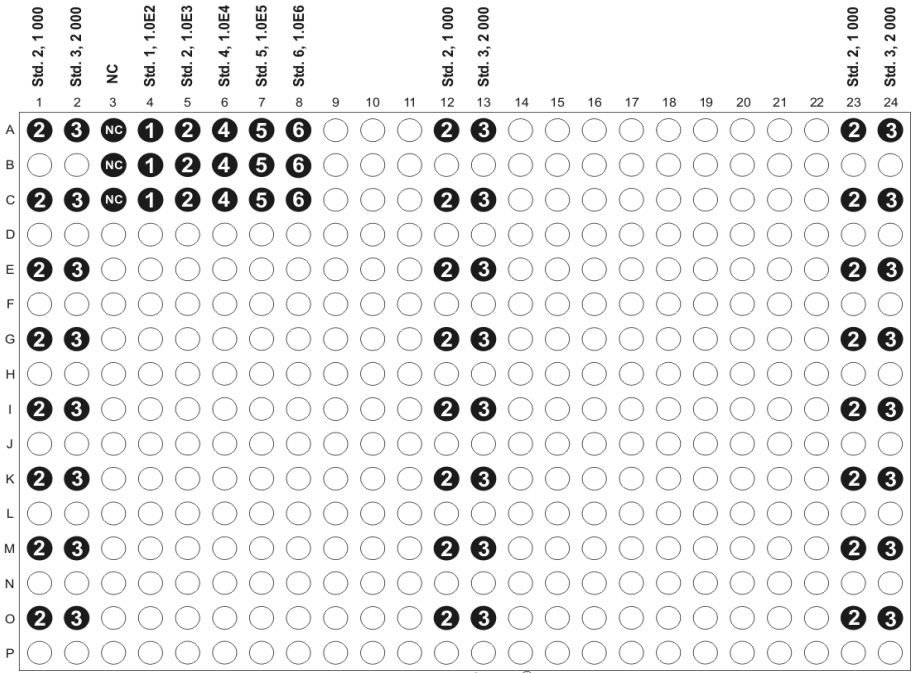

Fig. 1: Pipetting scheme for the LightCycler® 480 Multiwell Plate 384 used in procedure A. Positions are indicated for negative control and Standards 1, 2, 3, 4, 5, 6, respectively.

Evaluation

⚠ Make sure the concentrations of the standards in columns 4 – 8 are defined in the 'Sample Editor' in the 'Abs Quant' folder.

Under 'Analysis', open the 'Absolute Quantification' module, make sure channel FAM (483 - 533) is displayed, and click 'Calculate' to calculate the crossing points and standard curve.

For quantification of the internal control, switch to channel Red 610 (558 - 610).

A 144 bp fragment of the Cyp2C9 gene is amplified with specific primers from different types of plasmid DNAs (wild type, mutant, heterozygote). Amplification products are identified with HybProbe probes.

PCR Program

Program the LightCycler® 480 Instrument before preparing the reaction mixes.

A LightCycler® 480 protocol for procedure B using the LightCycler® 480 Probes Master and the LightCycler® 480 Control Kit contains the following programs:

- **Pre-Incubation** to activate FastStart Taq DNA polymerase and denature the DNA
- **Amplification** of the target DNA
- **Melting Curve** to identify the PCR product
- **Cooling** the plate

For details on how to program the experimental protocol, see the LightCycler® 480 Operator´s Manual.

Set-Up				
Detection Format	**Block Type**		**Reaction Volume**	
Mono Color HybProbe	384		20 µl	
Filter Setting	dynamic mode, Red 640 (483 - 640)			
Programs				
Program Name	**Cycles**		**Analysis Mode**	
Pre-Incubation	1		None	
Amplification	35		Quantification	
Melting Curve	1		Melting Curves	
Cooling	1		None	
Temperature Targets				
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**
Pre-Incubation				
95	None	00:05:00	4.8	–
Amplification				
Segment 1: 95	None	00:00:10	4.8	–
Segment 2: 55	Single	00:00:10	2.5	–
Segment 3: 72	None	00:00:10	4.8	–

Melting Curve				
Segment 1: 95	None	00:01:00	4.8	–
Segment 2: 40	None	00:01:00	2.0	–
Segment 3: 80	Continuous	–	–	2
Cooling				
40	None	00:00:30	2.5	-

Preparation of the PCR Mix

⚠ Do not touch the upper surface of the LightCycler® 480 Multiwell Plate when handling it.

❶ Thaw the following reagents, mix gently and store on ice:
- LightCycler® 480 Probes Master: vial 1
- LightCycler® 480 Control Kit: vials 5, 7, 8, 9, 10

❷ In a 1.5 ml reaction tube on ice, add the components in the order mentioned below, mix gently, close the tube and store on ice.

Component	Vol	Final conc.
H_2O, PCR grade	20 µl	–
LightCycler® 480 Probes Master	100 µl	1.33×
Primer Mix, 20× (vial 9)	10 µl	1.33×
Genotyping Probe, 10× (vial 10)	20 µl	1.33×

❸ Pipet 15 µl PCR mix into each of the wells on the plate that will contain standards (columns 14 through 16, as indicated on the pipetting scheme below; standards will be added in step 4).

❹ Add target DNA standards to the plate wells (5 µl/well), as indicated on the pipetting scheme below. The standards and their respective wells are:
- Wild Type (vial 5), to three wells in column 14
- Mutation (vial 7), to three wells in column 15
- Heterozygous (vial 8), to three wells in column 16

❺
- Seal the plate with LightCycler® 480 Sealing Foil.
- Place the multiwell plate in the centrifuge and balance it with a suitable counterweight (*e.g.*, another multiwell plate).
- Centrifuge at 1500 × *g* for 2 min.

❻ Transfer the multiwell plate into the plate holder of the LightCycler® 480 Instrument.

❼ Start the PCR program described above.

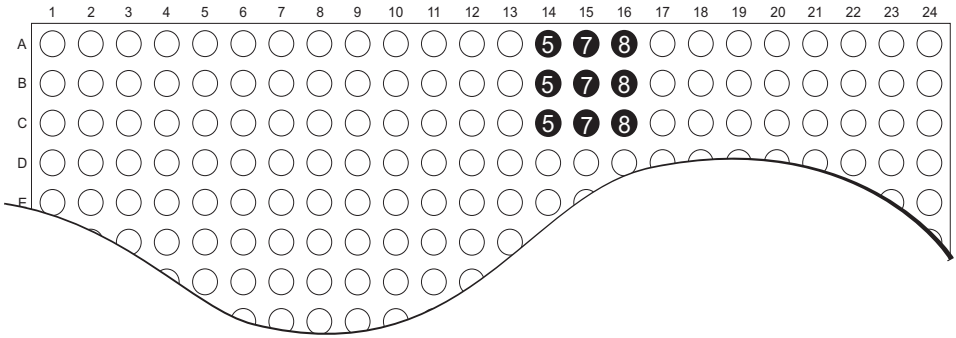

Fig. 2: Pipetting scheme for the LightCycler® 480 Multiwell Plate 384 used in procedure B. ❺ ❼ ❽ Positions for standards from vials 5, 7 and 8, respectively.

Evaluation

Data analysis is divided in two parts:

1. Under 'Analysis' open the 'Absolute Quantification' module and click 'Calculate' to calculate the fluorescence values versus cycle numbers.
2. Add the second Analysis with the 'Plus' button, choose the 'T_m calling' module and click 'Calculate' to calculate the T_m values.

3. Results

3.1 Typical Results Obtained in Procedure A

Quantification in Channel 530

The following amplification curves were obtained when procedure A was monitored in channel "483 - 533". The plot shows fluorescence versus cycle number.

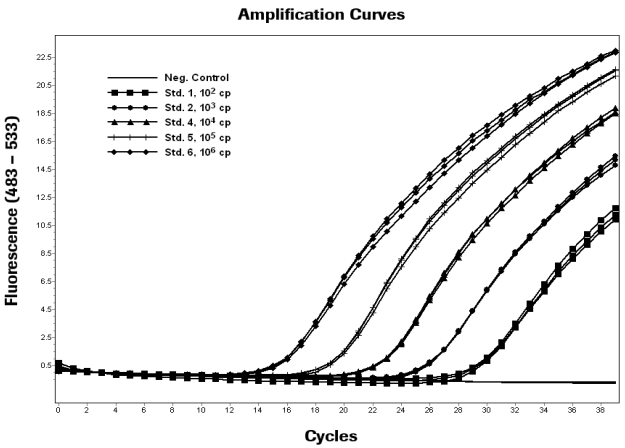

Fig. 3: Amplification curves of the standards in channel "483 - 533".

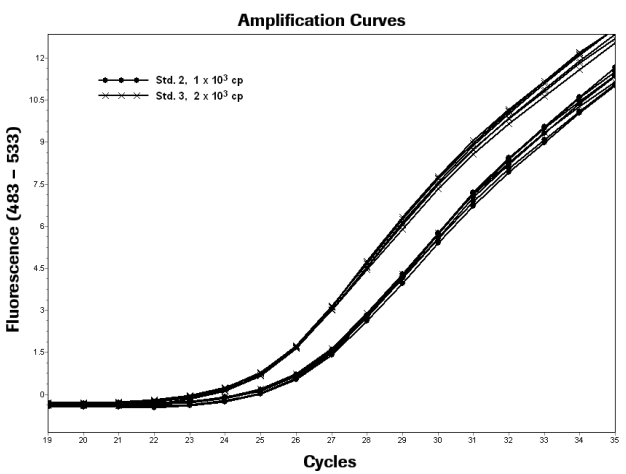

Fig. 4: The amplification curves for the replicates with 1000 or 2000 copies of target DNA are clearly separated in channel "483-533".

Quantification in Channel 610

The following amplification curves were obtained when procedure A was monitored in channel "558 - 610".

This channel is used to monitor the amplification of the internal control. The control template is present in each well at a constant concentration of about 100 copies.

Crossing points in each well are similar, because the amount of template was the same for each well. Due to competition between the target and the control PCR, the higher the amount of target DNA the lower the yield of PCR product from the internal control.

- If the target PCR in a particular well is negative or only weakly positive, the internal control can prove absence of PCR inhibition.
- If the target DNA concentration is high and the crossing point is early, the internal control may give a negative result, because target and control reaction compete for the same PCR reagents, but in this case there is no need to prove absence of inhibition.

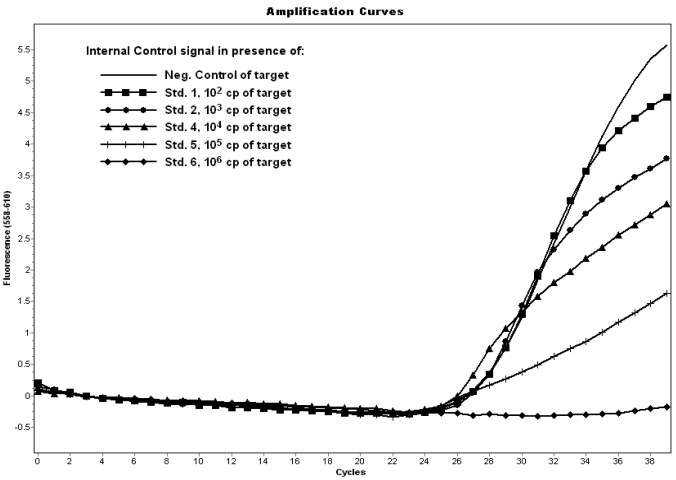

Fig. 5: Amplification curves of the internal control in channel "558 - 610".

Data analysis is divided into two parts:

1. Part 1: Quantification with Absolute Quantification module
2. Part 2: Melting curve analysis with T_m Calling module

Part 1: Quantification, Channel 640

The following amplification curves were obtained when procedure B was analyzed with the Absolute Quantification module in channel "483 - 640".

When there is a mismatch between the mutant DNA and the reporter probe, the annealing temperature during the PCR cycles is higher than the melting temperature of the probe-DNA hybrid. Hence, an amplification signal is only obtained from the wild type DNA.

The plot shows fluorescence versus cycle number.

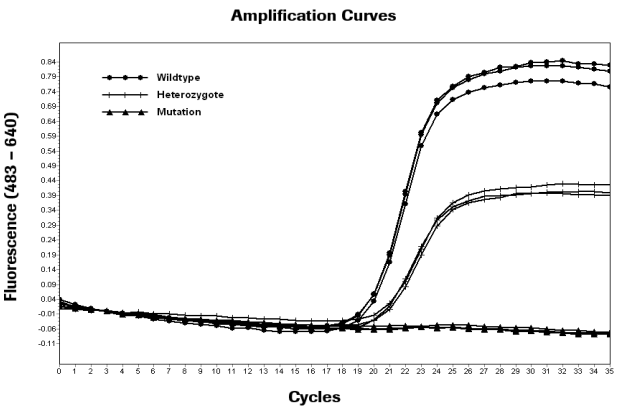

Fig. 6: Amplification curves of wild type and mutant target DNAs.

Part 2: Melting Curve Analysis, Channel 640

Melting curve analysis obtained when procedure B was analyzed with the T_m Calling module in channel "483 - 640".

The wild type melting peak can be clearly distinguished from the mutant peak, which has a melting temperature that is approx. 11°C lower because of the mismatch.

The plot shows (top) fluorescence versus temperature and (bottom) the first derivative of fluorescence versus temperature.

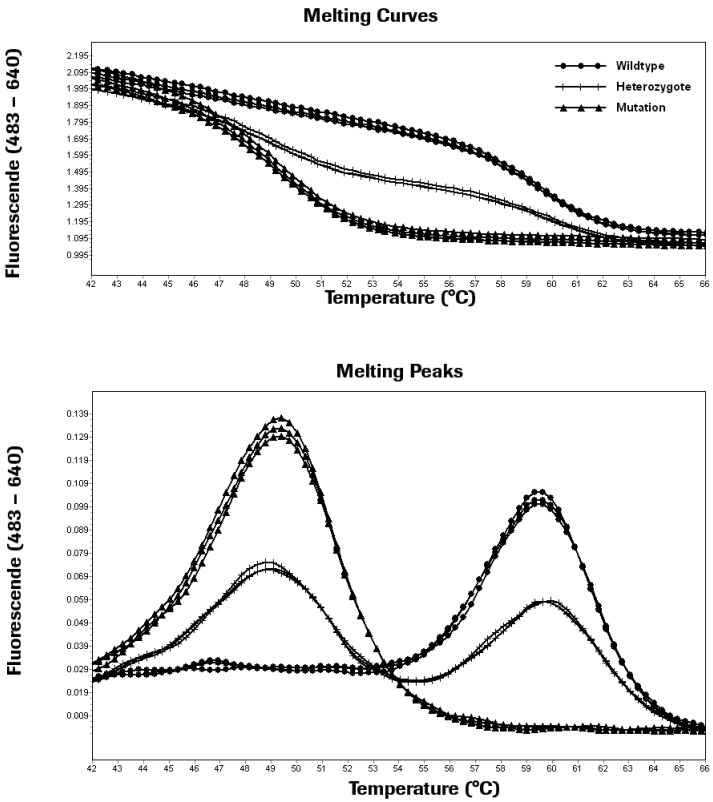

Fig. 8: Melting curve analysis of the PCR product with and without the mutation.

	Possible Cause	Recommendation
No amplification visible	Wrong channel was chosen for monitoring amplification.	Check the channel chosen on the programming screen.
	Pipetting errors or omitted reagents.	Check all reagents, especially for missing dye.
	Measurements do not occur.	Check the cycle programs. Choose “single” as acquisition mode at the end of the annealing phase for detection with hydrolysis probes and HybProbe probes.
Fluorescence intensity varies	Pipetting errors	Repeat experiment with improved pipetting accuracy or using an appropriate pipetting robot.
Negative control samples give positive values	Contamination	• Replace all critical solutions. • Pipette reagents on a clean bench. • Use heat-labile Uracil DNA-Glycosilase* (UNG) to eliminate carryover contamination from PCR products

Please refer to the package insert of your LightCycler® 480 System kits for further troubleshooting suggestions.

5. Additional Information on this Product

How this Product Works

Experiment A, Quantification:

A 144 bp fragment of the human CyP2C9 gene is amplified from plasmid DNA and detected with a short hydrolysis probe that is labeled with FAM (probe #80 from the Universal ProbeLibrary*). To test the precision of the LightCycler® 480 System, replicates with only 1000 or 2000 copies of target DNA per well are distributed throughout the plate and quantified with reference to a row of standards.

As an internal control (to prove absence of PCR inhibition), a small amount (about 100 copies) of an artificial DNA template is added to each well. This control is co-amplified with the target DNA. Its amplification is detected simultaneously with a LightCycler® Red 610-labeled hydrolysis probe. The results are displayed in a separate optical channel. The distances between the wavelengths of the two detection channels (483-533 and 558-610) are high enough that there is no need to use color compensation to correct for crosstalk.

Experiment B, Genotyping:

The same 144 bp fragment of the CyP2C9 gene is amplified from different samples of plasmid DNA. This gene is known to contain a single nucleotide polymorphism (SNP), and various samples included in the experiment contain the wild type sequence, the homozygous point mutation and heterozygote DNA with wild type and mutant strands. With HybProbe probes for detection, a subsequent melting curve analysis can be used for identification of the different genotypes, because the probe melts off the perfectly matched sequence and the mismatched sequence at different melting temperatures.

References

1 PCR Manual, Roche Diagnostics (1999) 2nd edition (1999) 2, 52-58.
2 Zipper H et al. (2004). Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nuc. Acid Res.* **32**, e103.
3 Kellogg DE et al. (1994). TaqStart Antibody: "hot start" PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. *Biotechniques* **16**, 1134-1137.

Quality Control

The LightCycler® 480 Control Kit is function tested with the LightCycler® 480 System and the LightCycler® 480 Probes Master, according to the protocols described above.

6. Supplementary Information

6.1 Conventions

Text Conventions

To make information consistent and memorable, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered stages labeled ①, ②, etc.	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ❶, ❷, etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

Symbols

In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

- Editorial changes

6.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, www.roche-applied-science.com, and our Special Interest Sites at:

- http://www.roche-applied-science.com\lightcycler480

	Product	Pack Size	Cat. No.
Instrument and Accessories	LightCycler® 480 Instrument	1 instrument plus accessories	12 011 468 001
	LightCycler® 480 Multiwell Plate 384	50 plates and sealing foils	04 729 749 001
Software	LightCycler® 480 Sealing Foil	5 × 10 foils	04 729 757 001
Associated Kits and Reagents	LightCycler® 480 Genotyping Master	1 kit (4 × 96 reactions, 20 µl each)	04 707 524 001
	LightCycler® 480 Probes Master	1 kit (5 × 100 reactions, 20 µl each)	04 707 494 001
	LightCycler® SYBR Green I Master	1 kit (5 × 100 reactions, 20 µl each)	04 707 516 001
	Universal ProbeLibrary, Probe #80		04 689 038 001

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