For life science research only. Not for use in diagnostic procedures.

Print Protocol | Print Changes to Previous Version | Print

LightCycler® 480 Control Kit

Version: 12

Content version: September 2016

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 1 kit
3 control reactions

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	yellow	LightCycler® 480 Control Kit Standard 1, 10^2 copies/5 µl	Target: wild type plasmid DNA	1 vial, >45 µl
2	yellow	LightCycler® 480 Control Kit Standard 2, 10^3 copies/5 µl	Target: wild type plasmid DNA	1 vial, >450 µl
3	yellow	LightCycler® 480 Control Kit Standard 3, 2 × 10^3 copies/5 µl	Target: wild type plasmid DNA	1 vial, >405 µl
4	yellow	LightCycler® 480 Control Kit Standard 4, 10^4 copies/5 µl	Target: wild type plasmid DNA	1 vial, >45 µl
5	yellow	LightCycler® 480 Control Kit Standard 5, 10^5 copies/5 µl	Target: wild type plasmid DNA	1 vial, >90 µl
6	yellow	LightCycler® 480 Control Kit Standard 6, 10^6 copies/5 µl	Target: wild type plasmid DNA	1 vial, >45 µl
7	yellow	LightCycler® 480 Control Kit Standard 7, Heterozygote	Target: heterozygous plasmid DNA	1 vial, >45 µl
8	yellow	LightCycler® 480 Control Kit Standard 8, Mutation	Target: mutant plasmid DNA	1 vial, >45 µl
9	blue	LightCycler® 480 Control Kit Primer Mix, 20x conc.	Mix of two target-specific primers	1 vial, 255 µl
10	red	LightCycler® 480 Control Kit Genotyping Probes, 10x conc.	▪ HybProbe probe mix ▪ Probe 1: Fluorescein-labeled at the 3' end ▪ Probe 2: LightCycler® Red 640-labeled at the 5' end	1 vial, 80 µl
11	green	LightCycler® 480 Control Kit Quantification Probe, 10x conc.	FAM-labeled hydrolysis probe	1 vial, 450 µl
12	purple	LightCycler® 480 Control Kit Internal Control, 10x conc.	Primer, probe, and template mix, containing LightCycler® Red 610-labeled hydrolysis probe for detection of control DNA sequence	1 vial, 450 µl
13	colorless	LightCycler® 480 Control Kit Water, PCR Grade	To adjust the final reaction volume	1 vial, 1,000 µl

1.2. Storage and Stability

Storage Conditions (Product)

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

⚠ ***Store the Genotyping Probes (Vial 10), the Quantification Probe (Vial 11), and the Internal Control (Vial 12) protected from light.***

⚠ ***Avoid repeated freezing and thawing.***

Vial / Bottle	Cap	Label	Storage
1	yellow	Standard 1	−15 to −25°C
2	yellow	Standard 2	
3	yellow	Standard 3	
4	yellow	Standard 4	
5	yellow	Standard 5	
6	yellow	Standard 6	
7	yellow	Standard 7	
8	yellow	Standard 8	
9	blue	Primer Mix	
10	red	Genotyping Probes	−15 to −25°C ⚠ ***Store protected from light.***
11	green	Quantification Probe	
12	purple	Internal Control	
13	colorless	Water, PCR Grade	−15 to −25°C

1.3. 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*, or LightCycler® 480 SYBR Green I Master*, or LightCycler® 480 Genotyping Master*
- LightCycler® 480 Multiwell Plate 96 or 384 with LightCycler® 480 Sealing Foil*
- Standard swinging-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors
- Nuclease free, aerosol-resistant pipette tips
- Pipettes
- Sterile 1.5 ml reaction tubes

1.4. 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 Procedures A and B), but it can also be used with the LightCycler® 480 SYBR Green I Master (Procedure A) or the LightCycler® 480 Genotyping Master (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 Instructions for Use is guaranteed only when it is used with the LightCycler® 480 System.***

1.5. Preparation Time

Assay Time/Hands-On-Time

Quantification with Hydrolysis Probes

Procedure	Time (min)
Prepare PCR mixes	10
Pipette into plate	15
PCR run	40
Total assay time	**1 h 5 min**

Genotyping with HybProbe Probes

Procedure	Time (min)
Prepare PCR mix	10
Pipette into plate	5
PCR run	50
Total assay time	**1 h 5 min**

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Template DNA is included in this kit.

General Considerations

Precautions

Always wear gloves when handling the PCR mixes and plates.

2.2. Protocols

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 A: for 24-fold replicates of 1,000 copies of target DNA
   – PCR Mix B: for 24-fold replicates of 2,000 copies of target DNA
   – PCR Mix C: 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.

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 the 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 below). In this case, no probes are necessary and there is no internal control applicable.

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.

Setup		
Block Type	Reaction Volume [µl]	
96 (384)	20 (20)	
Detection Format	Excitation Filter	Emission Filter
Customized Format for LightCycler® 480 Instrument I:		
FAM Red 610	483 558	533 610
Customized Format for LightCycler® 480 Instrument II:		
FAM Red 610	465 533	510 610
For new customized detection formats, set for all selected filters in the "Selected Filter Combination List" (under Tools), the following values:		
Melt Factor	1	
Quant Factor	10	
Max Integration Time (sec)	2	
Programs		
Program Name	Cycles	Analysis Mode
Pre-Incubation	1	None
Amplification	40	Quantification
Cooling	1	None

Setup					
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	95	None	00:00:10	4.4 (4.8)	–
	60	Single	00:00:30	2.2 (2.5)	–
	72 (optional)	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 the plate.***

1. 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, and 12.

2. Prepare PCR mixes.

   ⚠ ***Prepare all three PCR Mixes (A, B, C) before dispensing into 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, close the tube, mix gently, and store on ice.

Component	Volume	Final conc.
LightCycler® 480 Probes Master	270 µl	1x
Primer Mix, 20x (Vial 9)	27 µl	1x
Quantification Probe, 10x (Vial 11)	54 µl	1x
Internal Control, 10x (Vial 12)	54 µl	1x
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, close the tube, mix gently, and store on ice.

Component	Volume	Final conc.
LightCycler® 480 Probes Master	270 µl	1x
Primer Mix, 20x (Vial 9)	27 µl	1x
Quantification Probe, 10x (Vial 11)	54 µl	1x
Internal Control, 10x (Vial 12)	54 µl	1x
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, close the tube, mix gently, 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	Volume	Final conc.
LightCycler® 480 Probes Master	200 µl	1.33x
Primer Mix, 20x (Vial 9)	20 µl	1.33x
Quantification Probe, 10x (Vial 11)	40 µl	1.33x
Internal Control, 10x (Vial 12)	40 µl	1.33x

1. 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).

2. Dispense 15 µl from PCR Mix C into each of the wells that will contain a negative control and Standards 1, 2, 4, 5, 6 in triplicate (see pipetting scheme below).

3. Add 5 µl of each standard to three plate wells indicated on the pipetting scheme below:
   Negative control (NC): Water, PCR Grade (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)

4. Seal the multiwell plate with a LightCycler® 480 Sealing Foil.
   – Place the multiwell plate in a standard swinging-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 minutes.

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

6. Start the PCR program described above.

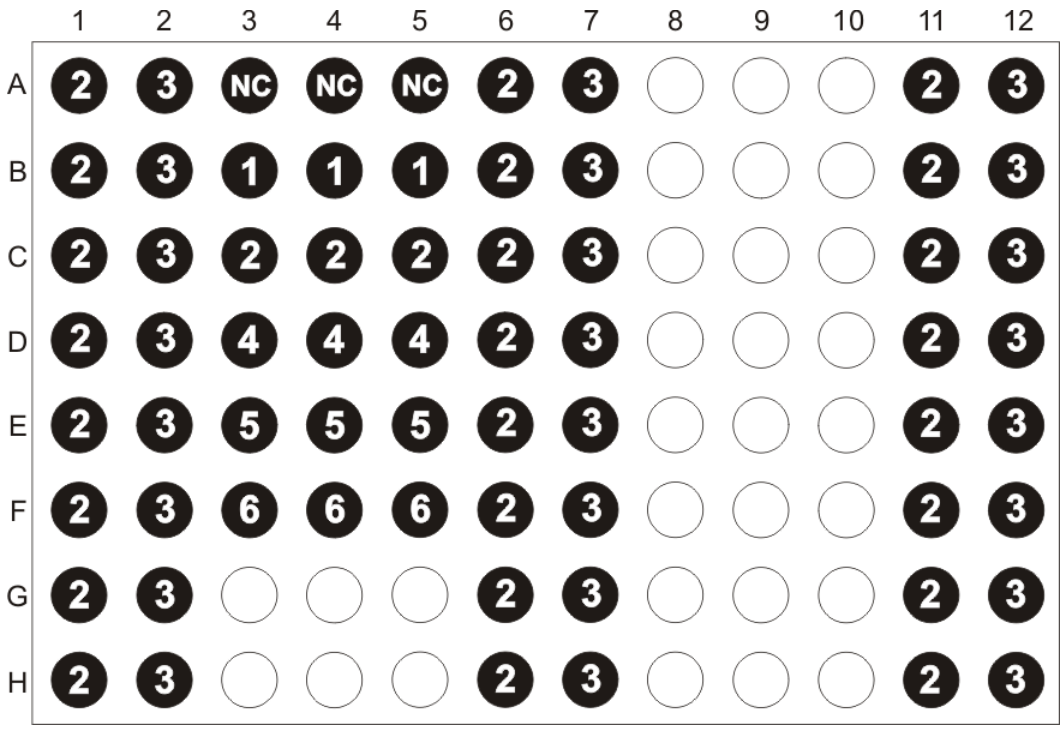

Fig. 1: Pipetting scheme for the LightCycler® 480 Multiwell Plate 96 used in Procedure A. Positions are indicated for negative control **NC** and standards **1, 2, 3, 4, 5, 6**, respectively.

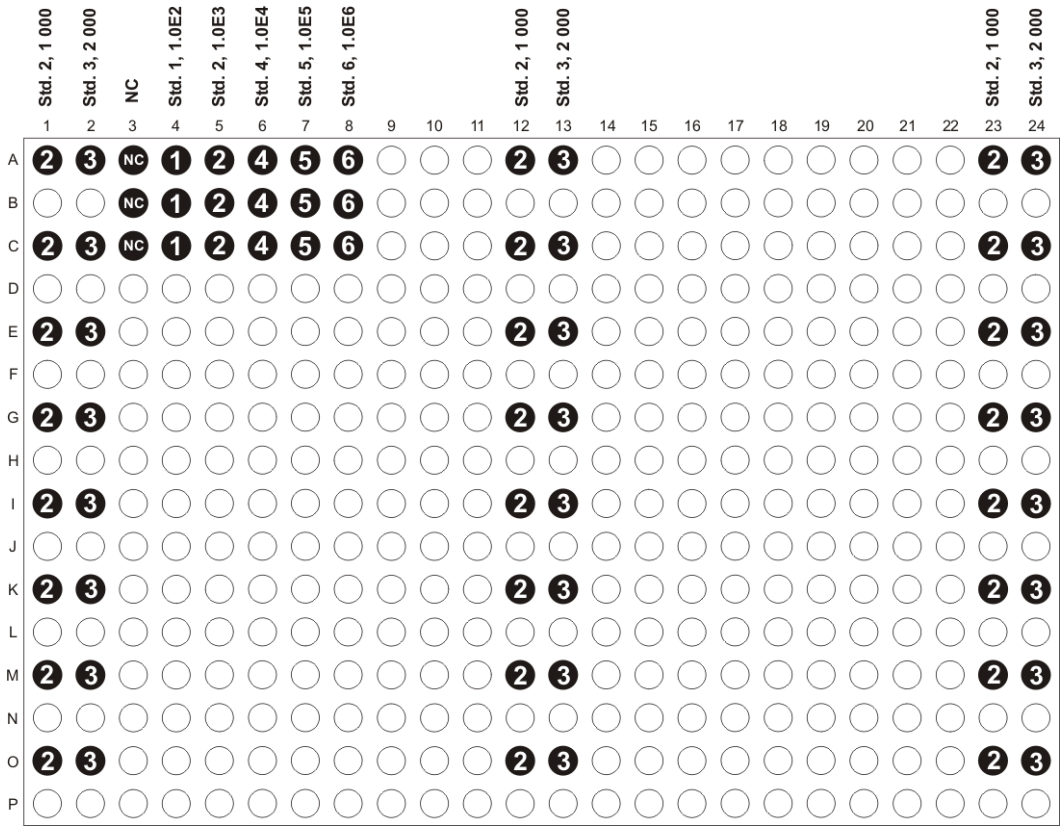

Fig. 2: Pipetting scheme for the LightCycler® 480 Multiwell Plate 384 used in Procedure A. Positions are indicated for negative control **NC** and standards **1, 2, 3, 4, 5, 6**, respectively.

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.***

1. 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.

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

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.

Setup					
Block Type			Reaction Volume [µl]		
96 (384)			20 (20)		
Detection Format	Excitation Filter		Emission Filter		
SYBR Green I / HRM Dye	465		510		
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	95	None	00:00:20	4.4 (4.8)	–
	60	None	00:00:15	2.2 (2.5)	–
	72	Single	00:00:15	4.4 (4.8)	–
Melting Curve	95	None	00:00:05	4.4 (4.8)	–
	70	None	00:01:00	2.2 (2.5)	–
	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 the plate.***

1. 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

---

2. Prepare PCR mixes

   ⚠ ***Prepare all three PCR mixes (A, B, C) before dispensing into 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 mentioned below, close the tube, mix gently, and store on ice.

Component	Volume	Final conc.
Water, PCR Grade (Vial 13)	108 µl	–
LightCycler® 480 SYBR Green I Master	270 µl	1x
Primer Mix, 20x (Vial 9)	27 µl	1x
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 mentioned below, close the tube, mix gently, and store on ice.

Component	Volume	Final conc.
Water, PCR Grade (Vial 13)	108 µl	–
LightCycler® 480 SYBR Green I Master	270 µl	1x
Primer Mix, 20x (Vial 9)	27 µl	1x
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 mentioned below, close the tube, mix gently, 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	Volume	Final conc.
Water, PCR Grade (Vial 13)	80 µl	–
LightCycler® 480 SYBR Green I Master	200 µl	1.33x
Primer Mix, 20x (Vial 9)	20 µl	1.33x

1. Dispense 20 µl from either PCR Mix A or PCR Mix B into each of the plate wells indicated on the pipetting scheme:
   – 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).

2. Dispense 15 µl from PCR Mix C into each of the wells that will contain a negative control and Standards 1, 2, 4, 5, 6 in triplicate (see pipetting scheme in Figures1 and 2).

3. Add 5 µl of each Standard to three plate wells as indicated in Figures 1 and 2 in the preceding Section:
   Negative control (NC): Water, PCR Grade (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)

4. Seal the multiwell plate with a LightCycler® 480 Sealing Foil.
   – Place the multiwell plate in a standard swinging-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 minutes.

5. Load the multiwell plate into the plate holder of the LightCycler® 480 Instrument.

6. 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.***

1. Under **Analysis**, open the **Absolute Quantification** module for this subset and click **Calculate** to calculate crossing points and standard curve.

2. Add a second analysis with the **Plus** button; choose the **Tm Calling** module for this subset and click **Calculate** to calculate Tm values.

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.

Setup				
Block Type		Reaction Volume [µl]		
96 (384)		20 (20)		
Detection Format	Excitation Filter		Emission Filter	
Mono Color HybProbe Red 640	498		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	95	None	00:00:10	4.4 (4.8)	–
	55	Single	00:00:10	2.2 (2.5)	–
	72	None	00:00:10	4.4 (4.8)	–
Melting Curve	95	None	00:01:00	4.4 (4.8)	–
	40	None	00:01:00	2.2 (2.5)	–
	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 the plate.***

1. 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).

2. In a 1.5 ml reaction tube on ice, add the components in the order mentioned below, close the tube, mix gently, and store on ice.
   If you use the LightCycler® 480 Probes Master:

Reagent	Volume	Final conc.
Water, PCR Grade	20 µl	–
LightCycler® 480 Probes Master	100 µl	1.33x
Primer Mix, 20x (Vial 9)	10 µl	1.33x
Genotyping Probe, 10x (Vial 10)	20 µl	1.33x

   Alternatively, using the LightCycler® 480 Genotyping Master:

Reagent	Volume	Final conc.
Water, PCR Grade	80 µl	–
LightCycler® 480 Genotyping Master	40 µl	1.33x
Primer Mix, 20x (Vial 9)	10 µl	1.33x
Genotyping Probe, 10x (Vial 10)	20 µl	1.33x

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

4. 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).

5. Seal the plate with a 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 minutes.

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

7. Start the PCR program described above.

Evaluation

1. In the **Subset Editor**, define a subset with the 9 used plate positions.

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

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

3. Results

Typical Results Obtained in Procedure A

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.

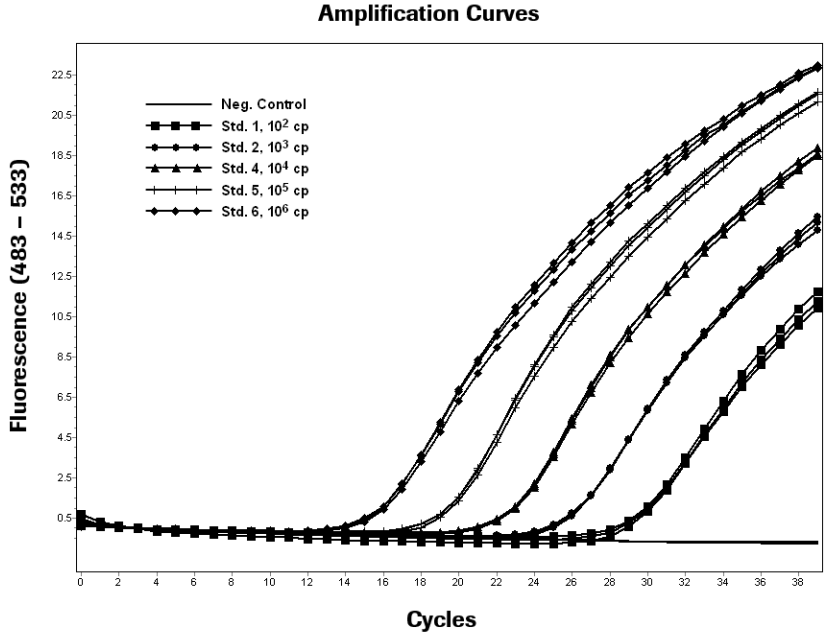

Fig. 3: Amplification curves of the standards in the FAM channel.

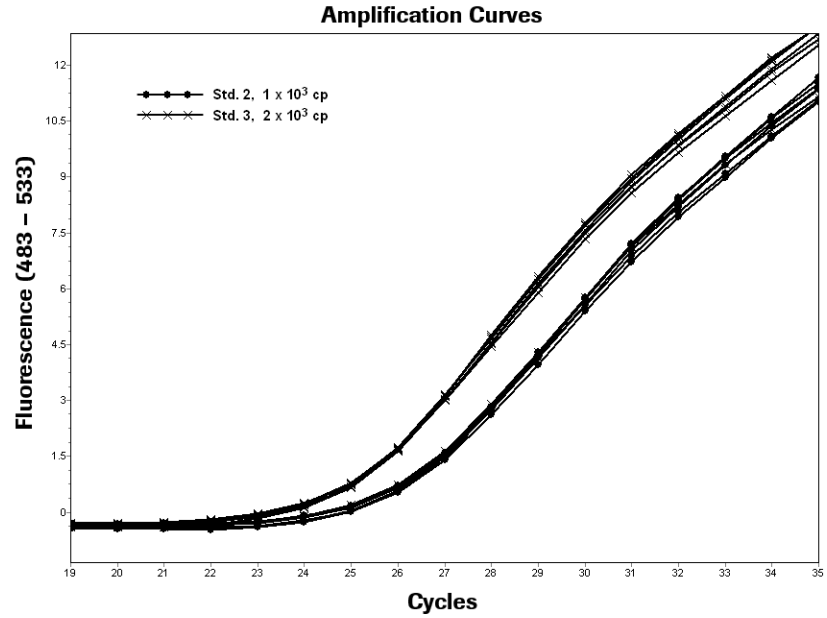

Fig. 4: The amplification curves of the replicates with 1,000 or 2,000 copies of target DNA are clearly separated in the FAM channel.

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.

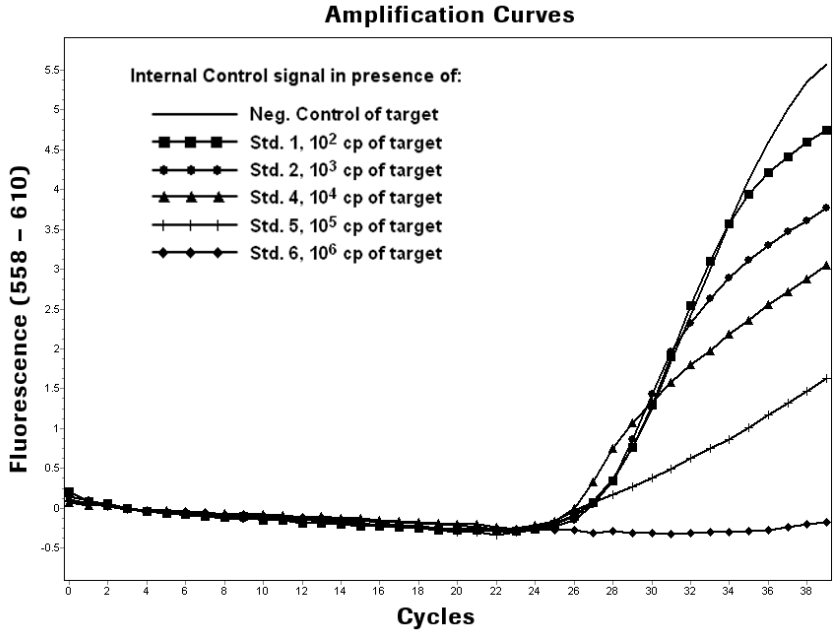

Fig. 5: Amplification curves of the Internal Control in the Red 610 channel.

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.

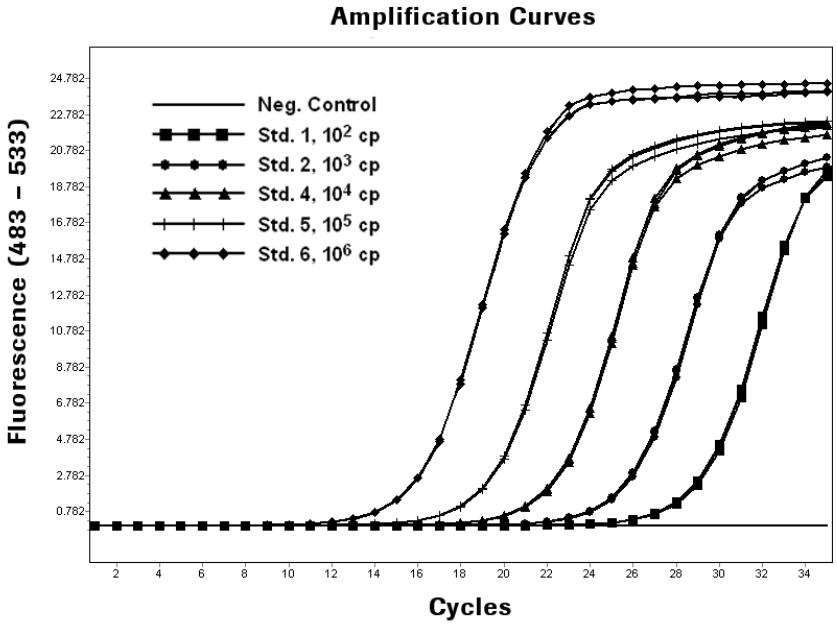

Fig. 6: Amplification curves of the standards detected with SYBR Green I.

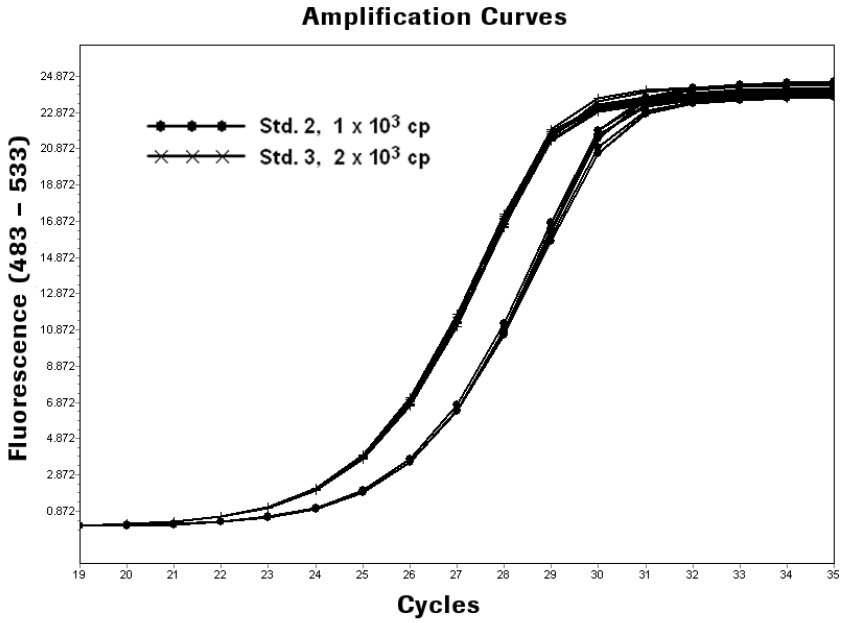

Fig. 7: The Amplification curves for the replicates with 1,000 or 2,000 copies of target DNA are clearly separated in the SYBR Green I channel.

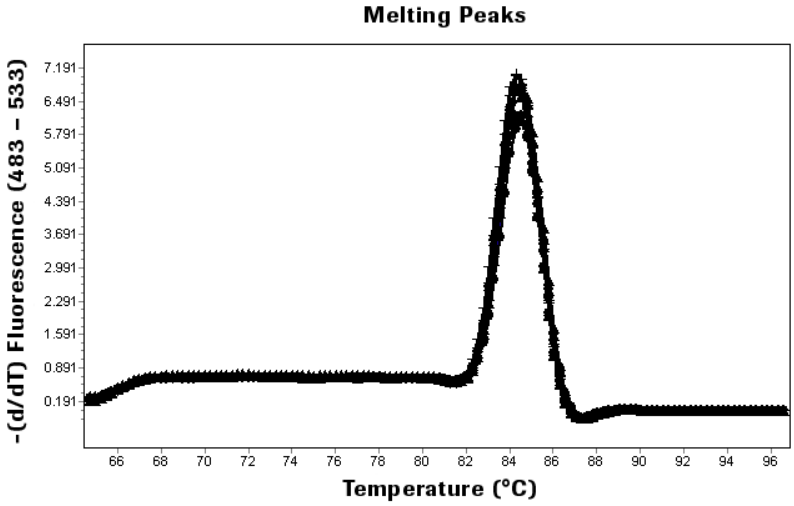

Fig. 8: Melting curve analysis results in a single peak for each positive sample, proving amplification of only the specific target sequence and no byproducts.

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 **Tm 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.

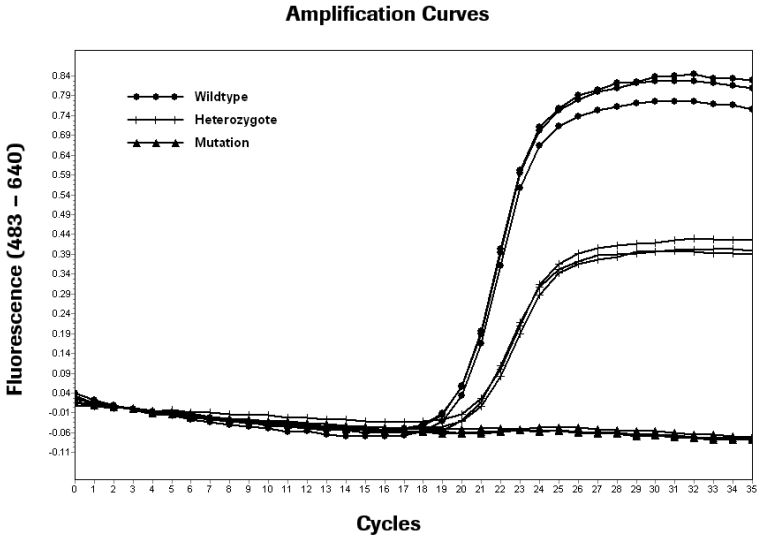

Fig. 9: Amplification curves of wild type and mutant target DNAs.
⚠ ***Using the LightCycler® 480 Genotyping Master, crossing points may occur about 5 cycles later. Data in this figure are produced with the LightCycler® 480 Probes Master.***

Part 2: Melting Curve Analysis

Melting curve analysis obtained when Procedure B was analyzed with the **Tm 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.

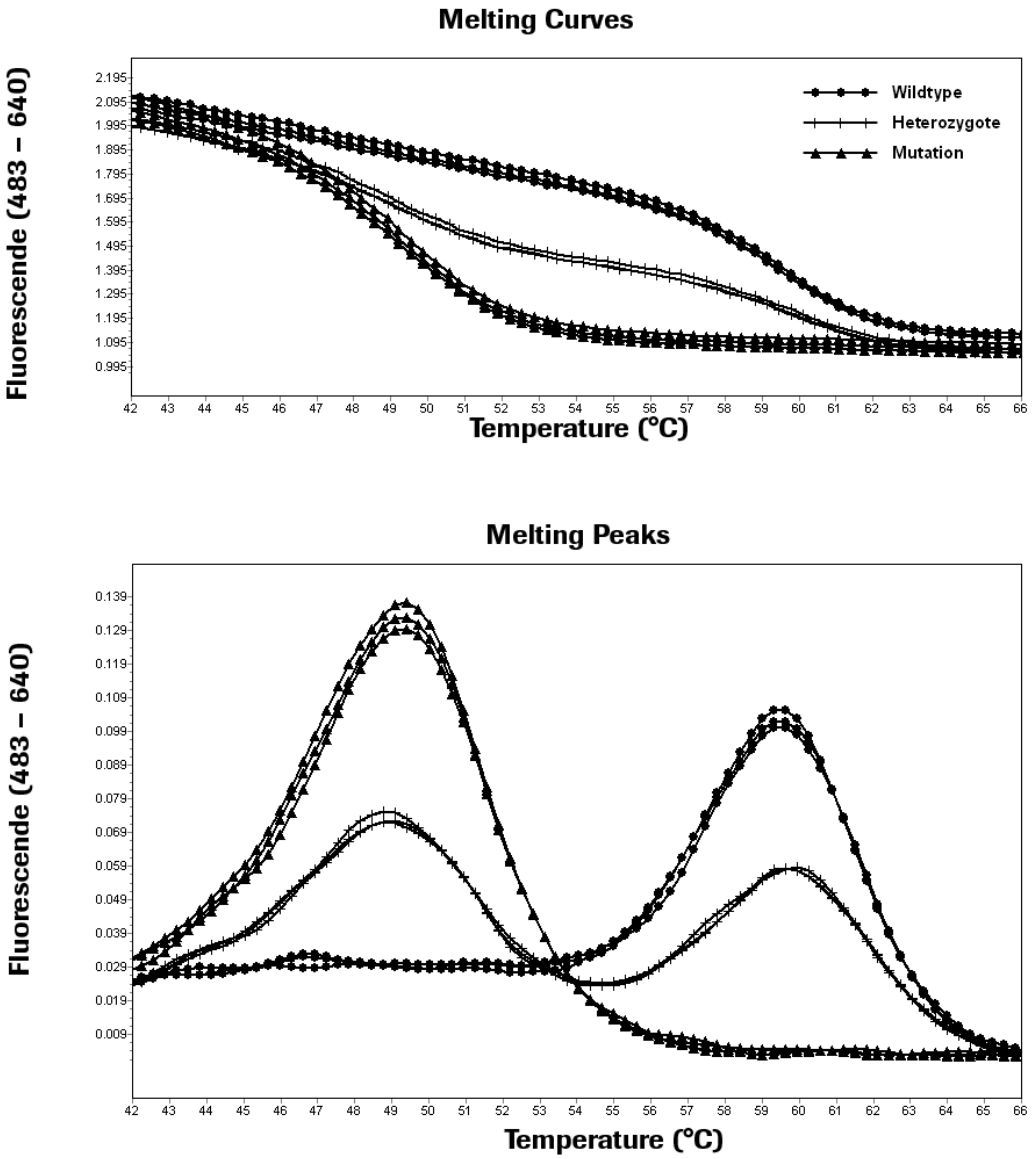

Fig. 10: Melting Curve analysis of the PCR product with and without the mutation.

4. Troubleshooting

Observation	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 reagets, 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-Glycosylase* (UNG) to eliminate carryover contamination from PCR products.

Refer to the Instructions for Use of your LightCycler® 480 System kits for further troubleshooting suggestions.

5. Additional Information on this Product

5.1. Test Principle

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 (approx. 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.

5.2. 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

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Layout changes.
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 homepage lifescience.roche.com.

Product	Pack Size	Cat. No.
Accessories general (hardware)		
LightCycler® 480 Block Kit 96 Silver	1 block kit	05 015 219 001
LightCycler® 480 Block Kit 384 Silver	1 block kit	05 015 197 001
Accessories software		
LightCycler® 480 LIMS Interface Module	1 software package	05 066 310 001
LightCycler® 480 Software, Version 1.5	1 software package	04 994 884 001
Consumables		
LightCycler® 480 Multiwell Plate 96, white	5 x 10 plates	04 729 692 001
LightCycler® 480 Multiwell Plate 384, white	5 x 10 plates	04 729 749 001
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
Instruments		
LightCycler® 480 Instrument II	1 instrument	05 015 278 001
	1 instrument	05 015 243 001
Reagents , kits		
LightCycler® 480 Probes Master	5 x 1 ml, 2x conc., 5 x 100 reactions of 20 µl final volume each	04 707 494 001
	10 x 5 ml, 2x conc., 10 x 500 reactions of 20 µl final volume each	04 887 301 001
	1 x 50 ml, 2x conc., 5,000 reactions of 20 µl final volume each	04 902 343 001
LightCycler® 480 SYBR Green I Master	5 x 1 ml, 2x conc., 5 x 100 reactions of 20 µl final volume each	04 707 516 001
	10 x 5 ml, 2x conc., 10 x 500 reactions of 20 µl final volume each	04 887 352 001
LightCycler® 480 Genotyping Master	4 x 384 µl, 5x conc., 384 reactions of 20 µl final volume each	04 707 524 001
Uracil-DNA Glycosylase, heat-labile	100 U	11 775 367 001
	500 U	11 775 375 001

6.4. Trademarks

FASTSTART, HYBPROBE and LIGHTCYCLER are trademarks of Roche.
SYBR is a trademark of Thermo Fisher Scientific Inc..
All third party product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For patent license limitations for individual products please refer to: http://technical-support.roche.com.

6.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

If you have questions or experience problems with this or any Roche product for Life Science, please contact our Technical Support staff. Our scientists are committed to providing rapid and effective help.
Please also contact us if you have suggestions for enhancing Roche product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to the research community worldwide.

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

Visit **lifescience.roche.com**, to download or request copies of the following **Materials**:
- Instructions for Use
- Safety Data Sheets
- Certificates of Analysis
- Information Material

To call, write, fax, or email us, visit **lifescience.roche.com** and select your home country to display country-specific contact information.

0916.04729510001 (12)

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany