LightMix® Kit *Alkhurma Virus*

Cat.-No. 40-0581-16 Roche SAP order n°05 945127001

Real-Time-PCR Kit with reagents for the detection of *Alkhurma virus* RNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II / 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°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 Instruments see pages 6-7

1. Introduction

Alkhurma virus is a member of the Flaviviridae virus family (class IV), contains a positive sense single stranded RNA genome and replicates in the cytoplasm of the infected host cell. The virus genome is able to mimic mRNA in host cells. Alkhurma virus encodes a single polyprotein which is cleaved to form mature proteins[1].

This virus was first isolated in Saudi Arabia in the 1990s and since then there have been reported 24 cases, with the case fatality-rate above 30%.

Alkhurma virus causes a type of tick-borne hemorrhagic fever with the symptoms including fever, headache, joint pain, muscle pain, vomiting and thrombocytopenia which lead to hemorrhagic fever and encephalitis which can result in death.

Camels and sheep are believed to be the natural hosts of this virus. It seems that there is more than one possible route of transmission seen in people who have become infected with this virus. These are a bite by an infected tick, ingestion of unpasteurized camel milk or entry via a skin wound.

[1] "Isolation of a flavivirus related to the tick-borne encephalitis complex from human cases in Saudi Arabia". Zaki AM, *Trans. R. Soc. Trop. Med. Hyg.* **91** (2): 179–81 (1997).

The LightMix® Kit *Alkhurma Virus* provides a fast, easy and accurate system to identify this target in a nucleic acid extract. A control amplification reaction acts as internal control (IC).

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 II Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

2. Description

A 110 bp fragment of the *Alkhurma Virus polyprotein gene* amplified with specific primers. The resulting PCR fragment is analyzed with hybridization probes labeled with LightCycler® Red 640 (detected in channel 640).
The PCR reaction is monitored by an additional PCR product of 278 bp, formed from the internal control. This control does not interfere with the *Alkhurma Virus* specific reactions. The amplification will usually fail in the presence of higher concentrated *Alkhurma Virus* DNA samples (1,000 copies or higher) while displaying an amplification signal in negative and low-concentrated samples. The hybridization probes are labeled with LightCycler® Red 690 (recorded in channel 705). The reactions do not interfere with each other. The IC is supplied separately to allow running the assay in the presence or absence of the IC.

The use of a color compensation file generated with the 'LightMix® Kit - Color Compensation HybProbe' is a prerequisite to run the reaction.

The supplied standard row DNA allows to determine the linear range of the reaction and to estimate the quantity of the target sequence in 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 blue caps containing lyophilized primers and probes for each 16 PCR reactions
6 Vials with white caps containing the internal control (IC)
1 Standard row with 6 lyophilized plasmid standards *Alkhurma* 10^1 - 10^6 target equivalents / rxn
1 Sealing foil for the standard row

4. Additional reagents and items required

TIB MOLBIOL:
LightMix® Kit – Color Compensation HybProbe Cat.-No. 40-0318-00

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

Transcriptor First Strand cDNA Synthesis Kit Cat.-No. 04 379 012 001

High Pure Viral Nucleic Acid Kit Cat.-No. 11 858 874 001

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 50 minutes (50 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (50 cycles and melting curve) with the LightCycler® 480 II Instrument.

Sensitivity
These reagents detect 10 copies of *Alkhurma virus pDNA* using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Alkhurma virus* genomic pDNA using the 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II 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 date o n the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days when stored protected from light and refrigerated (4°C).
- Dissolved reagents can be long-term stored frozen at -20°C. Avoid multiple thaw-freeze cycles.

6. Experimental protocol

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

Sample material: Use aqueous nucleic acid (e.g. Roche Diagnostics 'High Pure RNA Isolation 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.
For quantification always use a fresh solution prepared from the provided standard row (single use only).

6.1. Preparation of parameter-specific reagents and reagents for the IC (16 reactions):

One reagent vial with a **blue** cap contains primers and probes to run 16 LightCycler® reactions for *Alkhurma virus.*
One reagent vial with a **white** cap contains primers, probes and DNA to run 16 reactions for the IC.

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 t o light.

6.2. Preparation of the standard row DNA

The target DNA is provided in 6 different quantities to yield from 10^1 to10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. **Add 40 µl** PCR-grade water to each vial of the row. Mix the target DNA by pipetting the solution up and down 10 times.

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

This standard solution is not long-term stable and will lose sensitivity under prolonged storage. Use only fresh prepared solutions as quantification references. Older solutions can be used as a qualitative standard (positive control). For the control DNA provided with the kit please note that the relative amounts of 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**
2.6 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
2.4 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	**IC** mix (IC reagents containing primers, probes and DNA, see **6.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

To include the internal control **add 4 µl** of the IC reagent per reaction to the reaction mix.
To run the assay without the internal control substitute the 4 µl of IC with 4 µl PCR-grade water.

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 II Instrument).

Add 5 µl of sample or standard 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	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	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
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

(Melting not relevant for detection)

7.2. Data Analysis

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 'LightMix® Kit – 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 *Alkhurma virus* data in channel 640 Quantification mode. The negative control (NTC) must show no signal.

If the internal control (IC) is used, view IC data in channel 705 Quantification mode. The negative control and the low-concentrated *Alkhurma virus* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a Cp at approximately cycle 28.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Alkhurma virus* should have Cp values between cycles 18 and 35.

7.3. Sample Data – typical results

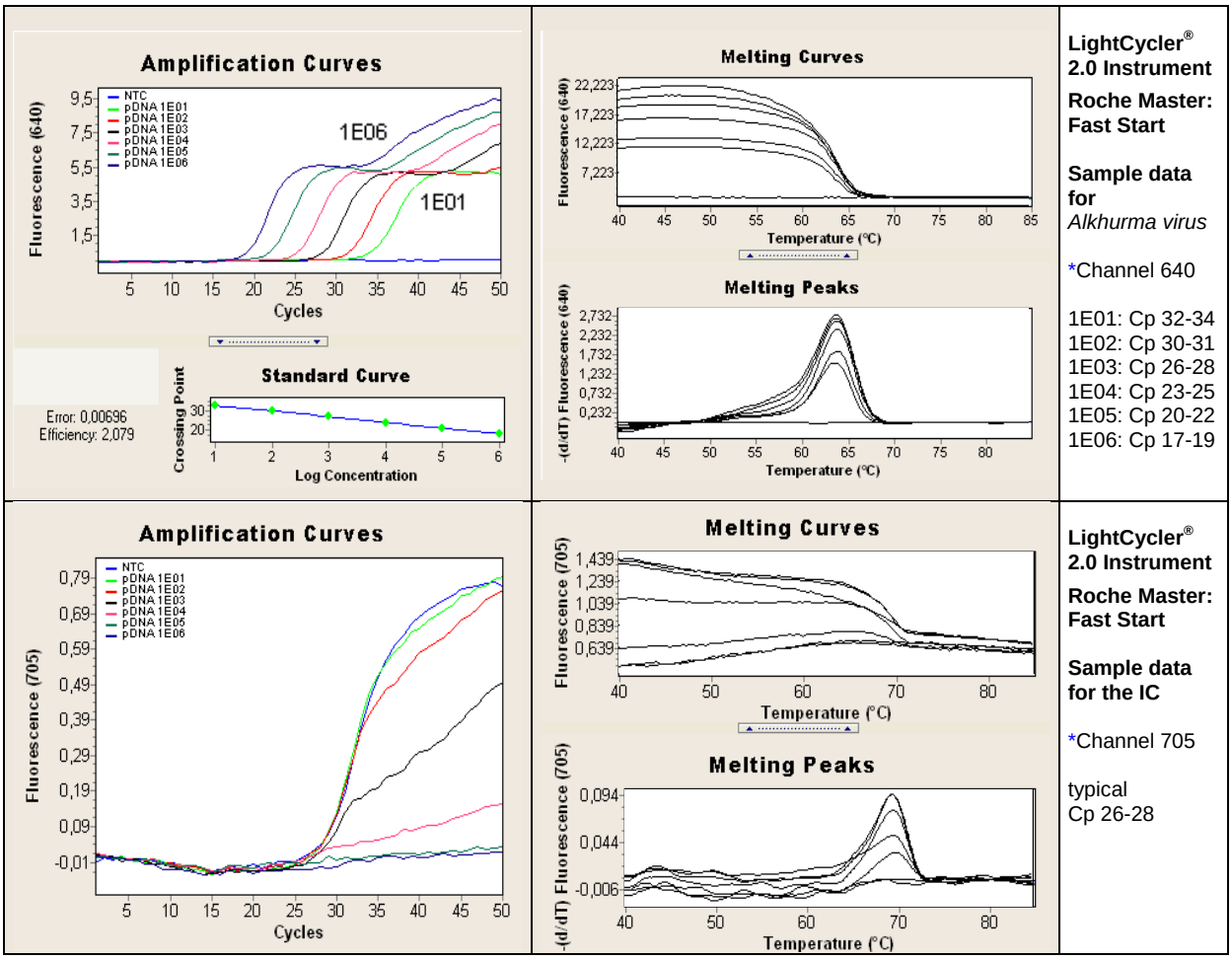

Fig.1. Sample data for the *Alkhurma virus* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *Alkhurma virus*. Right panel channel 640 melting analysis for *Alkhurma virus* (not relevant for detection).
Lower panels: Data from LightCycler® 2.0 Instrument. Left panel channel 705 quantification mode (Second Derivative Maximum) for the IC. Right panel channel 705 melting analysis for the IC (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

7.4. Interpretation of data

Alkhurma virus (sample)	Alkhurma virus (positive control)	IC (sample)	NTC	Result
no amplification	amplification signal	detectable	negative	Negative
amplification signal	amplification signal	not relevant	negative	Positive
no amplification	amplification signal	not detectable	not relevant	PCR failure, repeat experiment
not relevant	no amplification	not relevant	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat

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

8. LightCycler® 480 II / Cobas® Z480 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 II Instrument: 465-510, 498-640, 498-660
Cobas® Z480 Instrument: 465-510, 498-645, 498-700

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	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
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	3*	-

(Melting not relevant for detection)

8.2. Data Analysis

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

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of 'LightMix® Kit – 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 *Alkhurma virus* data with Filter Combination 498-640. The negative control (NTC) must show no signal.

If the internal control is used, view data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Alkhurma virus* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a Cp at approximately cycle 28.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Alkhurma virus* should have Cp values between cycles 18 and 35 (Cp values calculated with Second Derivative Maximum method).

8.3. Sample Data – typical results

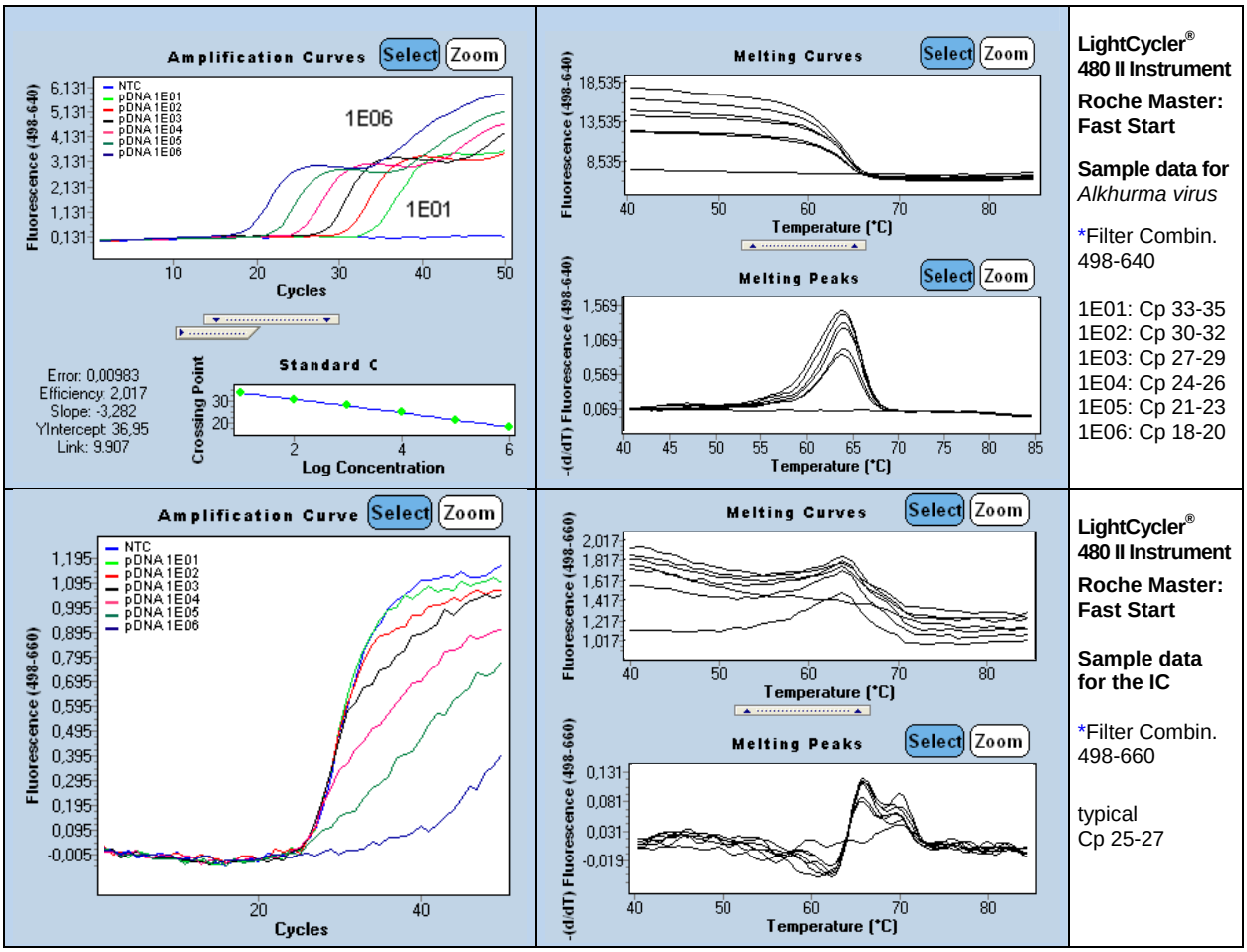

Fig.1. Sample data for the *Alkhurma virus* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *Alkhurma virus.* Right panel Filter Combination 498-640 melting analysis for *Alkhurma virus* (not relevant for detection).
Lower panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) for the IC. Right panel Filter Combination 498-660 melting analysis for the IC (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8.4. Interpretation of data

Alkhurma virus (sample)	Alkhurma virus (positive control)	IC (sample)	NTC	Result
no amplification	amplification signal	detectable	negative	Negative
amplification signal	amplification signal	not relevant	negative	Positive
no amplification	amplification signal	not detectable	not relevant	PCR failure, repeat experiment
not relevant	no amplification	not relevant	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat

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

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

V090630	Release version
V100824	Revised version
V130506	Cobas® Z480 Instruments included. MSDS included

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