LightMix® Kit *ApoE C112R R158C*

Cat.-No. 40-0445-32

Kit with reagents for the detection of the *human ApoE C112R R158C* polymorphism using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 and Cobas® Z480 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℃), do NOT freeze!

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 II / Cobas® Z480 Instrument see pages 6-7

1. Introduction

Apolipoprotein E (*ApoE*) plays an important role in the catabolism of lipoproteins and cholesterol. Three major alleles of *ApoE* are known: ***ApoE3*** (112C 158R, normal function), ***ApoE2*** (112C 158C, Type III Hyperlipidemia), ***ApoE4*** (112R 158R, increased cholesterol levels[1]). Homozygote *ApoE4* individuals (112R/R 158R/R) have an elevated risk for development of the Alzheimer disease[2].

In Western Europe the E3 allele is dominant, while E4 and E2 have a frequency of only 17% and 11%. The dominant haplotypes is E3/E3 (60%), followed by E3/E4 (23%) and E2/E3 (12%) whereas E2/E4, E4/E4 and E2/E2 are rare (1-2%).

The LightMix® Kit *ApoE C112R R158C* provides a fast, easy and accurate system to identify the genotype of these targets in a nucleic acid extract.

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

[1] Apolipoprotein E polymorphism and atherosclerosis. Davignon J, Gregg RE, Sing CF. *Arteriosclerosis* **8**:1-21(1988).

[2] Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer´s disease in late onset families. Corder EH, Saunders AM, Strittmatter WJ et al. *Science* **261**:828-9 (1993).

2. Description

A 228 bp fragment of the human *ApoE* gene is amplified with specific primers. The resulting PCR fragment is analyzed with a SimpleProbe® probe 519 (ApoE C112R, detected in channel 530) and with hybridization probes labeled with LightCycler® Red 640 (*ApoE R158C*, detected in channel 640).

The *ApoE* codon 112 exhibits in channel 530 a Tm of **55.0℃** for allele variant *112C* (wild type) and a Tm of **64.0℃** for allele variant *112R* (LightCycler® 2.0 Instruments).

The *ApoE* codon 158 exhibits in channel 640 a Tm of 63.0℃ for allele variant *158R* and a Tm of 53.0℃ for allele variant *158C* (LightCycler® 2.0 Instruments).

The use of a color compensation file generated with the ColorCompensation kit 40-0318 is a prerequisite to run the duplex reaction.

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

3 Vials with red caps containing lyophilized primers and probes for each 32 PCR reactions *ApoE*

1 Vial with colorless cap with control DNA (*ApoE 112C/C 158C/C*), 10^5 target equivalents per rxn
1 Vial with colorless cap with control DNA (*ApoE 112R/R 158R/R*), 10^5 target equivalents per rxn
1 Vial with colorless cap with control DNA (*ApoE 112C/R 158R/C*), 10^5 target equivalents per rxn

1 Vial with white cap containing DMSO.
Note: The vial contains some brown spheres of molecular sieveto keep the DMSO free of water.

4. Additional reagents and items required

TIB MOLBIOL:

ColorCompensation HybProbe 40-0318-00	Cat.-No. 05 997 704 001

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
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 Systems only)	Cat.-No. 04 729 749 001
LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Systems 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 / 480 Instruments.

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 / 480 Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment if stored protected from light at room temperature (18-25°C). See expiry date o n the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 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 Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. '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 (32 reactions):

One reagent vial with a **red** cap contains all primers and probes to run 32 reactions *ApoE.*

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

►**Use 2 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 t o light.

6.2. Preparation of the control DNA

Add **80 µl** PCR-grade water to each vial (16 x 10^5 target molecules) with a colorless cap. Mix 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 free ze 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	
Single reaction	**Component**
8.2 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
2.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
1.2 µl	**DMSO** (vial contains molecular sieve - pipett the colorless liquid only)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	**Volume of reaction mix**

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 Instruments

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 [℃]	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 [℃/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 use channel F2 instead of channel 640 and channel F1 instead of channel 530 for detection.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the TIB MOLBIOL Color Compensation HybProbe.

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 *ApoE C112R* data in channel 530 and *ApoE R158C* 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

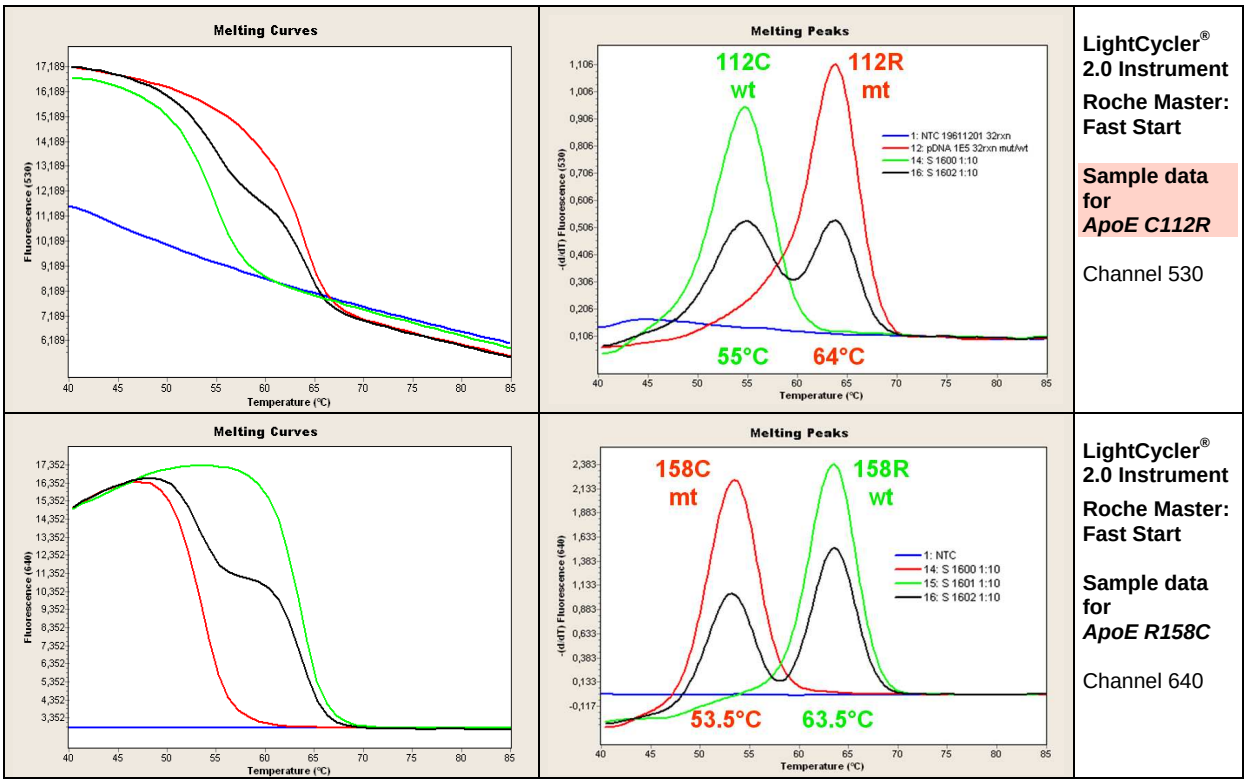

Fig.1. Sample data for the *ApoE C112R R158C* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 530 melting curves for *ApoE C112R*. Right panel channel 530 melting peaks for *ApoE C112R.* Wildtype (wt) corresponds with *ApoE 112C/C*, heterozygote corresponds with *ApoE 112C/R* and mutant (mt) corresponds with *ApoE 112R/R.*

Lower panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 melting curves for *ApoE R158C*. Right panel channel 640 melting peaks for *ApoE R158C.* Wildtype (wt) corresponds with *ApoE 158R/R*, heterozygote corresponds with *ApoE 158R/C* and mutant (mt) corresponds with *ApoE 158C/C.*

7.4. Interpretation of data

Allele (amino acid)	112 C/C 158 R/R	112 C/C 158 C/C	112 C/C 158 C/R	112 C/R 158 C/R	112 C/R 158 R/R	112 R/R 158 R/R
ApoE Type	*E3 / E3*	*E2 / E2*	*E2 / E3*	*E2 / E4*	*E3 / E4*	*E4 / E4*
Tm of peaks 530	55°C	55°C	55°C	55°C / 64°C	55°C / 64°C	64°C
Tm of peaks 640	63°C	53°C	53°C / 63°C	53°C / 63°C	63°C	63°C
Phenotype / Risk	normal wild type	Type III Hyperlipidemia				High risk for Alzheimer Disease
				E4 : Increased Cholesterol Levels		
Frequency	common 60%	rare	common 12%	rare	common 23%	rare

Tab. 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

***Notes**:* The values of the respective melting temperatures (T_M) may vary ±2.5°C between different experiments. Th e ΔT between the melting peaks for heterozygote genotypes may vary ±1.5°C. 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 II / Cobas® Z 480 Instruments

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: 483-533, 483-640
LightCycler® 480 II Instrument: 465-510, 498-640
Cobas® Z480 Instrument: 465-510, 498-645

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]	-	-	-	-	-	-	**1**	-

8.2. Data Analysis

Note: Cobas® Z480 Instruments signal levels are about 50% compared to LightCycler® 480 II results.

Note: Select Filter Combination as listed in the Detection Format table above.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the TIB MOLBIOL Color Compensation HybProbe.

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 *ApoE C112R* data with Filter Combination 483-533 (465-510) and *ApoE R158C* data with Filter Combination 483-640 (498-640/645), "Tm Calling" Analysis mode.

The negative control (NTC) must show no signal.

8.3. Sample Data – Typical Results

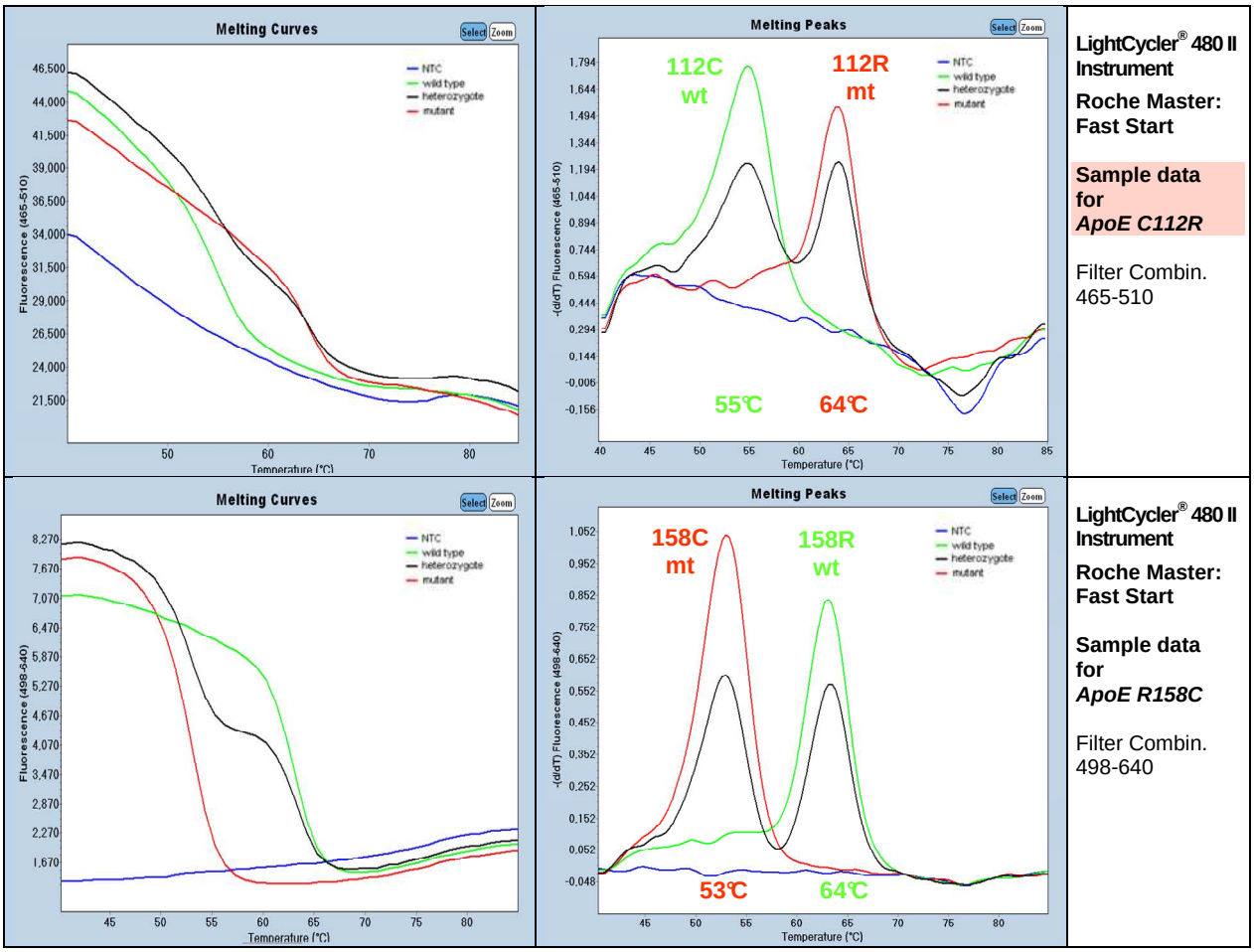

Fig.2. Sample data for the *ApoE C112R R158C* detection system.

Upper panels: Data from LightCycler® 480 Instrument. Left panel Filter Combination 465-510 melting curves for *ApoE C112R*. Right panel Filter Combination 465-510 melting peaks for *ApoE C112R*. Wildtype (wt) corresponds with *ApoE 112C/C*, heterozygote corresponds with *ApoE 112C/R* and mutant (mt) corresponds with *ApoE 112R/R*.

Lower panels: Data from LightCycler® 480 Instrument. Left panel Filter Combination 498-640 melting curves for *ApoE R158C*. Right panel Filter Combination 498-640 melting peaks for *ApoE R158C*. Wildtype (wt) corresponds with *ApoE 158R/R*, heterozygote corresponds with *ApoE 158R/C* and mutant (mt) corresponds with *ApoE 158C/C*.

8.4. Interpretation of Data

Allele (amino acid)	112 C/C 158 R/R	112 C/C 158 C/C	112 C/C 158 C/R	112 C/R 158 C/R	112 C/R 158 R/R	112 R/R 158 R/R
ApoE Type	*E3 / E3*	*E2 / E2*	*E2 / E3*	*E2 / E4*	*E3 / E4*	*E4 / E4*
Tm of peaks 530	55℃	55℃	55℃	55℃ / 64℃	55℃ / 64℃	64℃
Tm of peaks 640	64℃	53℃	53℃ / 64℃	53℃ / 64℃	64℃	64℃
Phenotype / Risk	normal wild type	Type III Hyperlipidemia				High risk for Alzheimer Disease
				E4 : Increased Cholesterol Levels		
Frequency	common 60%	rare	common 12%	rare	common 23%	rare

Tab. 3. Typical analysis results (LightCycler® 480 II Instrument, Roche Master: Fast Start)

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).

9. Version History

Modifications requiring changes in procedures are printed red.

V110125	Double amount of control DNA
V110511	32rxn per vial
V111027	Adjustment of melting temperatures for LC 480
V120702	Users reported low or missing signals in the 530 channel. Change of SimpleProbe sensor probe in order to increase the signals in channel 530 results in higher melting points.
V120704	Corrected Tm in interpretation table.
V130628	MSDS included, editorial changes
V141014	Editorial changes

10. Material Safety Data

According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

11. Additional Information

A diagnostic use CE-IVD marked kit version will be available from July 2013 (Cat.-No. 40-0445-64)

Roche SAP order n° 05997712001

Notice to Purchaser

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These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
SimpleProbe® probes and LightCycler® hybridization probes produced under license from Roche Diagnostics GmbH.