LightMix® Kit *ApoB-100 R3500Q*

Cat.-No. 40-0519-16 **Version 05/2011: double amount of control DNA per vial**

Kit with reagents for the detection of the human *ApoB-100 R3500Q* polymorphism using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 Instruments.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do N OT freeze!**

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

1. Introduction

Apolipoprotein B-100 (ApoB-100) plays an important role in the catabolism of cholesterol. ApoB-100 is responsible for the solubility of cholesterol in blood and the removal from circulation. The most important polymorphism of ApoB-100 is *R3500Q* which decreases the affinity of ApoB-100 to LDL resulting in increased cholesterol levels in blood. Arteriosclerotic vascular damage is the consequence[1]. The melting curve based analysis of this genetic variation has been published by Aslanidis et al.[2].

The LightMix® Kit *ApoB-100 R3500Q* provides a fast, easy and accurate system to identify the genotype of this target in a nucleic acid extract.

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

[1] Major apolipoprotein B-100 mutations in lipoprotein metabolism and atherosclerosis. Vrablik M, Ceska R, Horinek A. *Physiol Res.* 2001;50(4):337-43

[2] High-speed apolipoprotein E genotyping and apolipoprotein B3500 mutation detection using real-time fluorescence PCR and melting curves. Aslanidis C, Schmitz G. *Clin Chem* 1999 Jul;45(7):1094-7

2. Description

A 352 bp fragment of the human *ApoB-100* gene is amplified with specific primers. The resulting PCR fragments are analyzed with hybridization probes labeled with LightCycler® Red 640 (detected in channel 640). The genotype is identified by running a melting curve with specific melting points (Tm). The wildtype *ApoB-100 R3500* exhibits a Tm of 53.5°C in channel 640. The mutant *ApoB-100 3500Q* exhibits a Tm of 61.0°C in channel 640.

The supplied control DNA allows for the accurate comparison with unknown samples.

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

3. Set contents

6 Vials with red caps containing premixed lyophilized primers and probes for 16 PCR reactions each of *ApoB-100 R3500Q*
3 Vials with colorless caps containing control DNA (*ApoB-100 R3500Q:* mt, wt, hetero), 10^5 target equivalents per reaction

4. Additional reagents and items required

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
or LightCycler® 480 Genotyping Master (LightCycler® 480 Instrument only)	Cat.-No. 04 707 524 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments only)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument only)	Cat.-No. 04 729 749 001
LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument only)	Cat.-No. 04 729 692 001

5. Product characteristics

PCR results are obtained within 45 minutes (45 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 75 minutes (45 cycles and melting curve) with the LightCycler® 480 Instrument.

Sensitivity
These reagents detect 1 ng of *human* genomic DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler 1.x / 2.0 Instruments and if using the Roche 'LightCycler® 480 Genotyping Master' with the LightCycler 480 Instrument.

Measuring range
The measuring range of the assay is 1 ng to 100 ng of *human* genomic DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 Instruments and whenusing the Roche 'LightCycler® 480 Genotyping Master' with the LightCycler® 480 Instrument.

Storage and Stability
- Lyophilized reagents are stable for at least 3 months after shipment when stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days when stored protected from light and refrigerated (4°C).

6. Experimental protocol

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

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure PCR Template Preparation Kit').

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

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

6.1. Preparation of parameter-specific reagents (16 reactions):

One reagent vial with a **red** cap contains all primers and probes to run 16 LightCycler® reactions for *ApoB-100 R3500Q*.

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

►**Use 4 µl** reagent for a 20 µl PCR reaction.

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

6.2. Preparation of the control DNA

Add 80 µl PCR-grade water to each vial (1.6 x 10^6 target molecules). Mix the target DNA by pipetting the solution up and down 10 times (final concentration: 10^5 target molecules in 5 µl).

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

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

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

6.3. Preparation of the LightCycler® reaction mix

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

For use with the Roche FastStart Master		**For use with the Roche Genotyping Master**
Single reaction	**Component**	**Single reaction**
7.8 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)	7.0 µl
1.2 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	--
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)	4.0 µl
2.0 µl	Roche Master (red cap, for preparation see Roche manual)	4.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

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

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

Start run.

7. LightCycler® 1.x / 2.0 Instrument

7.1. Programming

The protocol consists of four program steps

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

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

7.2. Data Analysis

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640 for detection.

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

View *ApoB-100 R3500Q* data in channel 640 "Tm Calling" Analysis mode (LightCycler® 2.0 Instrument) or Melting Curves mode (LightCycler® 1.x Instrument). The negative control (NTC) must show no signal.

7.3. Sample Data – typical results

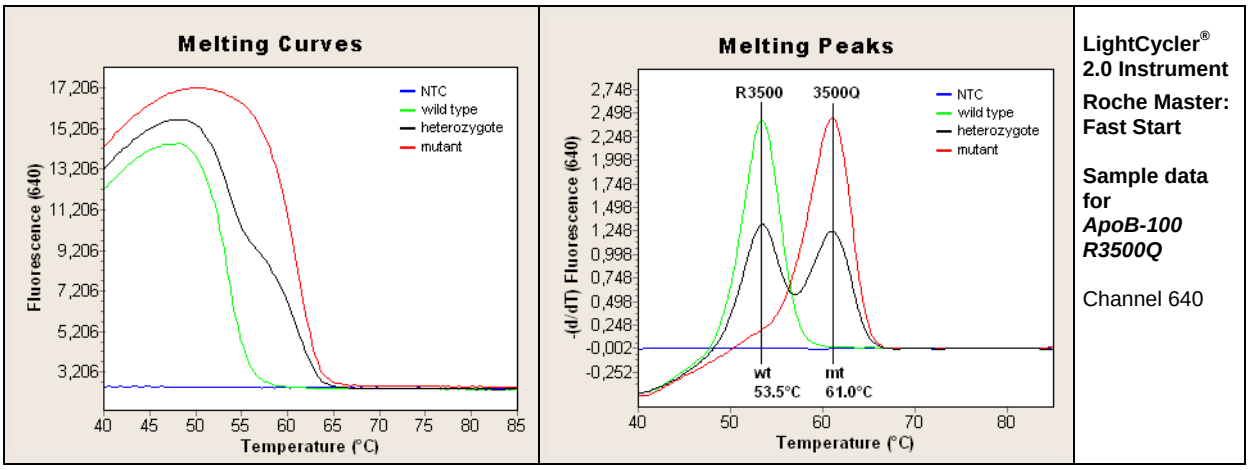

Fig.1. Sample data for the *ApoB-100 R3500Q* detection system.

Data from LightCycler® 2.0 Instrument. Left panel channel 640 melting curves for *ApoB-100 R3500Q*. Right panel channel 640 melting peaks for *ApoB-100 R3500Q*. Wildtype (wt) corresponds with *ApoB-100 3500R/R*, heterozygote corresponds with *ApoB-100 3500R/Q* and mutant (mt) corresponds with *ApoB-100 3500Q/Q*.

7.4. Interpretation of data

Genotype:	wild type homozygote *ApoB-100 3500R/R*	heterozygote *ApoB-100 3500R/Q*	mutant homozygote *ApoB-100 3500RQ/Q*
Number of melting peaks (color)	1 (green)	2 (black)	1 (red)
Melting temperature of peaks	53.5℃	53.5℃ and 61.0 ℃	61.0℃
Temperature difference between peaks	---	7.5℃	---
Phenotype	Normal LDL affinity	Decreased LDL affinity	Strongly decreased LDL affinity

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

8. LightCycler® 480 Instrument

8.1. Programming

The protocol consists of four program steps

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

Detection Format:
LightCycler® 480 Instrument: 483-640
LightCycler® 480 II Instrument: 498-640

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	**3**	-

8.2. Data Analysis

Note: For use on LightCycler® 480 II Instruments select Filter Combination 498-640 instead of Filter Combination 483-640, for detection.

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

View *ApoB-100 R3500Q* data with Filter Combination 483-640 "Tm Calling" Analysis mode. The negative control (NTC) must show no signal.

8.3. Sample Data – typical results

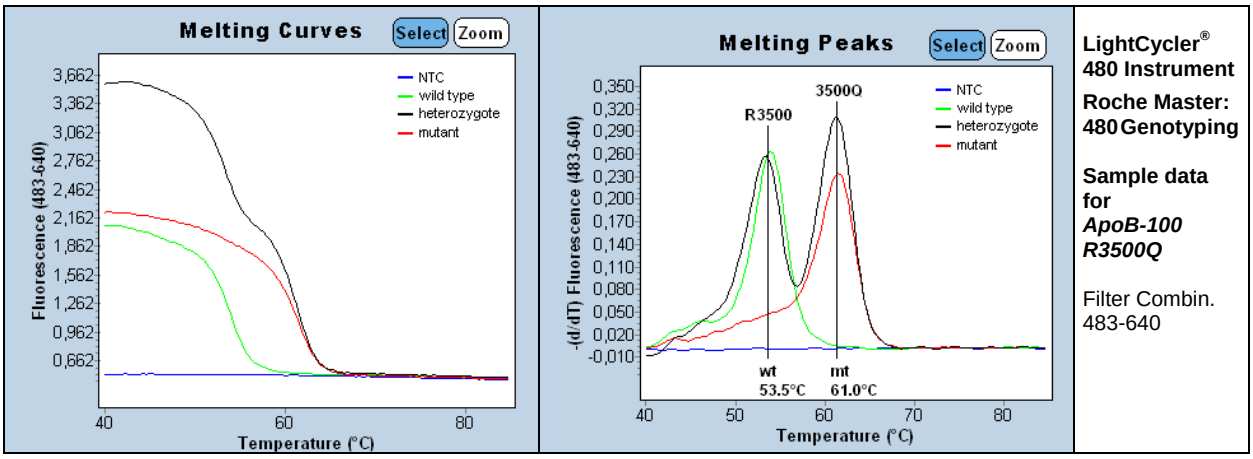

Fig.1. Sample data for the *ApoB-100 R3500Q* detection system.

Data from LightCycler® 480 Instrument. Left panel Filter Combination 483-640 melting curves for *ApoB-100 R3500Q*. Right panel Filter Combination 483-640 melting peaks for *ApoB-100 R3500Q.* Wildtype (wt) corresponds with *ApoB-100 3500R/R*, heterozygote corresponds with *ApoB-100 3500R/Q* and mutant (mt) corresponds with *ApoB-100 3500Q/Q.*

8.4. Interpretation of data

Genotype:	wild type homozygote *ApoB-100 3500R/R*	heterozygote *ApoB-100 3500R/Q*	mutant homozygote *ApoB-100 3500RQ/Q*
Number of melting peaks (color)	1 (green)	2 (black)	1 (red)
Melting temperature of peaks	53.5℃	53.5℃ and 61.0 ℃	61.0℃
Temperature difference between peaks	---	7.5℃	---
Phenotype	Normal LDL affinity	Decreased LDL affinity	Strongly decreased LDL affinity

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

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