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

Roche

Roche Applied Science

LightCycler® 480 Control Kit

Version December 2005

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 the LightCycler® 480 genotyping probes (vial 10), quantification probe (vial 11) and internal control (vial 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, containing 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

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

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

** 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 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 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: Gene quantification

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

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.
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.

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	–

Set-Up					
Cooling					
	40	None	00:00:30	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 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 Load the multiwell plate into the LightCycler® 480 Instrument.

❽ Start the PCR program described above.

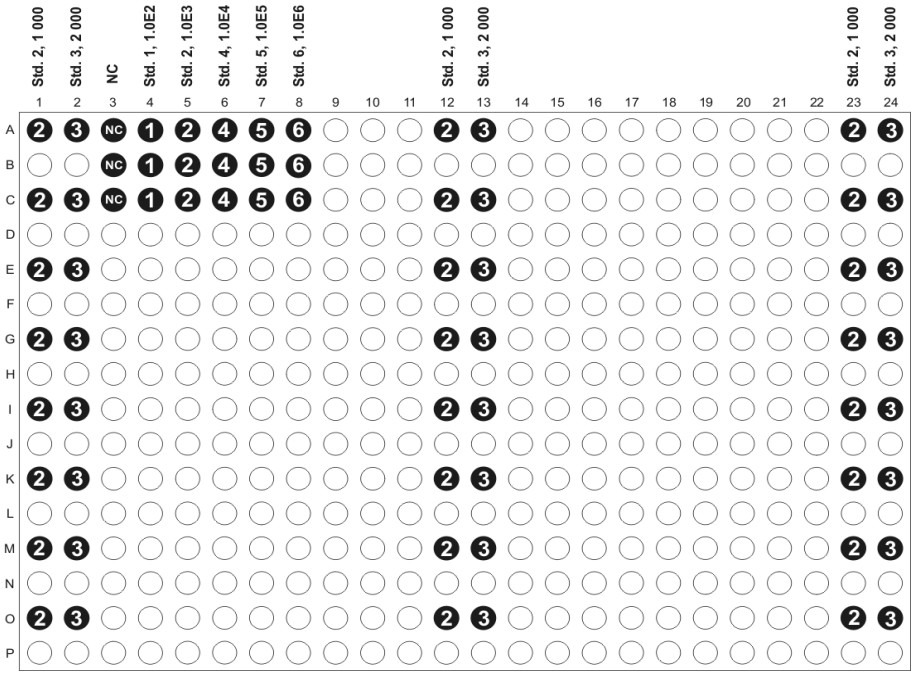

Fig. 1: Pipetting scheme for the LightCycler® 480 Multiwell Plate 384 used in procedure A. Positions are indicated for and standards ❶ ❷ ❸ ❹ ❺ ❻, standards 1, 2, 3, 4, 5, and 6, respectively and negative control K

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 filter combination FAM (483 – 533) is displayed, and click 'Calculate' to calculate the crossing points and standard curve.
- For quantification of the internal control, switch to filter combination Red 610 (558 - 610).

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.

Set-Up				
Detection Format		**Block Type**		**Reaction Volume**
SYBR Green I		384		20 µl
Filter Setting		dynamic mode, SYBR Green I (483 – 533)		
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	00:00:20	4.8	–
Segment 2:	60	None	00:00:15	2.5	–
Segment 3:	72	Single	00:00:15	4.8	–
Melting Curve					
Segment 1:	95	None	00:00:05	4.8	–
Segment 2:	70	None	00:01:00	2.5	–
Segment 3:	95	Continuous	-	-	2
Cooling					
	40	None	00:00:30	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 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.
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	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.
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	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.
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 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 as indicated in figure 1 in the preceding chapter: 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-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 1500 × *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 in columns 4 – 8 are defined in the 'Sample Editor' in the 'Abs Quant' folder.

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

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. 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.

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	–

Set-Up					
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	2.0	–

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 and store on ice.
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 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	Load the multiwell plate into the LightCycler® 480 Instrument.
❼	Start the PCR program described above.

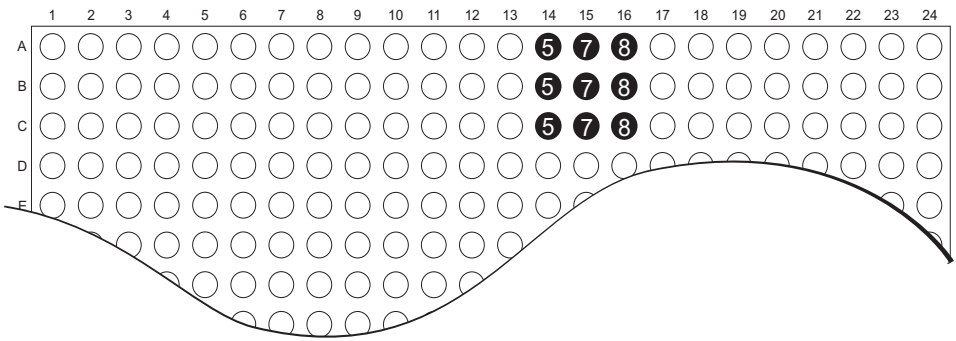

Fig. 2: Pipetting scheme for the LightCycler® 480 Multiwell Plate 384 used in procedure B.
❺ ❼ ❽ Positions for standards from vials 5, 7 and 8, respectively.

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

3.1.1 Quantification with Hydrolysis Probes

Quantification using Filter Combination (483 – 533)

The following amplification curves were obtained when procedure A was monitored by filter combination (483 - 533). The plot shows fluorescence versus cycle number.

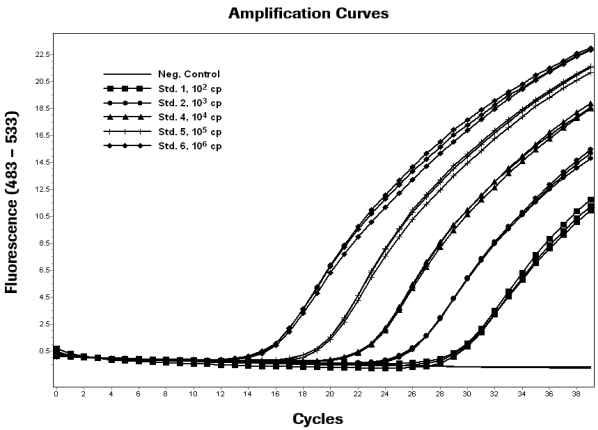

Fig. 3: Amplification curves of the standards using filter combination (483 - 533).

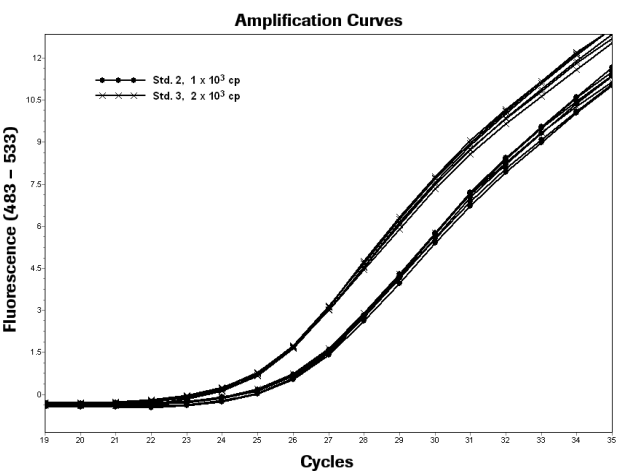

Fig. 4: The amplification curves for the replicates with 1000 or 2000 copies of target DNA are clearly separated using filter combination (483 - 533).

Quantification using Filter Combination (558 – 610)

The following amplification curves were obtained when procedure A was monitored by filter combination (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.

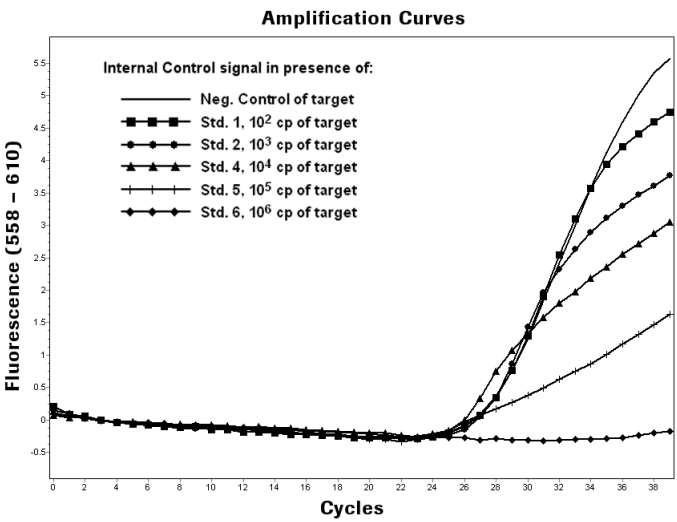

Fig. 5: Amplification curves of the internal control using filter combination (558 - 610).

3.1.2 Quantification with SYBR Green I

Quantification

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

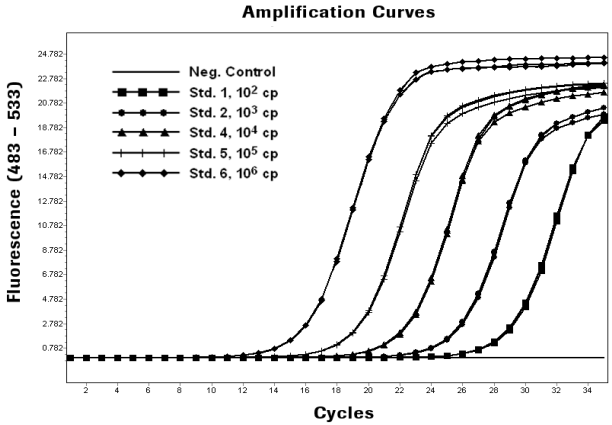

Fig. 6: Amplification curves of the standards detected with SYBR Green I using the filter combination (483 – 533).

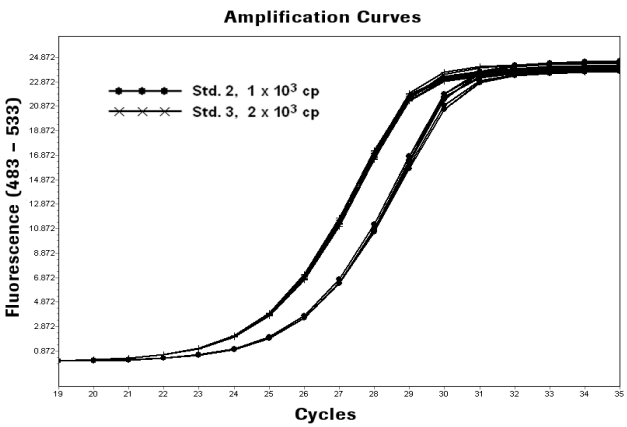

Fig. 7: The amplification curves for the replicates with 1000 or 2000 copies of target DNA are clearly separated detected with SYBR Green I using the filter combination (483 – 533).

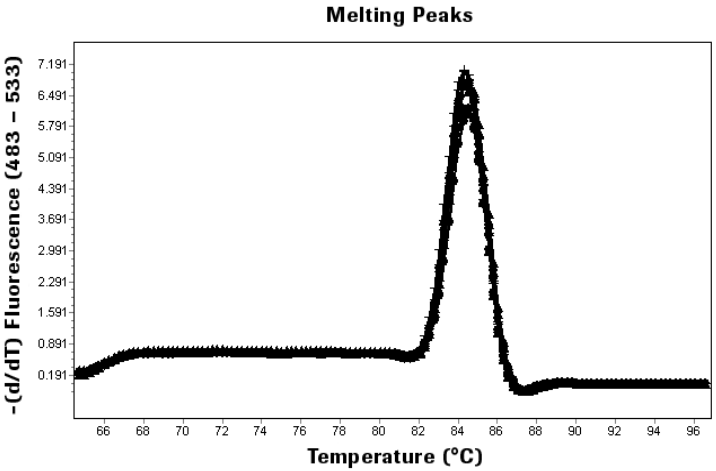

Fig. 8: Melting curve analysis results in a single peak for each positive sample, proving amplification of only the specific target sequence and no byproducts.

Data analysis is divided into two parts:

Part 1: Quantification with Absolute Quantification module, filter combination (483 – 640).

Part 2: Melting curve analysis with T_m Calling module, filter combination (483 - 640).

Part 1: Quantification using Filter Combination (483 – 640)

The following amplification curves were obtained when procedure B was analyzed with the Absolute Quantification module using filter combination (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.

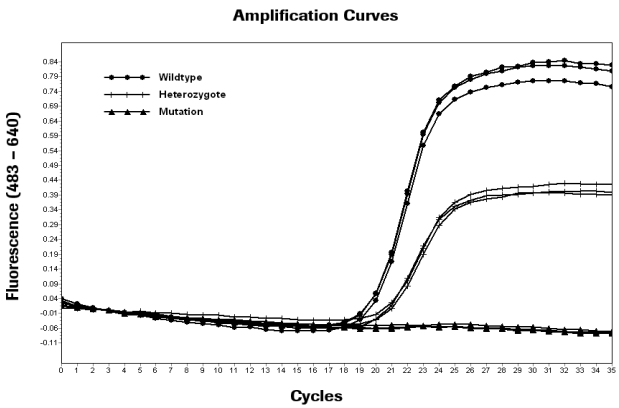

Fig. 9: Amplification curves of wild type and mutant target DNAs.

⚠ 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 using Filter Combination (483 – 640)

Melting curve analysis obtained when procedure B was analyzed with the T_m Calling module using filter combination (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.

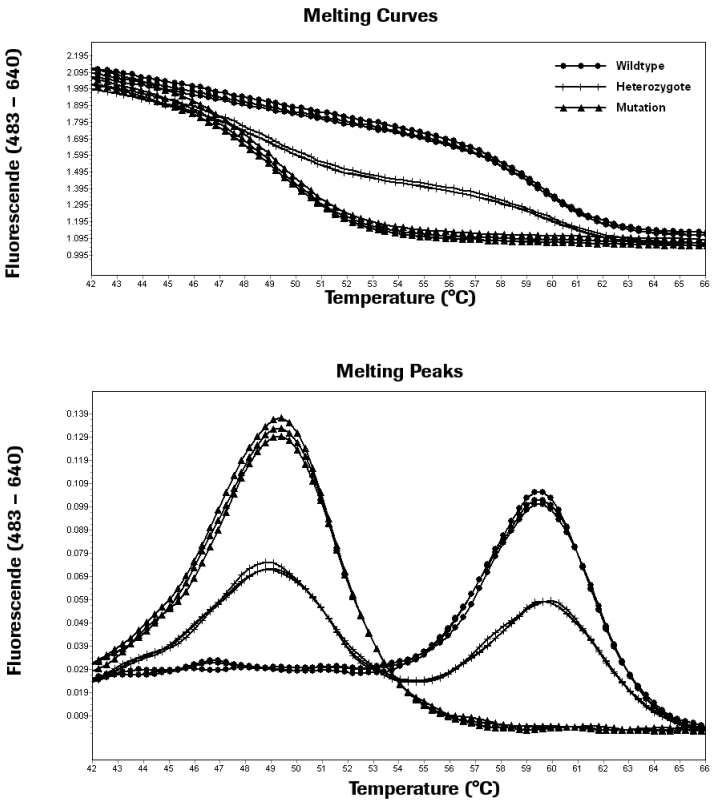

Fig. 10: Melting curve analysis of the PCR product with and without the mutation.

	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 refe-rence 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
- Addition of new products

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, 384-well	1 instrument with data workstation and accessories	04 545 885 001
	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

6.4 Disclaimer of License

Notice to Purchaser

A license to perform the 5' nuclease process for research requires the use of a Licensed 5' Nuclease Kit (containing Licensed Probe), or the combination of an Authorized Core Kit plus Licensed Probe, or license rights that may be purchased from Applied Biosystems. This product contains Licensed Probe. Its purchase price includes a limited, non transferable immunity from suit under U.S. Patents Nos. 6,214,979 and 5,804,375 (claims 1-12 only) and corresponding patent claims outside the United States, owned by Roche Molecular Systems, Inc. or F. Hoffmann-La Roche Ltd ("Roche"), for using only this amount of probe in the practice of the 5' nuclease process solely for the purchaser's own internal research and development activities. This product is also a Licensed Probe for use with service sublicenses available from Applied Biosystems. This product conveys no rights under U.S. Patents Nos. 5,210,015 and 5,487,972, which claim 5' nuclease processes, or U.S. Patents Nos. 5,476,774 and 5,219,727, which claim quantification methodology, and corresponding patent claims outside the United States of any of the foregoing patents and no right under any other patent claims (such as apparatus or system claims in U.S. Patent No. 6,814,934) and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted expressly, by implication, or by estoppel. This product is for research purposes only. Diagnostic uses require a separate license from Roche. Further information regarding the 5' nuclease licensing program may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

NOTICE TO PURCHASER: DISCLAIMER OF LICENSE

This product is compatible for use in the Polymerase Chain Reaction (PCR) process claimed in patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd. No license under these patents is conveyed expressly, by implication, by estoppel or otherwise to the purchaser by the purchase of this product. A license to use these patented processes for certain research and development activities accompanies the purchase of certain reagents of Applied Biosystems and other licensed suppliers when used in conjunction with an authorized thermal cycler, or is available from Applied Biosystems.

The product is covered in-part by US 5,871,908, co-exclusively licensed from Evotec OAI AG.

Parts of the Software used for the LightCycler® 480 System are licensed from Idaho Technology Inc., Salt Lake City, UT, USA.
The technology used for the LightCycler® System is licensed from Idaho Technology Inc., Salt Lake City, UT, USA.

Trademarks

LIGHTCYCLER, LC, and HYBPROBE are trademarks of Roche.
SYBR is a trademark of Molecular Probes, Inc., Eugene, OR, USA.

Contact and Support

If you have questions or experience problems with this or any Roche Applied Science (RAS) product, please contact our Technical Support staff. Our scientists commit themselves to providing rapid and effective help.

We also want you to contact us if you have suggestions for enhancing RAS product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to RAS and the world-wide research community.

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support** Site at:

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country.
Country-specific contact information will be displayed.
On the Roche Applied Science home page select **Printed Materials** to find:

- in-depth Technical Manuals
- Lab FAQS: Protocols and references for life science research
- our quarterly Biochemica Newsletter
- Material Safety Data Sheets
- Pack Inserts and Product Instructions

or to request hard copies of printed materials.

1205.**04729510001**®

Roche Diagnostics

Roche Diagnostics GmbH
Roche Applied Science
68298 Mannheim
Germany