LightMix® Kit for the detection of *Bacillus anthracis*

Cat.-No. 40-0252-16 New Version: working on the LightCycler® 1.x / 2.0 /480 II Instruments

Kit with reagents for the detection of *Bacillus anthracis* 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

Bacillus anthracis, a Gram positive spore forming microorganism causes splenic fever or anthrax. Nearly all warm blooded organism are susceptible to an anthrax disease. *B. anthracis* has been used as a biological warfare agent.

The RNA polymerase *rpoB* gene is a chromosomal marker which can be used for identification of the species *B. anthracis* and for discrimination against *B. cereus* and *B. thuringiensis* [1,2].

Two plasmids (pX01 and pX02) are necessary for a full pathogenicity of *Bacillus anthracis*. pX01 contains the genes *pagA*, *lef* and *cya* encoding for the three toxin proteins, pX02 contains the genes *capA, capB and capC* which are necessary for capsule formation. The gene pagA is a preferred target for Real-Time-PCR detection of *B. anthracis* [2,3].

The LightMix® Kit *Bacillus anthracis* detects one chromosomal and one plasmid marker and 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'.

[1] Utilization of the rpoB Gene as a Specific Chromosomal Marker for Real-Time PCR Detection of Bacillus anthracis. Qi Y, Patra G, Liang X, Williams LE, Rose S, Redkar RJ, DelVecchio VG. Appl Environ Microbiol 67 (2001) 3720-3727

[2] Rapid and sensitive identication of pathogenic and apathogenic Bacillus anthracis by real-time PCR. Ellerbrock H. , Nattermann, H., Özel, M., Neutin, L., Appel, B., and Pauli, H. FEMS Microbiology Letters 214 (2002) 51-59

[3] High throughput screening for spores and vegetative forms of pathogenic B. anthracis by an internally controlled real-time PCR assay with automated DNA preparation. Panning, M., Kramme, S. Petersen N and Drosten, C. Medical Microbiology and Immunology, Volume 196, Number 1, March 2007 , pp. 41-50

2. Description

This LightMix® detects one genomic and one plasmid encoded target from *Bacillus anthracis*. A control amplification reaction acts as internal positive control (IC).

A 173 bp fragment (pagA) and a 174 bp fragment (rpoB) are amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (detected in channel 640). The PCR products are identified by running a melting curve with a specific melting point (Tm) of 58°C (rpoB) and a specific melting point of 65°C (pagA) in channel 64 0.

The PCR reaction is monitored by an additional PCR product of 281 bp, formed from the internal control. This control does not interfere with the *B. anthracis* specific reactions and will usually fail in the presence of higher concentrated *B. anthracis* 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 IC is supplied separately.

The use of a color compensation file generated with TIB MOLBIOL 'LightMix® Kit - Color Compensation 530/640/690' or the Roche Diagnostics 'LightCycler®-Color Compensation Set' or with the Roche Diagnostics '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 *Bacillus anthracis*
6 Vials with white caps containing the internal control (IC)
1 Standard row with 6 lyophilized cloned plasmid standards of *Bacillus anthracis* 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:

LightMix® Kit – Color Compensation 530/640/690	Cat.-No. 40-0318-00

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Multicolor Demo Set	Cat.-No. 03 624 854 001
or LightCycler® Color Compensation Set (LightCycler® 1.x Instrument)	Cat.-No. 12 158 850 001
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 Instruments)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instruments)	Cat.-No. 04 729 692 001

5. Product characteristics

PCR results are obtained within 50 minutes (55 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (55 cycles and melting curve) with the LightCycler® 480 II Instrument.

Sensitivity
These reagents detect 10 copies of *Bacillus anthracis* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments.

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Bacillus anthracis* 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 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 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 primers and probes to run 16 LightCycler® reactions for *Bacillus anthracis.*
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 to 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). 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 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	55			1			1
Target [℃]	95	95	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:08	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

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 'LightMix® Kit – Color Compensation 530/640/690' or the Roche Diagnostics 'LightCycler® – Color Compensation Kit' (LightCycler® 1.x Instrument) or '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 *Bacillus anthracis* data in channel 640 Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Bacillus anthracis* data in channel 640 Melting Curves mode.

If the internal control (IC) is used view IC data in channel 705 Quantification mode. The negative control and the low-concentrated *Bacillus anthracis* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 30.

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 *Bacillus anthracis* should have CPs between cycles 18 and 36.

7.3. Sample Data – typical results

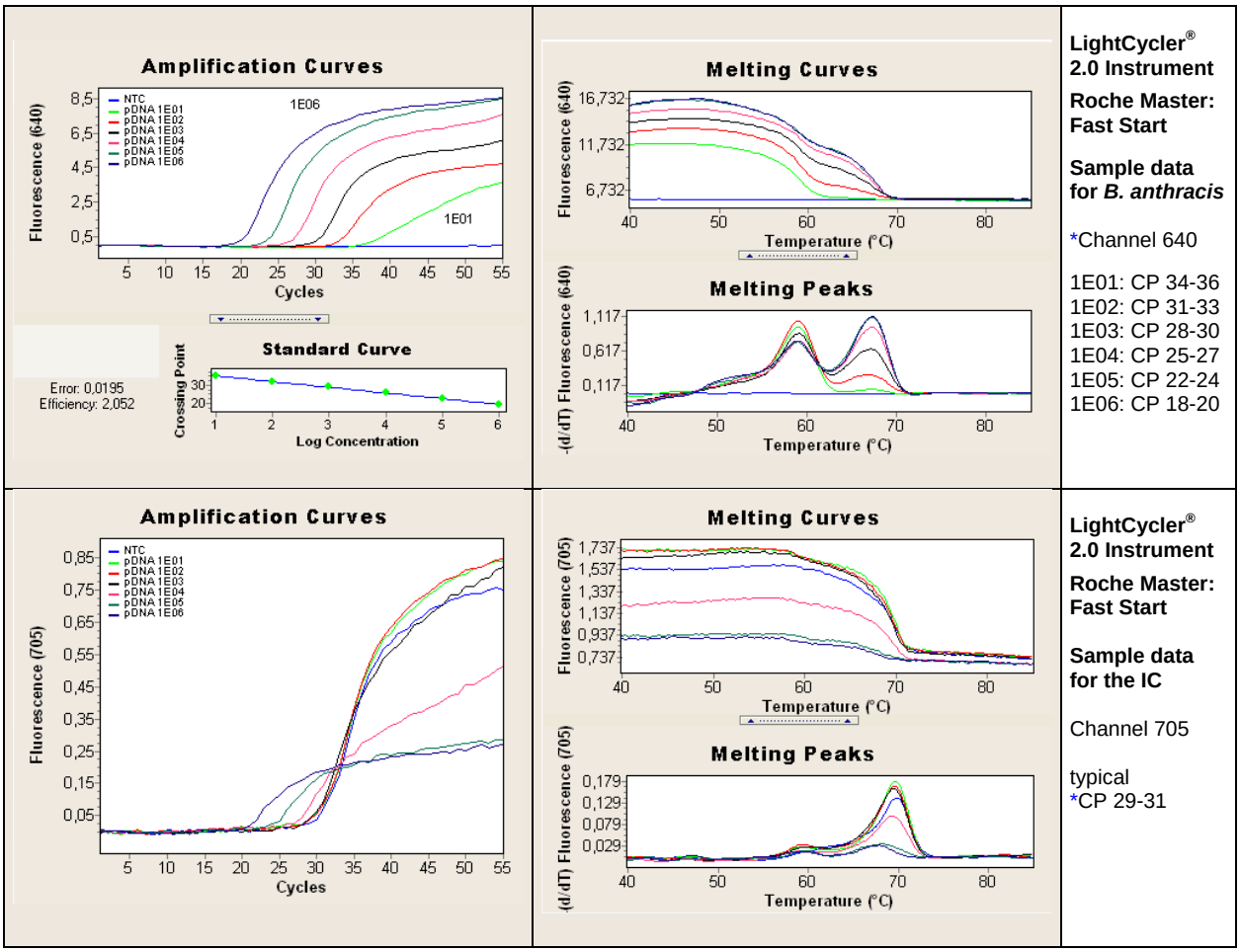

Fig.1. Sample data for the *Bacillus anthracis* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *Bacillus anthracis* . Right panel channel 640 melting analysis for *Bacillus anthracis* . (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/peaks 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

Bacillus anthracis **(sample) Quantification 640**	***Bacillus anthracis*** **(sample) Melting Analysis 640**	**Internal Control (sample) Quantification 705**	**NTC (control sample) Quantification 640**	**Result**
no amplification	no melting peak	detectable	negative	**Negative**
amplification signal	2 peaks 58°C + 65°C	not relevant	negative	**Positive for *B. anthracis* (BA)**
amplification signal	1 melting peak only	not relevant	negative	**Probably positive BA**
no amplification	no melting peak	not detectable	not relevant	**PCR failure, repeat**
amplification signal	2 peaks 61°C & 69°C	not relevant	positive	**Contamination, repeat**

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

8. LightCycler® 480 II 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 II Instrument: 465-510, 498-640, 498-660

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	55			1			1
Target [°C]	95	95	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:08	00:00:15	00:00:30	00:2:00	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

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 'LightMix® Kit – Color Compensation 530/640/690' or 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 *Bacillus anthracis* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Bacillus anthracis* data with Filter Combination 498-640, Melting Curves mode.

If the internal control is used, view data with Filter Combination 498-640, Quantification mode, and the IC with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Bacillus anthracis* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 30.

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 *Bacillus anthracis* should have CPs between cycles 18 and 35.

8.3. Sample Data – typical results

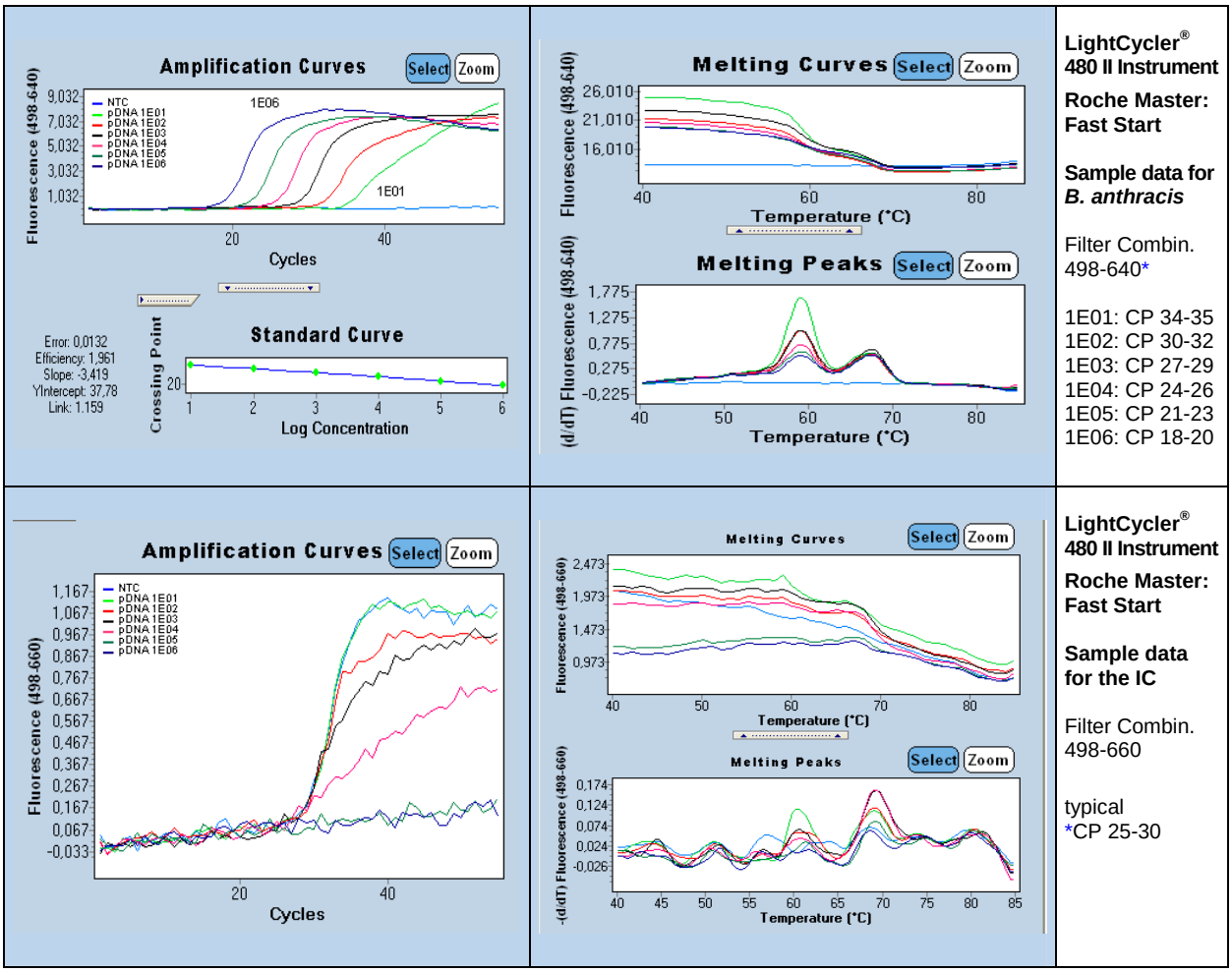

Fig.1. Sample data for the *Bacillus anthracis* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *Bacillus anthracis.* Right panel Filter Combination 498-640 melting analysis for *Bacillus anthracis* (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.

* **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

Bacillus anthracis **(sample) Quantification 640**	***Bacillus anthracis*** **(sample) Melting Analysis 640**	**Internal Control (sample) Quantification 705**	**NTC (control sample) Quantification 640**	**Result**
no amplification	no melting peak	detectable	negative	**Negative**
amplification signal	2 peaks 58°C + 65°C	not relevant	negative	**Positive for *B. anthracis* (BA)**
amplification signal	1 melting peak only	not relevant	negative	**Probably positive BA**
no amplification	no melting peak	not detectable	not relevant	**PCR failure, repeat**
amplification signal	2 peaks 61°C & 69°C	not relevant	positive	**Contamination, repeat**

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

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