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

TIB MOLBIOL

LightMix® Kit *Listeria monocytogenes*

Cat.-No. 40-0417-16

New Version: for the LightCycler® 1.x / 2.0 /480 II Instruments
Not suitable for the LightCycler® 480 Instrument (Version 1)

Kit with reagents for the detection of *Listeria monocytogenes* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II 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 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 Instrument see pages 6-7

1. Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen causing listeriosis, which may develop meningitis and sepsis. The disease affects primarily pregnant women, newborns, persons of advanced age and immunocompromised patients. The disease is common in domestic animals. The virulence is linked to the listeriolysin protein which is encoded by the hemolysin gene (hly)[1)].

The conventional detection method is based on culture (§ 35 LMBG DIN EN ISO-Method 11290-1). Preferred targets for the PCR based detection are the internalin (inlA)[2], listeriolysin (hlayA)[3] and the virulence gene regulatory factor A (prfA) [4] genes.

The LightMix® Kit *Listeria monocytogenes* 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® 2.0 / 480 II Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

[1] Cossart P: Listeriology (1926–2007): The rise of a model pathogen. In: *Microbes Infect*. May 6th (2007).

[2] A PCR protocol using inl gene as a target for specific detection of L. monocytogenes. Almeida. Food Ctrl 11 (2000) 97-101

[3] Application of 5'-nuclease PCR for quantitative detection of Listeria monocytogenes in pure cultures, water, skim milk, and unpasteurized whole milk. Nogva et al. Appl Environ Microbiol 66 (2000) 4266-71

[4] Quantification of L. mono. in fermented sausages by MPN-PCR method. Martin et al. L Appl Microbiol.39 (2004) 290-5

2. Description

A 146 bp fragment of the *hlyA* gene of the *Listeria monocytogenes* genome is 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 349 bp, formed from the internal control. This control does not interfere with the *Listeria monocytogenes* specific reactions. The amplification will usually fail in the presence of higher concentrated *Listeria monocytogenes* DNA samples (1,000 copies or higher) while displaying an amplification signal in negative and low-concentrated samples. Detection is recorded in channel 705.
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 CC_530/640/690 or Roche Diagnostics 'LightCycler®-Color Compensation Set' or 'LightCycler® Multicolor Demo Set' is a prerequisite to run the duplex reaction.

The supplied standard row 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 green caps containing premixed lyophilized primers and probes for 16 PCR reactions each of *Listeria monocytogenes*
6 Vials with white caps containing premixed reagents for 16 reactions of the internal control (IC)
1 Standard row with 6 lyophilized cloned plasmid standards of *Listeria monocytogenes* from 10^1 to 10^6 target equivalents per reaction
1 Sealing foil for the standard row

4. Additional reagents and items required

TIB MOLBIOL:	**Cat.-No.**
Color compensation Kit 530/640/690	40-0318-00
Roche Diagnostics:	
LightCycler® FastStart DNA Master HybProbe	03 003 248 001
LightCycler® Multicolor Demo Set	03 624 854 001
or LightCycler® Color Compensation Set (LightCycler® 1.x Instrument)	12 158 850 001
High Pure PCR Template Preparation Kit	11 796 828 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments only)	04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 II Instrument)	04 729 749 001
LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 II Instrument)	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 *Listeria monocytogenes* DNA 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 *Listeria monocytogenes* DNA using the Roche '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 3 months after shipment if stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if 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 II 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.
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 **green** cap contains all primers and probes to run 16 LightCycler® reactions for *Listeria monocytogenes.*
One reagent vial with a **white** cap contains all primers, probes and DNA to run 16 LightCycler® reactions for the IC.

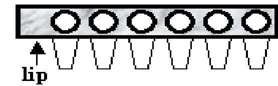
Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**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

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 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). After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation.
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**

Table 1

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 components with 4 µl PCR-grade water.

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary or plate.

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

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. Color Compensation

LightCycler® 1.2 and 1.5 instruments	Use the standard Color Compensation (Roche).
LightCycler® 2.0 instruments	Use the LightMix® 640/690 Color Compensation Blank (water) 515 640 705 Standard (Roche Diagnostics) color compensation works for the LightMix® 640/690 kits but the LC640 signals might be visible in the 705 channel (internal control).

7.2. 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) Table 2

7.3. Data Analysis

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640 and channel F3 instead of channel 705 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 TIB MOLBIOL Color compensation Kit 530/640/690 or the Roche Diagnostics 'LightCycler® – Color Compensation Kit' (LightCycler® 1.x Instrument) / 'LightCycler® Multicolor Demo Set' (LightCycler® 2.0 Instrument).

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 *Listeria monocytogenes* data in channel 640, Quantification mode. The negative control (NTC) must show no signal.

When using the internal control (IC), view IC data in channel 705 Quantification mode. The negative control and the low-concentrated *Listeria monocytogenes* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 31.

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 *Listeria monocytogenes* should have CPs between cycles 18 and 35 (CPs calculated with Second Derivative Maximum method).

7.4. Sample Data – typical results

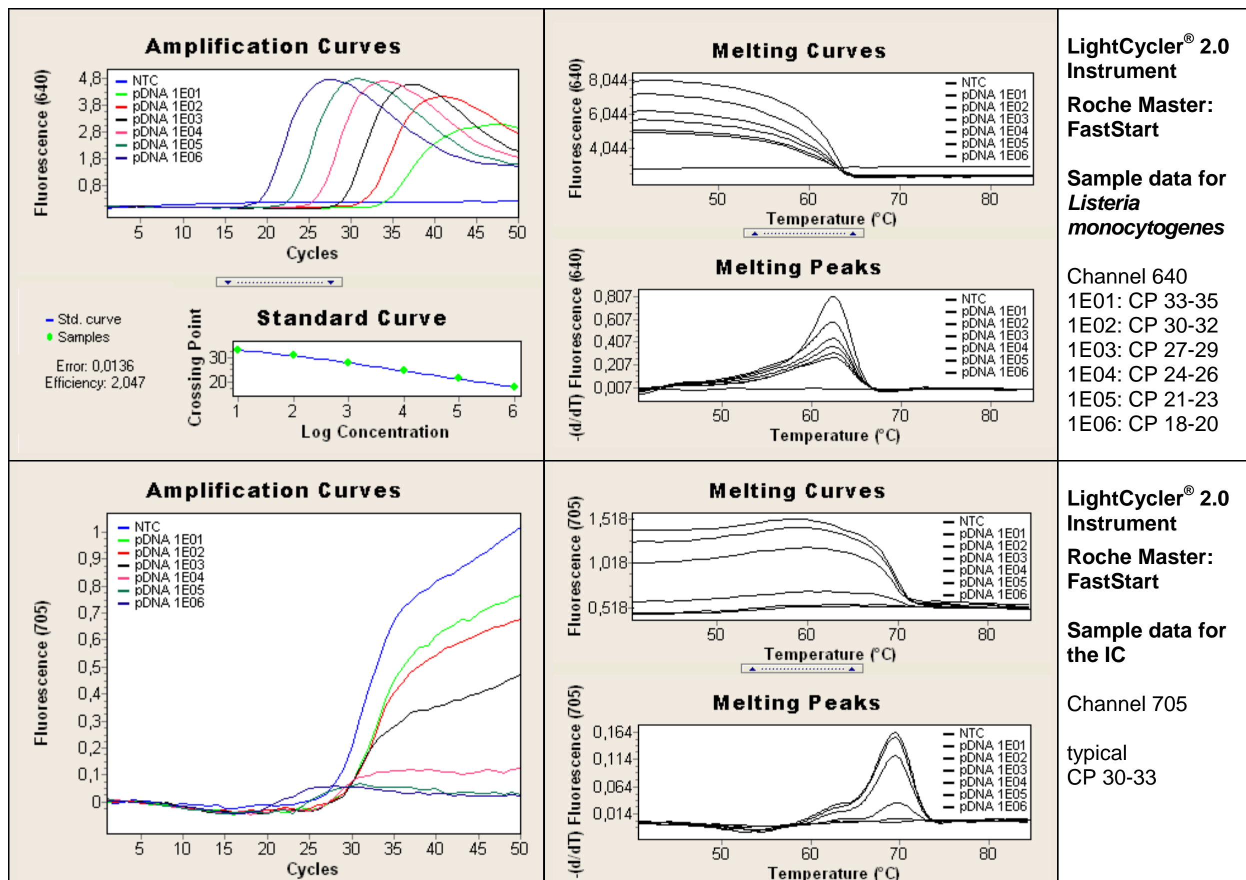


Fig.1. Sample data for the *Listeria monocytogenes* detection system (Roche Diagnostics Master: FastStart)

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with standard curve for *Listeria monocytogenes*. Right panel channel 640 melting analysis for *Listeria monocytogenes* (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). *Listeria* positive samples might yield a low level signal in the 705 channel (due to an inaccurateness of the Color Compensation)

7.5. Interpretation of data

Read channel 640 (F2) first. The positive control must give an amplification while the negative control must stay negative (yellow shaded). Analyze the unknown samples to identify *Listeria* positives and negatives (bold).

Read channel 705 (F3). Negative samples must show amplification for the internal control (green).

L. monocytogenes **(sample)**	**IC (sample)**	**Positive Control**	**Negative Control (NTC)**	**Result (warnings)**
640 (F2)	**705 (F3)**	640 (F2)	640 (F2)	
no amplification	detectable	amplification	negative	**Negative (not detectable)**
amplification signal	not relevant	amplification	negative	**Positive**
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Tab. 3. Typical analysis results (LightCycler® 1.x / 2.0 Instruments, Roche Diagnostics Master: FastStart)

8. LightCycler® 480 II Instrument

8.1. Color Compensation

LightCycler® 480 II instruments

Use the LightMix® 640/690 Color Compensation
Blank (water) 515 640 705

Standard (Roche Diagnostics) color compensation works for the LightMix® 640/690 kits but the LC640 signals might be visible in the 705 channel (internal control).

8.2. 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: 465-510, 498-640, 498-660

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
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]	-	-	-	-	-	-	1	-

(Melting not relevant for detection) Table 4

8.3. 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 Roche Diagnostics 'LightCycler® Multicolor Demo Set'.

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 *Listeria monocytogenes* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal.

When using the internal control (IC), view IC data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Listeria monocytogenes* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 29-31.

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 *Listeria monocytogenes* should have CPs between cycles 18 and 35 (CPs calculated with Second Derivative Maximum method).

8.4. Sample Data – typical results

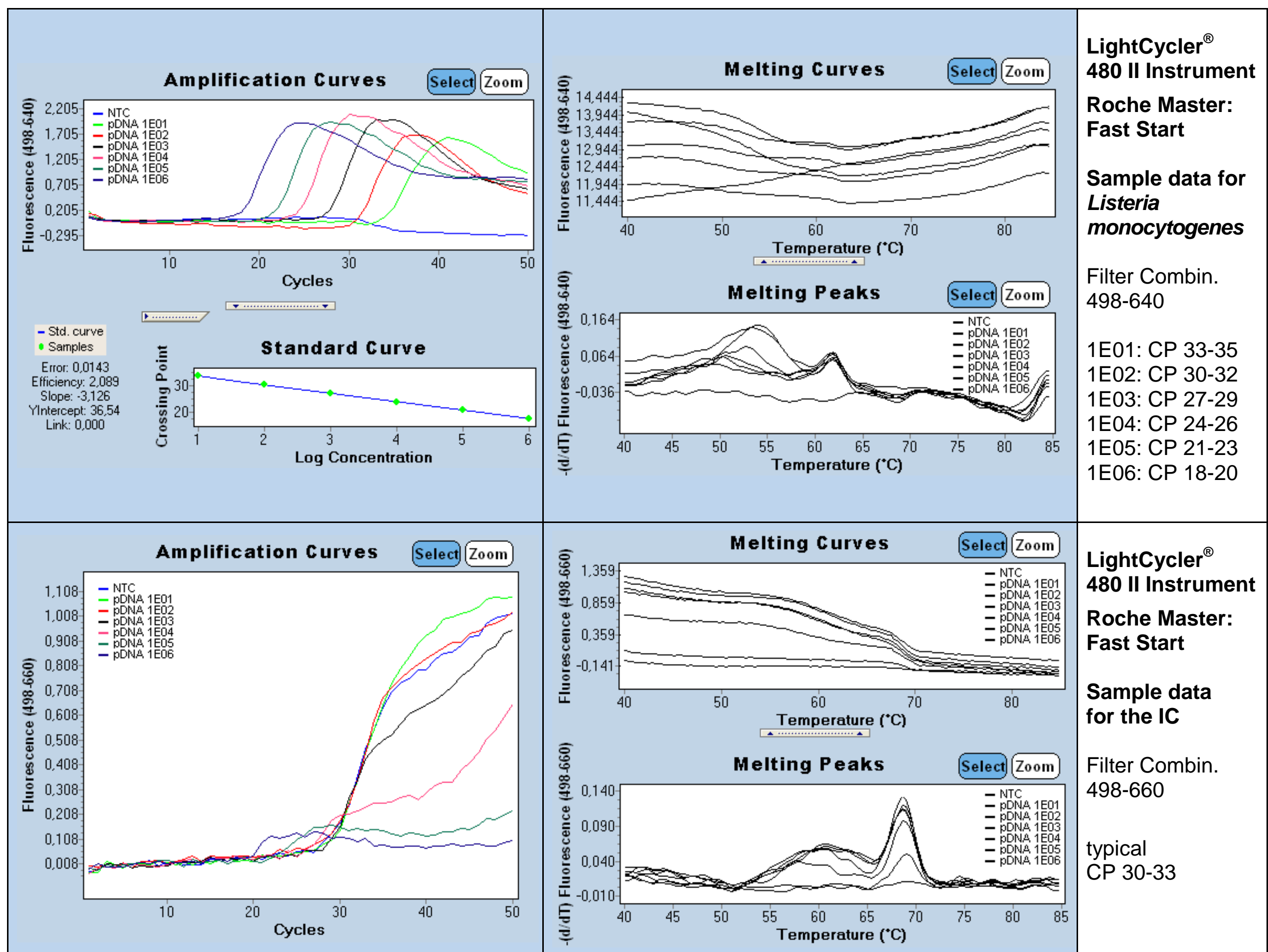


Fig.1. Sample data for the *Listeria monocytogenes* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with standard curve for *Listeria monocytogenes*. Right panel Filter Combination 498-640 melting analysis peaks for *Listeria monocytogenes* (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). *Listeria* positive samples might yield a low level signal in the 498-660 channel (inaccurateness of the Color Compensation). The LightCycler® 480 version 1 instrument will be not able to detect the internal control.

8.5. Interpretation of data

Read channel 498-640 first. The positive control must give an amplification while the negative control must stay negative (yellow shaded). Analyze the unknown samples to identify *Listeria* positives and negatives (bold).

Read channel 498-660. Negative samples must show amplification for the internal control (green).

L. monocytogenes **(sample)**	**IC (sample)**	**Positive Control**	**Negative Control (NTC)**	**Result(warnings)**
498-640	**498-660**	498-640	498-640	
no amplification	detectable	amplification	negative	**Negative (not detectable)**
amplification signal	not relevant	amplification	negative	**Positive**
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Tab. 5. Typical analysis results (LightCycler® 480 II Instrument, Roche Diagnostics Master: FastStart)

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These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
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