LightMix® Kit *MTHFR A1298C*

Cat.-No. 40-0269-32

Kit with reagents for the detection of the *human MTHFR A1298C* 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 II / Cobas® Z480 Instrument see pages 6-7

Note: CE-IVD marked LightMix® Kit, order no. 40-0269-64 available - please consider to change.

1. Introduction

Methylenetetrahydrofolate reductase (*MTHFR*) catalyzes the conversion of 5,10-methylen-etetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for the remethylation of homocysteine to methionine. The absence of 5-methyltetrahydrofolate induces an increase of homocysteine in plasma. Several polymorphisms of *MTHFR* are known. The A1298C transversion in the MTHFR gene, leading to an amino acid exchange from glutamate to alanine, in combination with the transition mutation C677T of the same gene has a significant effect on homocysteine levels.

The LightMix® Kit *MTHFR A1298C* 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 /480 Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

2. Description

A 163 bp fragment of the human *MTHFR* 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 *MTHFR A1298* DNA exhibits a Tm of 64°C to 65.0°C (dependent of L ightCycler® Instrument and melting program) in channel 640. The mutant *MTHFR 1298C* exhibits a Tm of 58.5°C to 59.5°C (dependent of LightCycler ® Instrument and melting program) 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

3 Vials with **red** cap containing lyophilized primers and probes for 32 PCR rxns *MTHFR A1298C*

1 Vial with colorless cap containing wild type control DNA, 10^5 target equivalents per reaction
1 Vial with colorless cap containing variant control DNA (*1298C*), 10^5 target equivalents per rxn
1 Vial with colorless cap with mixed control DNA (hetero), 10^5 target equivalents per rxn

4. Additional Reagents and items required

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)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	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 when stored protected from light at room temperature (18-25°C). See expiry on 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 Prep. Kit').

Negative control: Always run at least one no-template control (NTC) – replace the 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 primers and probes to run 32 rxns *MTHFR A1298C*.

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 (1.6 x 10^6 target molecules) with a colorless cap. Mix the target DNA by pipetting the solution up and down 10 times (final concentration: 10^5 targets in 5 µl).

►**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**
9.8 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
1.2 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.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	**Volume of reaction mix**

Table 1

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 ultiwall 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

Table 2

"Modified" Melting Program avoiding shoulders in Melting Peak profile (see 7.3. Sample Data) :

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	72	95	40	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:30	00:00:20	00:00:01	00:00:30	00:00:01	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	0.2	20	0.5	20
Acquisition Mode	None	None	Single	None	None	None	None	None	Step	None

Table 3

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.

View *MTHFR A1298C* 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

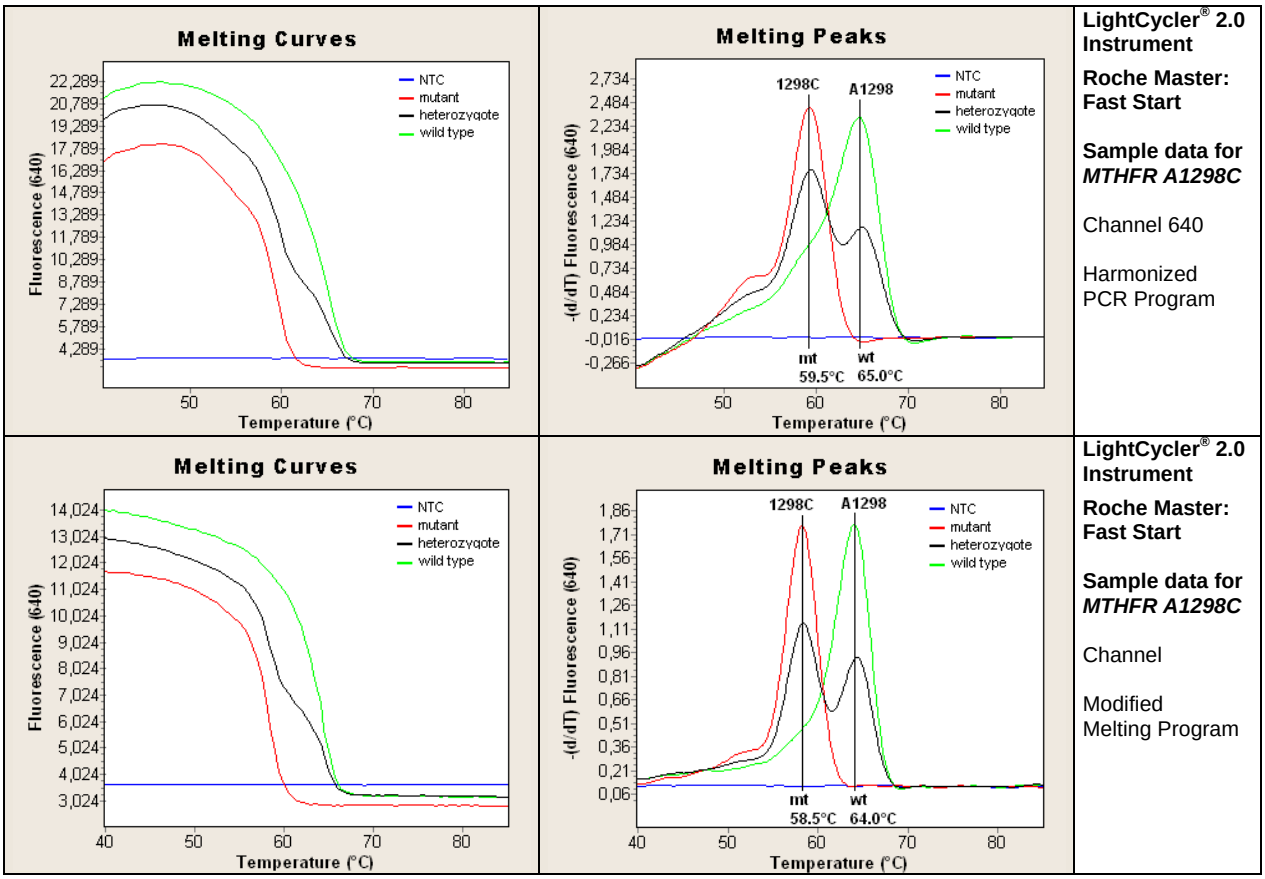

Fig.1. LightCycler® 2.0 sample data for the *MTHFR A1298C* detection system.

Upper panels: Data obtained with the Harmonized PCR Program. Left panel channel 640 melting curves for *MTHFR A1298C*. Right panel channel 640 melting peaks for *MTHFR A1298C*. Wildtype (wt) corresponds with *MTHFR 1298A/A*, heterozygote (hetero) corresponds with *MTHFR 1298A/C* and mutant (mt) corresponds with *MTHFR 1298C/C.*
Lower panels: Data obtained with the Modified Melting Program. Left panel channel 640 melting curves for *MTHFR A1298C*. Right panel channel 640 melting peaks for *MTHFR A1298C*. Wildtype (wt) corresponds with *MTHFR 1298A/A*, heterozygote (hetero) corresponds with *MTHFR 1298A/C* and mutant (mt) corresponds with *MTHFR 1298C/C.*

7.4. Interpretation of Data

Genotype:	mutant homozygote *MTHFR 1298C/C*	heterozygote *MTHFR 1298A/C*	wild type homozygote *MTHFR 1298A/A*
Number of melting peaks (color)	1 (red)	2 (black)	1 (green)
Melting temperature of peaks	59.5℃	59.5℃ and 65.0℃	65.0℃
Temperature difference between peaks	---	5.5℃	---
Phenotype	Hyperhomocysteinemia	depends on 677 status	Asymptomatic

Table 4. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start, Harmonized PCR Program)

***Notes**:* The values of the respective melting temperatures ™ may vary ±2.5℃ between different experiments. The 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).

*[!] No symptoms are observed only if the second allele is free of additional SNPs which are also associated with a reduced enzyme activity of MTHFR (e.g C677T).

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: 483-640
LightCycler® 480 II Instrument: 498-640
Cobas® Z480 Instrument: 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]	-	-	-	-	-	-	**3**	-

Table 5

"Modified" Melting Program (see 7.3. Sample Data)

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	55	45	40	75	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:60	00:00:30	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	2.2	1.5	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.4	2.0	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	None	Continu.	Continu.	None
Acquisitions [per °C]	-	-	-	-	-	-	-	**3**	**5**	-

Table 6

8.2. Data Analysis

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

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.

View *MTHFR A1298C* data with Filter Combination 483-640 "Tm Calling" Analysis mode.

The negative control (NTC) must show no signal.

8.3. Sample Data – Typical Results

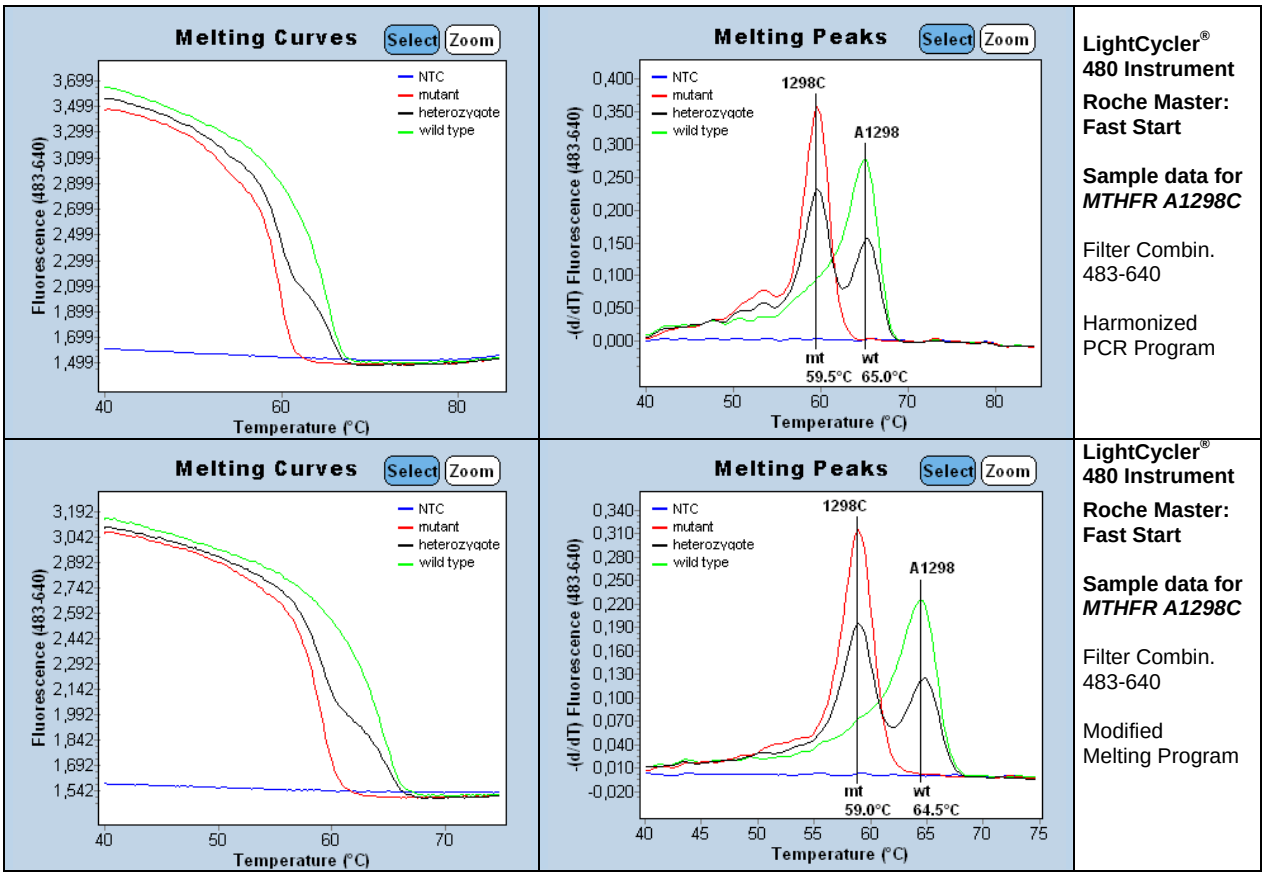

Fig.1. LightCycler® 480 sample data for the *MTHFR A1298C* detection system.

Upper panels: Data obtained with the Harmonized PCR Program. Left panel channel 640 melting curves for *MTHFR A1298C*. Right panel channel 640 melting peaks for *MTHFR A1298C*. Wildtype (wt) corresponds with *MTHFR 1298A/A*, heterozygote (hetero) corresponds with *MTHFR 1298A/C* and mutant (mt) corresponds with *MTHFR 1298C/C.*
Lower panels: Data obtained with the Modified Melting Program. Left panel channel 640 melting curves for *MTHFR A1298C*. Right panel channel 640 melting peaks for *MTHFR A1298C*. Wildtype (wt) corresponds with *MTHFR 1298A/A*, heterozygote (hetero) corresponds with *MTHFR 1298A/C* and mutant (mt) corresponds with *MTHFR 1298C/C.*

8.4. Interpretation of Data

Genotype:	mutant homozygote *MTHFR 1298C/C*	heterozygote *MTHFR 1298A/C*	wild type homozygote *MTHFR 1298A/A*
Number of melting peaks (color)	1 (red)	2 (black)	1 (green)
Melting temperature of peaks	59.5℃	59.5℃ and 65.0℃	65.0℃
Temperature difference between peaks	---	5.5℃	---
Phenotype	Hyperhomocysteinemia	depends on 677 status	Asymptomatic

Table 7. Typical analysis results (LightCycler® 480 Instrument, Roche Master: Fast Start, Harmonized PCR Program)

***Notes**:* The values of the respective melting temperatures ™ may vary ±2.5℃ between different experiments. The 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).

*[!] No symptoms are observed only if the second allele is free of additional SNPs which are also associated with a reduced enzyme activity of MTHFR (e.g C677T).

9. 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.

10. Version History

Notes in red mark events require to change procedures

V100819	32rxn per vial
V120710	Change in 6.2. Preparation of the control DNA: Add now 80 µl PCR-grade water to each vial Change in 6.3. Preparation of the LightCycler® reaction mix: use now 1.2µl $MgCl_2$;
V130410	Editorial changes
V130913	Remark regarding the CE-IVD version included. MSDS included. Editorial changes.

Roche SAP order n° 05945810001

Notice to Purchaser

A license under U.S. Patents 4,683,202, 4,683,195 and 4,965,188 or their foreign counterparts, owned by Hoffmann-La Roche Inc. and F. Hoffmann-La Roche Ltd ("Roche"), has an up-front fee component and a running-royalty component. The purchase price of this product includes limited, nontransferable rights under the running-royalty component to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents solely for the research and development activities of the purchaser when this product is used in conjunction with a thermal cycler whose use is covered by the up-front fee component. Rights to the up-front fee component must be obtained by the end user in order to have a complete license. These rights under the upfront fee component may be purchased from Perkin-Elmer or obtained by purchasing an authorized thermal cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process for research applications may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Center Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501. The purchase of this product does not convey any right for its use in clinical diagnostic applications. No rights for TaqMan technology under U.S. Patents 5,210,015 and 5,487,972 are hereby conveyed.

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

4260159330762