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

TIB MOLBIOL

LightMix® Kit *Brucella Genus*

Cat.-No. 40-0249-16 New Version: working on the LightCycler® 1.x / 2.0 /480 II Instruments

Kit with reagents for the detection of *Brucella Genus* 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℃), 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

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella*, a gram-negative, non-spore forming, facultative intracellular, nonmotile coccobacillus. The genus is divided according to antigenic properties and host specificity into six species, but only *B. abortus*, *B. melitensis* and *B. suis* are considered to be human pathogen. The bacterium is found in farm animals (e.g. cattle, goat, sheep) especially in the Mediterranean basin, the Middle East, Africa, India, and Central and South America. Brucellosis is acquired by direct contact with infected animals and through contaminated milk or milk products. The human disease includes intermittent fever, chills, weakness and weight loss, but is rarely fatal; the mortality without treatment is less than 2%. Bacteria of the genus *Brucella* have the potential of being used as a biological warfare agent.

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

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

2. Description

A 207 bp fragment of IS711 from the *Brucella Genus* 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 278 bp, formed from the internal positive control. This control will not interfere with the *Brucella Genus* specific reactions. The amplification will usually fail in the presence of higher concentrated *Brucella Genus* DNA samples (1,000 - 10,000 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 to allow running the assay in the presence or absence of the IC.

The use of a color compensation file generated with the TIB MOLBIOL 'LightMix® Kit - Color Compensation 530/640/690' or the Roche Diagnostics 'LightCycler®-Color Compensation 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 reactions each of *Brucella Genus.*
6 Vials with white caps containing the internal positive control (IC)
1 Row with 6 lyophilized cloned plasmid standards of *Brucella Genus* from 10^1 to 10^6 target equivalents per reaction
1 Sealing foil for the standard row Foil for sealing 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 *Brucella Genus* 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 *Brucella Genus* 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℃).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days when stored protected from light and refrigerated (4℃).

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):

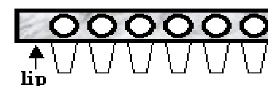
One reagent vial with a **green** cap contains all primers and probes to run 16 LightCycler® reactions for *Brucella Genus.*
One reagent vial with a **white** cap contains all primers, probes and DNA to run 16 LightCycler® reactions for the IC

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℃. 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.
> 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	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

(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 TIB MOLBIOL 'LightMix® Kit – Color Compensation 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 *Brucella* data in channel 640 Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Brucella* 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 *Brucella* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 26.

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 *Brucella* should have CPs between cycles 18 and 35.

7.3. Sample Data – typical results

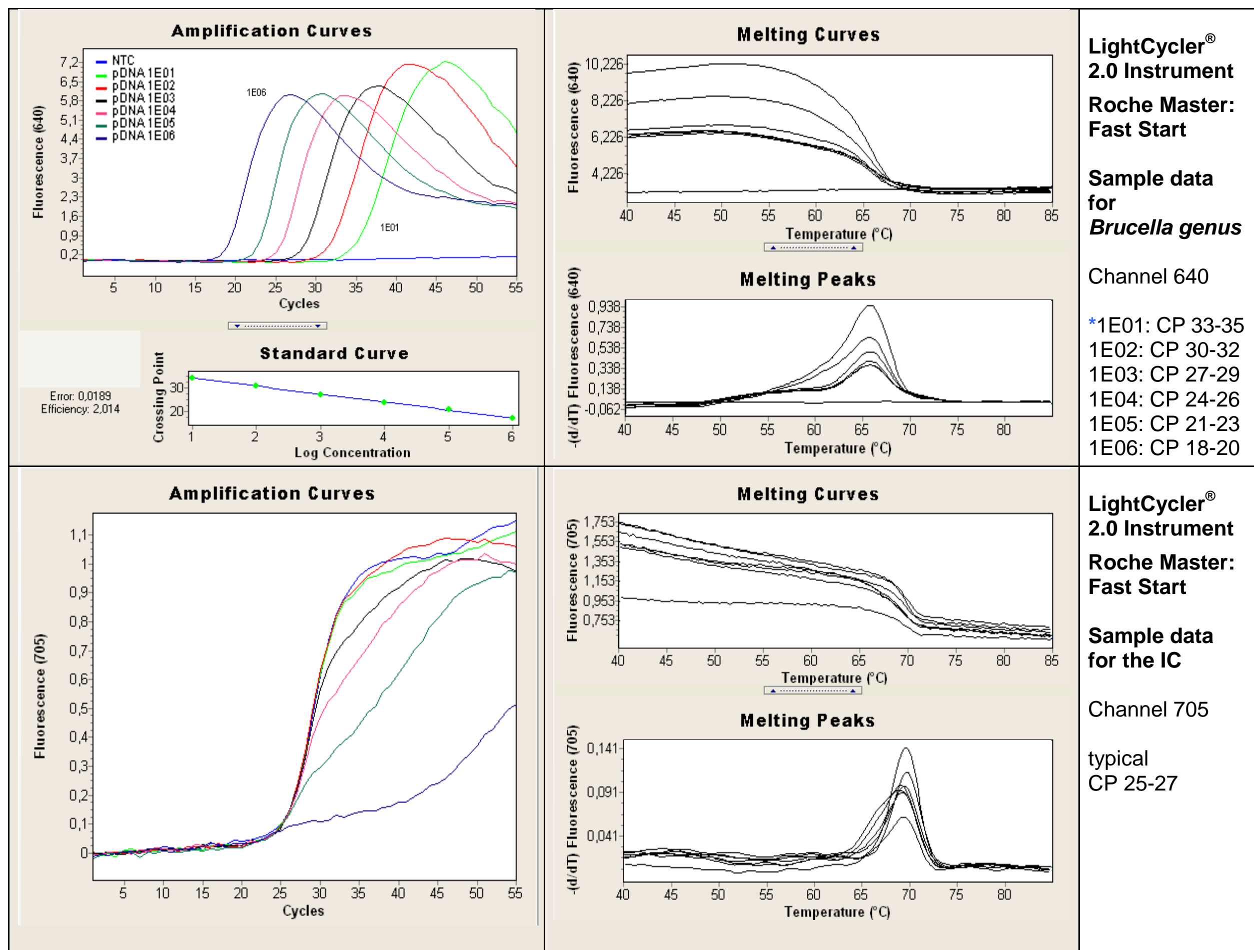


Fig.1. Sample data for the *Brucella genus* detection system.

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

Brucella genus **(sample)**	**IC (sample)**	**NTC**	**Result**
no amplification	detectable	negative	Negative
amplification signal	not relevant	negative	Positive
no amplification	not detectable	not relevant	PCR failure, repeat experiment
amplification signal	not relevant	positive	Contamination, repeat experiment

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 [℃]	95	95	55	72	95	40	80	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:08	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [℃/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [℃/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 ℃]	-	-	-	-	-	-	1	-

(Melting not relevant for detection)

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 the TIB MOLBIOL 'LightMix® Kit – Color Compensation 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 *Brucella* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal.

If the internal control is used, view IC data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Brucella* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 26.

The provided standard row of cloned and purified DNA with concentrations in the range from10^6 copies/rxn to 10^1 copies/rxn of *Brucella* should have CPs between cycles 19 and 35

8.3. Sample Data – typical results

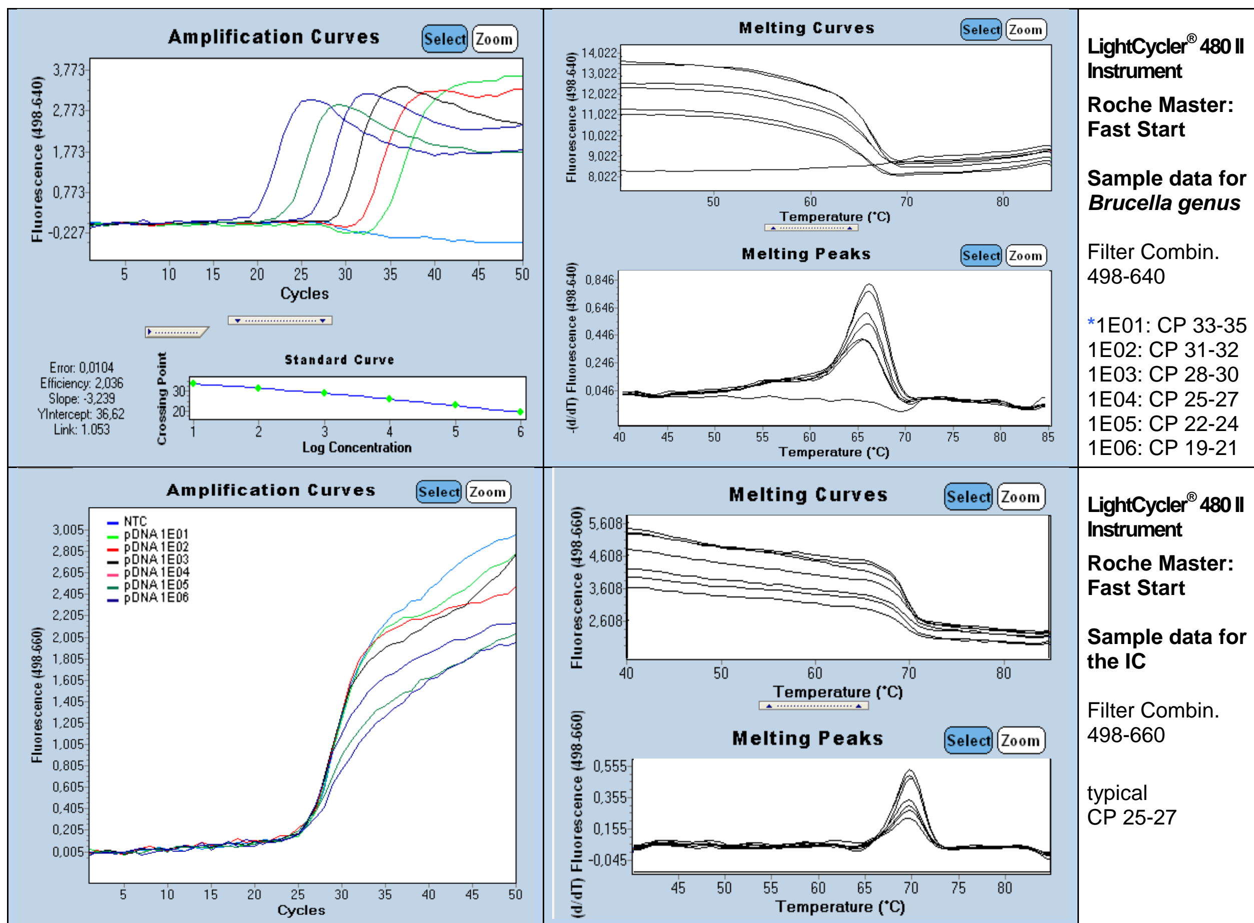


Fig.1. Sample data for the *Brucella genus* detection system.

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

Brucella genus **(sample)**	**IC (sample)**	**NTC**	**Result**
no amplification	detectable	negative	Negative
amplification signal	not relevant	negative	Positive
no amplification	not detectable	not relevant	PCR failure, repeat
amplification signal	not relevant	positive	Contamination, repeat

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

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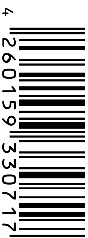

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
LightCycler® hybridization probes produced under license from Roche Diagnostics.