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LightCycler® 480 Control Kit

Version 11

Content version: July 2014

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

Cat. No. 04 710 924 001 Kit for 3 control reactions

Store the kit at −15 to −25°C

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

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PROTOCOL

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	• > 450 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.	• 255 µl • mix of two target-specific primers
10 red	Genotyping Probes 10× conc.	• 80 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, containing LightCycler® Red 610-labeled hydrolysis probe for detection of control DNA sequence
13 colorless	H_2O, PCR grade	1,000 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

Additional reagents and equipment required to perform reactions with the LightCycler® 480 Control Kit using the LightCycler® 480 System include:

- LightCycler® 480 Instrument, 96 or 384 well*
- LightCycler® 480 Probes Master*
- LightCycler® 480 Multiwell Plate 96 or 384 with 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

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 different analysis types are provided with the kit:

- Procedure A: Gene quantification
- Procedure B: Genotyping

The following procedures show how to use the LightCycler® 480 Control Kit.

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

Procedure A: Gene quantification

①	Set up instrument.
②	Prepare 3 reaction mixes: • PCR mix 1: for 24-fold replicates of 1,000 copies of target DNA • PCR mix 2: for 24-fold replicates of 2,000 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

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

2.3 Procedure A: Quantification

A 136 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 1,000 or 2,000 copies of target DNA per well are distributed throughout the plate and quantified with reference to a row of standards.

If the target PCR in a particular well is negative or only weakly positive, an internal control can prove absence of PCR inhibition. Therefore an additional DNA target is amplified simultaneously in each well and detected with a LightCycler® Red 610-labeled probe in a separate optical channel.

Alternatively, quantification of the target DNA can be achieved by detection with SYBR Green I, using the LightCycler® SYBR Green I Master instead of the LightCycler® Probes Master (see 2.3.2). In this case no probes are necessary and there is no internal control applicable.

2.3.1 Quantification with Hydrolysis Probes

LightCycler® 480 Instrument Protocol

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

A LightCycler® 480 Instrument 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.

Settings for the 384-well block type that differ from the 96-well settings are given in brackets.

Set-Up		
Detection Format	**Block Type**	**Reaction Volume**
Multi Color hydrolysis probes (for LightCycler®480 Instrument Version I) **or** Customized format (for Light Cycler®480 Instrument Version II)	96 (384)	20 µl
Customize	dynamic mode, FAM and Red 610 For LightCycler® 480 Instrument Version I: 483–533, and 558–610 For LightCycler® 480 Instrument Version II: 465–510, and 533–610, Quant factors = 10	

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	4.4 (4.8)	–
Amplification					
Segment 1:	95	None	00:00:10	4.4 (4.8)	–
Segment 2:	60	Single	00:00:30	2.2 (2.5)	–
Segment 3: (optional)	72	None	00:00:01	4.4 (4.8)	–
Cooling					
	40	None	00:00:30	1.5 (2.0)	–

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 1,000 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	1,000 copies/20 µl

PCR Mix B

(24-fold replicates of a standard containing 2,000 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	2,000 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, 6 and 11 (1, 12 and 23) • PCR mix B (with Standard 3) into the indicated wells of columns 2, 7 and 12 (2, 13 and 24)
❹	Dispense 15 µl from PCR mix C into each of the wells that will contain negative control and Standards 1, 2, 4, 5, 6 in triplicate (see pipetting scheme below).
❺	Add 5 µl of each standard to three plate wells indicated on the pipetting scheme below: Negative control (NC): H_2O, PCR grade (from vial 13). Standard curve: • Standard 1 (10^2 copies of DNA) • Standard 2 (10^3 copies of DNA) • Standard 4 (10^4 copies of DNA) • Standard 5 (10^5 copies of DNA) • Standard 6 (10^6 copies of DNA)
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 1,500 × *g* for 2 min.
❼	Load the multiwell plate into the LightCycler® 480 Instrument.
❽	Start the PCR program described above.

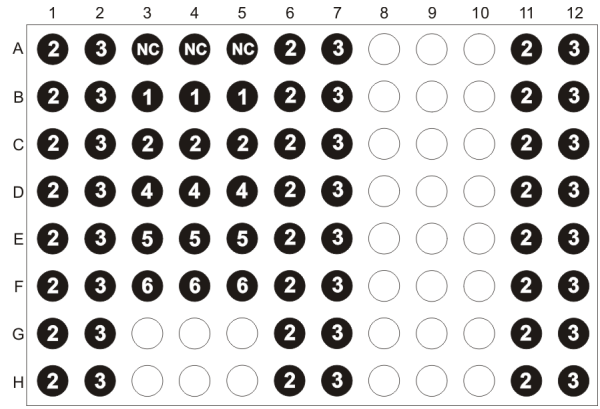

Fig. 1: Pipetting scheme for the LightCycler® 480 Multiwell Plate 96 used in procedure A. Positions are indicated for negative control ❿ and standards ❶ ❷ ❸ ❹ ❺ ❻, respectively.

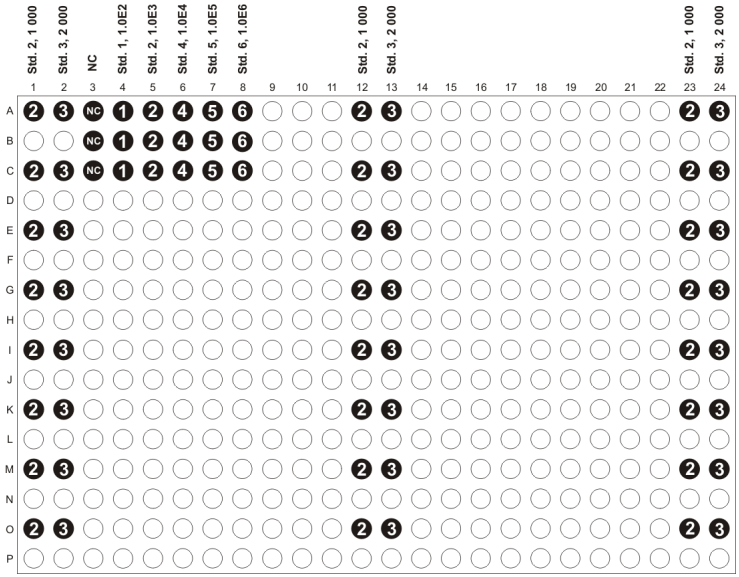

Fig. 2: Pipetting scheme for the LightCycler® 480 Multiwell Plate 384 used in procedure A. Positions are indicated for negative control ⓃⒸ and standards ❶ ❷ ❸ ❹ ❺ ❻, respectively.

Evaluation

⚠ Make sure the concentrations of the standards (three replicates each) are defined in the 'Sample Editor' in the 'Abs Quant' folder. For faster evaluation, use 'Subset Editor' and define a subset for the used plate positions.

❶ Under 'Analysis', open the 'Absolute Quantification' module for this subset, make sure filter combination FAM (483 – 533, LightCycler® 480 Instrument I; 465 – 510, LightCycler® 480 Instrument II) is displayed, and click 'Calculate' to calculate the crossing points and standard curve.

❷ For analysis of the internal control, switch to filter combination Red 610 (558 - 610, LightCycler® 480 Instrument I; 533 – 610, LightCycler® 480 Instrument II).

2.3.2 Quantification with SYBR Green I (optional)

LightCycler® 480 Instrument Protocol

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

A LightCycler® 480 protocol for procedure A using the LightCycler® 480 SYBR Green I 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 PCR products
- **Cooling** the multiwell plate

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

Settings for the 384-well block type that differ from the 96-well settings are given in brackets.

Set-Up		
Detection Format	**Block Type**	**Reaction Volume**
SYBR Green I	96 (384)	20 µl
Customize	dynamic mode, SYBR Green I	
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	4.4 (4.8)	–
Amplification					
Segment 1:	95	None	00:00:20	4.4 (4.8)	–
Segment 2:	60	None	00:00:15	2.2 (2.5)	–
Segment 3:	72	Single	00:00:15	4.4 (4.8)	–
Melting Curve					
Segment 1:	95	None	00:00:05	4.4 (4.8)	–
Segment 2:	70	None	00:01:00	2.2 (2.5)	–
Segment 3:	95	Continuous	-	-	2
Cooling					
	40	None	00:00:30	1.5 (2.0)	-

Preparation of the PCR Mixes

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

❶ Thaw the following reagents, mix gently and store on ice:

- LightCycler® 480 SYBR Green I Master: vial 1
- LightCycler® 480 Control Kit: vials 1, 2, 3, 4, 5, 6, 9, 13

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 1,000 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.
Water PCR grade (vial 13)	108 µl	–
LightCycler® 480 SYBR Green I Master	270 µl	1×
Primer Mix, 20× (vial 9)	27 µl	1×
Standard 2 (vial 2)	135 µl	1,000 copies/20 µl

PCR Mix B

(24-fold replicates of a standard containing 2,000 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.
Water PCR grade (vial 13)	108 µl	–
LightCycler® 480 SYBR Green I Master	270 µl	1×
Primer Mix, 20× (vial 9)	27 µl	1×
Standard 3 (vial 3)	135 µl	2,000 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.
Water, PCR grade (vial 13)	80 µl	-
LightCycler® 480 SYBR Green I Master	200 µl	1.33×
Primer Mix, 20× (vial 9)	20 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 in chapter 2.3.1: • PCR mix A (with Standard 2) into the indicated wells of columns 1, 6 and 11 (1, 12 and 23) • PCR mix B (with Standard 3) into the indicated wells of columns 2, 7, and 12 (2, 3 and 24)
❹	Dispense 15 µl from PCR mix C into each of the wells that will contain negative control and Standards 1, 2, 4, 5, 6 in triplicate (see pipetting scheme in figure 1 and 2).
❺	Add 5 µl of each Standard to three plate wells as indicated in figures 1 and 2 in the preceding chapter: Negative control (NC): H_2O, PCR grade (from vial 13). Standard curve: • Standard 1 (10^2 copies of DNA) • Standard 2 (10^3 copies of DNA) • Standard 4 (10^4 copies of DNA) • Standard 5 (10^5 copies of DNA) • Standard 6 (10^6 copies of DNA)
❻	• Seal the multiwell plate with LightCycler® 480 Sealing Foil. • Place the multiwell plate in a standard swing-bucked 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 1,500 × *g* for 2 min.
❼	Load the multiwell plate into the plate holder of the LightCycler® 480 Instrument.
❽	Start the PCR program described above.

Evaluation

⚠ Make sure the concentrations of the standards (three replicates each) are defined in the 'Sample Editor' in the 'Abs Quant' folder. For faster evaluation, use 'Subset Editor' and define a subset for the used plate positions.

❶	Under 'Analysis', open the 'Absolute Quantification' module for this subset and click 'Calculate' to calculate crossing points and standard curve.
❷	Add a second analysis with the 'Plus' button, choose the 'T_m Calling' module for this subset and click 'Calculate' to calculate T_m values.

2.4 Procedure B: Genotyping with HybProbe probes

A 136 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.
Alternatively, the LightCycler® 480 Probes Master or the LightCycler® 480 Genotyping Master can be used with the same protocol.

LightCycler® 480 Instrument Protocol

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

A LightCycler® 480 Instrument protocol that uses the LightCycler® 480 Probes Master and the LightCycler® 480 Control Kit with procedure B 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.

Settings for the 384-well block type that differ from the 96-well settings are given in brackets

Set-Up					
Detection Format		**Block Type**		**Reaction Volume**	
Mono Color HybProbe		96 (384)		20 µl	
Customize		dynamic mode, Red 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	4.4 (4.8)	–
Amplification					
Segment 1:	95	None	00:00:10	4.4 (4.8)	–
Segment 2:	55	Single	00:00:10	2.2 (2.5)	–
Segment 3:	72	None	00:00:10	4.4 (4.8)	–
Melting Curve					
Segment 1:	95	None	00:01:00	4.4 (4.8)	–
Segment 2:	40	None	00:01:00	2.2 (2.5)	–
Segment 3:	80	Continuous	—	—	2
Cooling					
	40	None	00:00:30	1.5 (2.0)	–

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 or LightCycler® 480 Genotyping 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.

If you use the LightCycler® 480 Probes Master:

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×

Alternatively, using LightCycler® 480 Genotyping Master:

Component	Vol	Final conc.
H_2O, PCR grade	80 µl	–
LightCycler® 480 Genotyping Master	40 µl	1.33×
Primer Mix, 20× (vial 9)	10 µl	1.33×
Genotyping Probe, 10× (vial 10)	20 µl	1.33×

❸ Choose 9 wells of the plate and pipette 15 µl PCR mix into each of these wells.

❹ Add standard DNA to these 9 wells, 5 µl/well, each standard in triplicate:
- three wells Wild Type (vial 5)
- three wells Heterozygous (vial 7)
- three wells Mutation (vial 8)

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 1,500 × *g* for 2 min.

❻ Load the multiwell plate into the LightCycler® 480 Instrument.

❼ Start the PCR program described above.

Evaluation

❶ In the 'Subset Editor', define a subset with the 9 used plate positions.

❷ Under 'Analysis' open the 'Absolute Quantification' module for this subset and click 'Calculate' to calculate the fluorescence values versus cycle numbers.

❸ Add the second analysis with the 'Plus' button, choose the 'Tm Calling' module for this subset and click 'Calculate' to calculate the T_m values.

3. Results

3.1 Typical Results Obtained in Procedure A

3.1.1 Quantification with Hydrolysis Probes

Filter Combination for the Quantification Target

The following amplification curves were optained when procedure A was monitored in the FAM channel. The plot shows fluorescence versus cycle number.

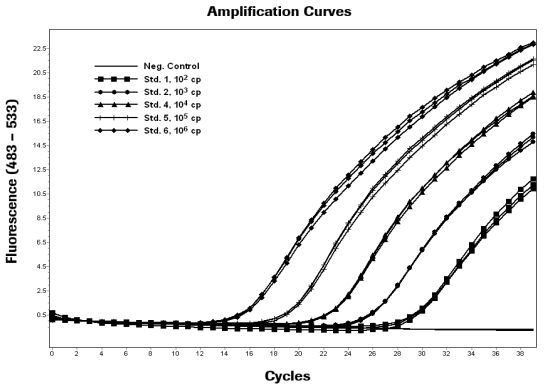

Fig. 3: Amplification curves of the standards in the FAM channel

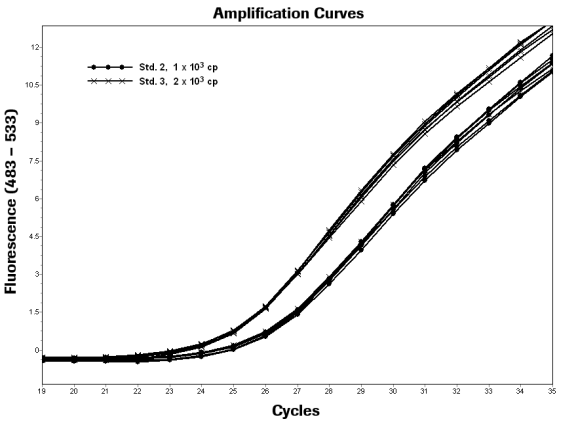

Fig. 4: The amplification curves of the replicates with 1,000 or 2,000 copies of target DNA are clearly separated in the FAM channel.

Filter Combination for the Internal Control

The following amplification curves of the internal control were optained when procedure A was monitored in the channel for LightCycler® Red 610. 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.

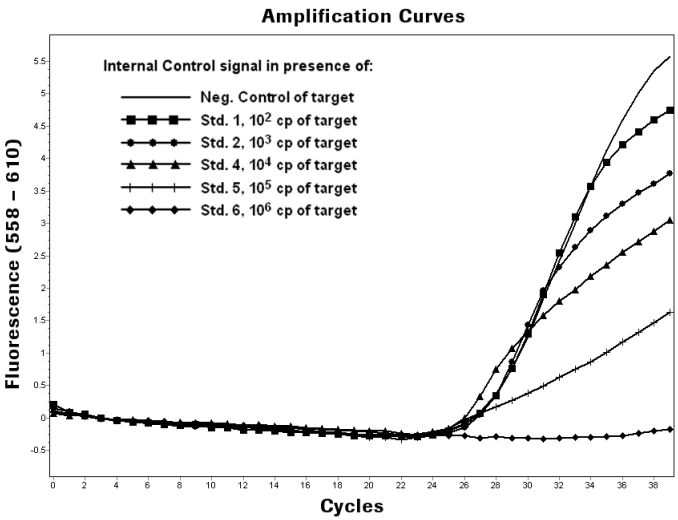

Fig. 5: Amplification curves of the internal control in the Red 610 channel

3.1.2 Quantification with SYBR Green I

Quantification

The following amplification curves were optained when procedure A was monitored in the channel for SYBR Green I. The plot shows fluorescence versus cycle number.

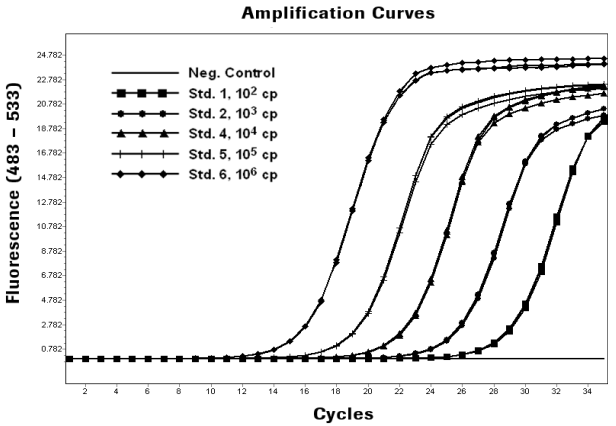

Fig. 6: Amplification curves of the standards detected with SYBR Green

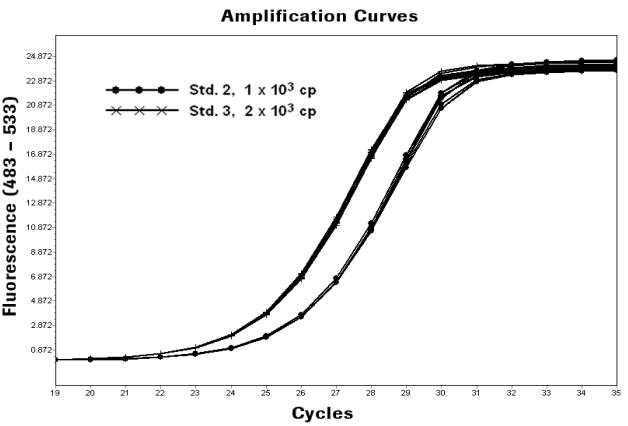

Fig. 7: The amplification curves for the replicates with 1,000 or 2,000 copies of target DNA are clearly separated detected with SYBR Green I

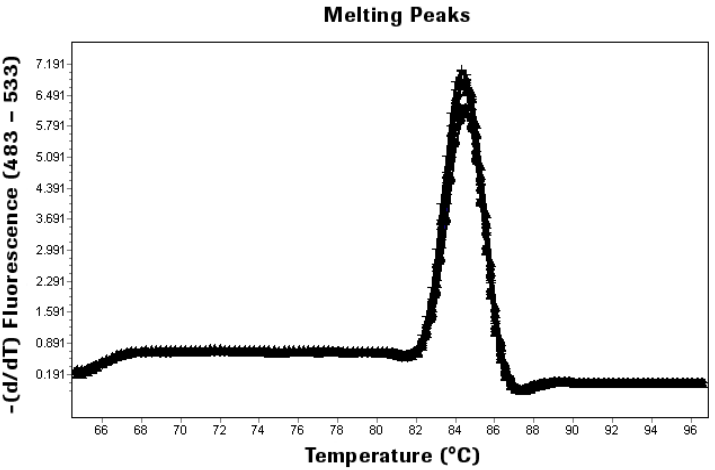

Fig. 8: Melting curve analysis results in a single peak for each positive sample, proving amplification of only the specific target sequence and no byproducts.

3.2 Typical Results Obtained in Procedure B

Data analysis is divided into two parts:
Part 1: Quantification with Absolute Quantification module
Part 2: Melting curve analysis with T_m Calling module,

Part 1: Quantification

The following amplification curves were obtained when procedure B was analyzed with the Absolute Quantification module using filter combination (483 - 640, LightCycler® 480 Instrument I, or 498 - 640 for LightCycler® 480 Instrument II).

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.

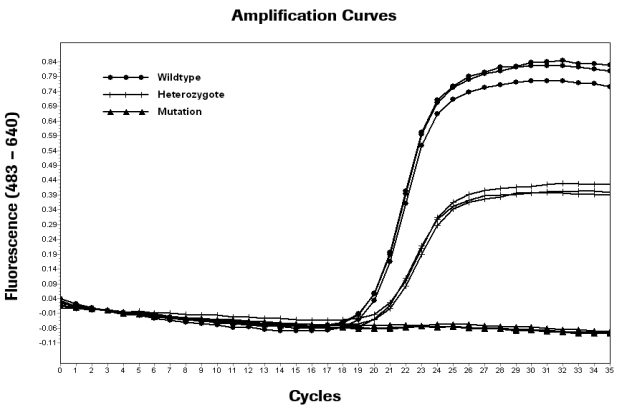

Fig. 9: Amplification curves of wild type and mutant target DNAs.

⚠ Using the LightCycler® 480 Genotyping Master, crossing points may occur about 5 cycles later. Data in this figure are produced with the LightCycler® 480 Probes Master.

Part 2: Melting Curve Analysis

Melting curve analysis obtained when procedure B was analyzed with the T_m Calling module using filter combination.(483 - 640, LightCycler® 480 Instrument I, or 498 - 640 for LightCycler® 480 Instrument II)

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.

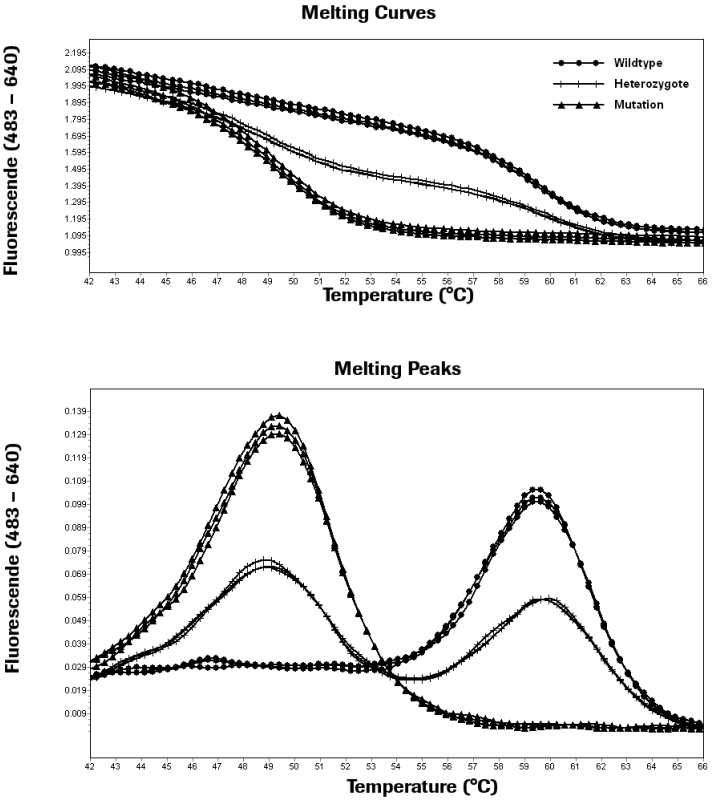

Fig. 10: Melting curve analysis of the PCR product with and without the mutation.

4. Troubleshooting

	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 136 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 1,000 or 2,000 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, LightCycler® 480 Instrument I; 465 – 510 and 533 - 610, LightCycler® 480 Instrument II) are high enough that there is no need to use color compensation to correct for crosstalk.

Alternatively, the target amplification can be detected using SYBR Green I. By subsequent melting curve analysis of the PCR product, the specificity of the reaction can be proven.

Experiment B, Genotyping:

The same 136 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 document:

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 Diagnostics

Symbols

In this document, 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

- Correction of the filling volume
- Update of Ordering Information, License Disclaimer and Trademarks,
- Editorial changes

6.3 Ordering Information

Roche 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.lifescience.roche.com.

	Product	Pack Size	Cat. No.
Instruments	LightCycler® 480 Instrument II, 96-well	1 instrument with control unit and accessories	05 015 278 001
	LightCycler® 480 Instrument II, 384-well	1 instrument with control unit and accessories	05 015 243 001
Software	LightCycler® 480 Software, Version 1.5	1 software package	04 994 884 001
	LightCycler® 480 LIMS Interface Module	1 software package	05 066 310 001
	LightCycler® 480 Gene Scanning Software	1 software package	05 103 908 001

	Product	Pack Size	Cat. No.
Accessories	LightCycler® 480 Thermal Block Cycler Unit (96-well) Silver	96-well thermal block cycler unit, including block cycler cover, storage box and loading device	05 015 219 001
	LightCycler® 480 Thermal Block Cycler Unit (384-well) Silver	384-well thermal block cycler unit, including block cycler cover, storage box and loading device	05 015 197 001
	LightCycler® 480 Multiwell Plate 96	50 plates with 50 sealing foils	04 729 692 001
	LightCycler® 480 Multiwell Plate 384	50 plates with 50 sealing foils	04 729 749 001
	LightCycler® 480 Multiwell Plate 96, clear	50 plates with 50 sealing foils	05 102 413 001
	LightCycler® 480 Multiwell Plate 384, clear	50 plates with 50 sealing foils	05 102 430 001
	LightCycler® 480 Sealing Foil	1 × 50 foils	04 729 757 001
PCR Reagents	LightCycler® 480 SYBR Green I Master	5 × 1 ml (5 × 100 reactions, 20 µl each) 10 × 5 ml (10 × 500 reactions, 20 µl each)	04 707 516 001 04 887 352 001
	LightCycler® 480 High Resolution Melting Master	1 kit (5 × 100 reactions, 20 µl each)	04 909 631 001
	LightCycler® 480 Probes Master	1 kit (5 × 100 reactions, 20 µl each) 1 kit (10 × 500 reactions, 20 µl each) 1 kit (1 × 5,000 reactions, 20 µl each)	04 707 494 001 04887 301 001 04 902 343 001
	LightCycler® 480 Genotyping Master	1 kit (384 reactions, 20 µl each)	04 707 524 001
	LightCycler® 480 RNA Master Hydrolysis Probe	1 kit (5 × 100 reactions)	04 991 885 001

6.4 Disclaimer of License

NOTICE TO PURCHASER

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany