LightMix® Kit *Borrelia* spp. EC

Cat.-No. 40-0295-32 2014: Internal Ctrl changed to spiked Extraction Ctrl, 32 rxsn/vial

Kit with reagents for the detection of *Borrelia* spp. *genomic* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer.

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 / cobas z 480 Instruments see pages 6-7

1. Introduction

Borreliosis (Lyme disease) is caused by Gram-negative spirochetal bacteria from the genus *Borrelia*. Typical symptoms include fever, headache, fatigue and erythema migrans, a characteristic skin rash. *Borrelia* is usually transmitted by tick bites. From the five important human pathogenic genospecies *Borrelia burgdorferi* sensu stricto is the predominant species in North America, while *Borrelia afzelii*, *B. spielmanii, B. garinii* and *B. lusitaniae* are the more prevalent species found in Europe.

Molecular detection of *Borrelia* is often based on the flagellin gene, while rpoB, Hbb, recA and ospA genes have been commonly used for the identification of the different species. This kit uses the plasmid encoded ospA gene similar to the publication from Rauter et al., 2002 [1], including additional primers to capture more *Borrelia* species and one additional probe to detect also *B. lusitaniae*.

2. Description

This kit provides a fast and accurate system to detect and identify *Borrelia* genomic DNA in a nucleic acid extract. An about 160 bp long PCR fragment from the ospA gene is amplified with specific primers and detected with LightCycler® Red 640 labeled hybridization probes. The species are distinguished by running a melting curve analysis of the PCR product, resulting in melting points (Tm) of typically about 71 °C for *Borrelia afzelii*, 67°C for *B. garinii* or *B. lusitaniae*, 62°C for *B. burgdorferi*, and 58°C for *B. spielmanii*, as shown for exemplary samples (fig 2). However, as the target region is known to be variable, the Tm shall be except the highest Tm (*B.afzelii*) not used for an identification of the species.

The control reaction generates an additional product of 125 bp from the PhHV target, using with LightCycler® Red 690 labeled hybridization probes. This second PCR has no visible impact on the *Borrelia* specific reaction and will even fail in the presence of higher amounts of target (1,000 copies and more) while displaying an amplification signal in negative and low-concentrated samples.

The former internal control (IC) using Lambda DNA has been changed to a spiked extraction control (sEC) based on a segment of the PhHV virus in order to monitor a successful extraction and to demonstrate the ability to run a PCR reaction (absence of inhibition).
We recommend to use the 'Extraction Control' procedure; for compatibility with the former procedure the use as IC is also described. The extraction control target ^{n}ECT contains Lamdba and PhHV DNA and may be used also for other LightMix kits, without adding the ECT provided with the other kit(s).

Note: With a MagNA Pure Compact extraction the recovery rate for the EC target can be very low or the DNA target even gets lost.

The use of a color compensation file generated with the LightMix® Color Compensation HybProbe 530/640/690 kit (order no. 40-0318-00) is a prerequisite to detect the control 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.

The kit is tested with 'LightCycler® FastStart DNA Master HybProbe' only.

3. Set Contents

3 Vials with **green** cap containing premixed primers and probes for 32 PCR reactions
3 Vials with **white** cap containing premixed primers and probes for 32 control reactions

1 Vial with **white** cap containing Extraction Control Target (ECT): 4.8 x 10^6 copies (total)
1 Vial with **black** cap containing the stabilizer for preparation of the 'No Template Control' (NTC)

1 Standard row with 6 dried standards *B. burgdorferi* sensu stricto DNA 10-10^6 copies per rxn
1 Sealing foil for the standard row

1 Certicate of Analysis (CoA) with lot-specific data

4. Additional Reagents and Items Required

	Roche Diagnostics
Color Compensation HybProbe order n°40-0318-00	Cat .-No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
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

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.

4.1. Optional Additional Reagents

High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001

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 plate based Instruments.

Sensitivity
These reagents detect 10 copies of *Borrelia* DNA per reaction, using Roche 'LightCycler® FastStart DNA Master HybProbe' (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 / reaction of *Borrelia* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe'.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25℃). See expiry date 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℃).

6. Experimental Protocol

Start programming before preparing the solutions. See the Instrument operator's manual for details.

6.1. Preparation of Parameter-Specific Reagents (PSR) and Control Reaction :

One reagent vial with a **green** cap contains primers and probes to run 32 reactions *Borrelia.*
One reagent vial with a **white** cap contains primers and probes to run 32 reactions control reaction.

Check for the colored pellet, then **add 66 µl** PCR-grade water, mix (vortex) and spin down.

►**Use 2 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least ten days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of Extraction Control Target (ECT):

Add **1,200 µl** PCR-grade water to the vial with **white** cap containing the Extraction Control Target

6.3. Sample Preparation:

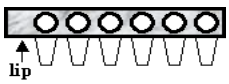
Before extraction add **10 µl** of ECT (vial **white** cap) to the sample; the amount may has to be adapted to the extraction method to get a Cp value in the range of 28-32. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

6.4. Preparation of No Template Control (NTC) including the Extraction Control Target

Add **900 µl** PCR-grade water to the **NTC** (vial **black** cap) and
add **100 µl** of ECT (vial **white** cap).

►Use **5 µl** No Template Control DNA for a 20 µl PCR reaction.

6.5. Preparation of the Standard Row:



The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 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** of **NTC** (vial **black** cap) to each well. Use water if the IC procedure is selected. 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. After adding the target DNA to reaction mix, use the provided sealing foil to close the vials in order to avoid contaminations.

6.6. Preparation of the Reaction Mix

Include Positive Controls and at least one 'No Template Control' (NTC). In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes by the number of reactions plus one reserve :

sEC Procedure	**For use with the Roche FastStart Master**	**IC Procedure**
Single reaction	**Component**	**Single reaction**
7.4 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	7.9 µl
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	1.6 µl
2.0 µl	**PSR** mix (parameter specific reagents, primers and probes, see **6.1.**)	2.0 µl
2.0 µl	**Control Reaction** (see **6.1.**)	2.0 µl
---- µl	ECT Control Target (vials **white** cap)	0.5 µl
2.0 µl	Roche Master (red cap, for preparation see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

Table 1

Mix gently, spin down and **transfer 15 µl** of the reaction mix to a capillary or well.

Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to the multiwell plate and seal, and spin down.

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

Table 2

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 Color Compensation Kit HybProbe 530/640/690.

Perform data analysis, as described in the 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 *Borrelia* data in channel 640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Borrelia* data in channel 640, Melt. Curves mode.

For the Control Reaction view channel 705 data. The negative control and the low-concentrated DNA samples (10 to 1,000 copies) will show an amplification curve with a Cp at approximately cycle 32-36.

The provided standard row of cloned *B. burgdorferi* sensu stricto target DNA with concentrations from 10^6 to 10 copies/reaction target should yield Cp values between cycles 19 and 37 (see figure 1).

For use in LightCycler® 1.x / 2.0 Instruments select channel F2 instead of channel 640, channel F3 instead of channel 705 for detection.

7.3. Sample Data – Typical Results

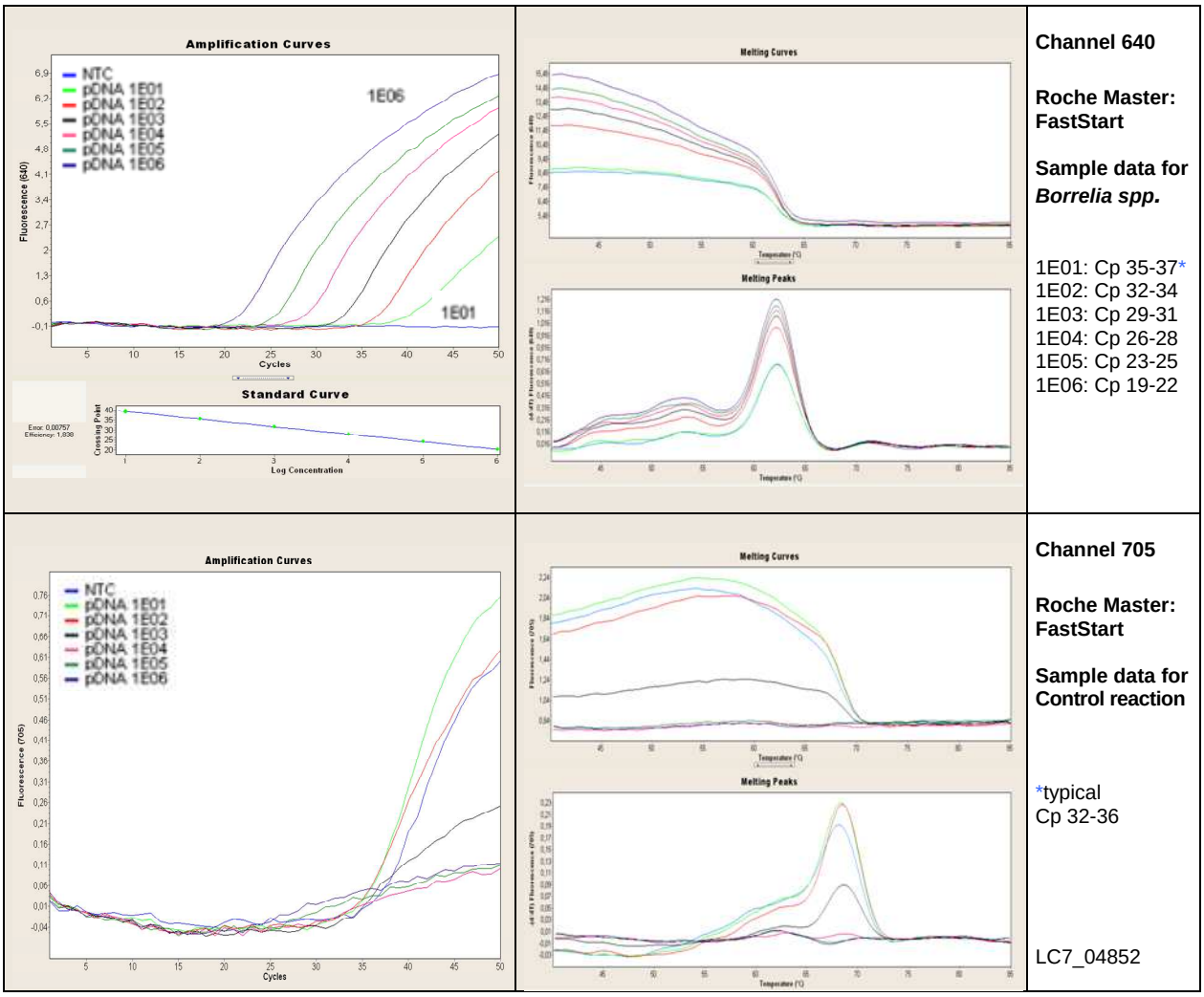


Fig.1. LightCycler® 2.0 sample data for the *Borrelia spp.* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with standard curve for *Borrelia spp.* Right panel channel 640 melting peaks for *B. burgdorferi sensu stricto;* see specific melting point described in legend of Fig 2.
Lower panels: Left: channel 705 quantification mode (Sec. Der. Maximum).Right: channel 705 melting peaks control reaction.

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

7.4. Interpretation of Data

Sample 640 *Borrelia*	**Melting Tm**	**Sample 705** *Ctrl. Reaction*	**Channel 640** *Pos. Control*	Channel 640 *NTC*	*Result (warnings)*
no amplification	**-**	detectable	amplification	negative	**Negative (not detectable)**
Cp < 40+	≈ 70℃	not relevant	amplification	negative	***Borrelia* positive** Most likely *Borrelia afzelii*
Cp < 40+	< 70℃	not relevant	amplification	negative	**Pos. for other *Borrelia***
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

Table 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: FastStart)

+ The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies.

8. LightCycler® 480 II Instrument and cobas z 480 Analyzer

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: 565-510, 498-640, 498-660
cobas z 480 Analyzer: 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	80	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:01:00	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]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**

Table 4

8.2. Data Analysis

Note: cobas z 480 Analyzer 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 'LightMix® Kit – Color Compensation HybProbe.

Perform data analysis, as described in the 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 *Borrelia* data with Filter Combination 498-640, Quantification mode. The negative control (NTC) must show no signal.

Use the Melting Curves analysis (Tm Calling) for the identification of the PCR product, view *Borrelia* data with Filter Combination 498-640 (see figure 2).

For the Control Reaction view Filter Combination 498-660 data, Quantification mode. The negative control and the low-concentrated DNA samples (10 to 1,000 copies) will show an amplification curve with a Cp at approximately cycle 29-32.

The provided standard row of cloned *B. burgdorferi* sensu stricto target DNA with concentrations from 10^6 to 10 copies/reaction target should yield Cp values between cycles 19 and 36 (see figure 2).

8.3. Sample Data – Typical Results

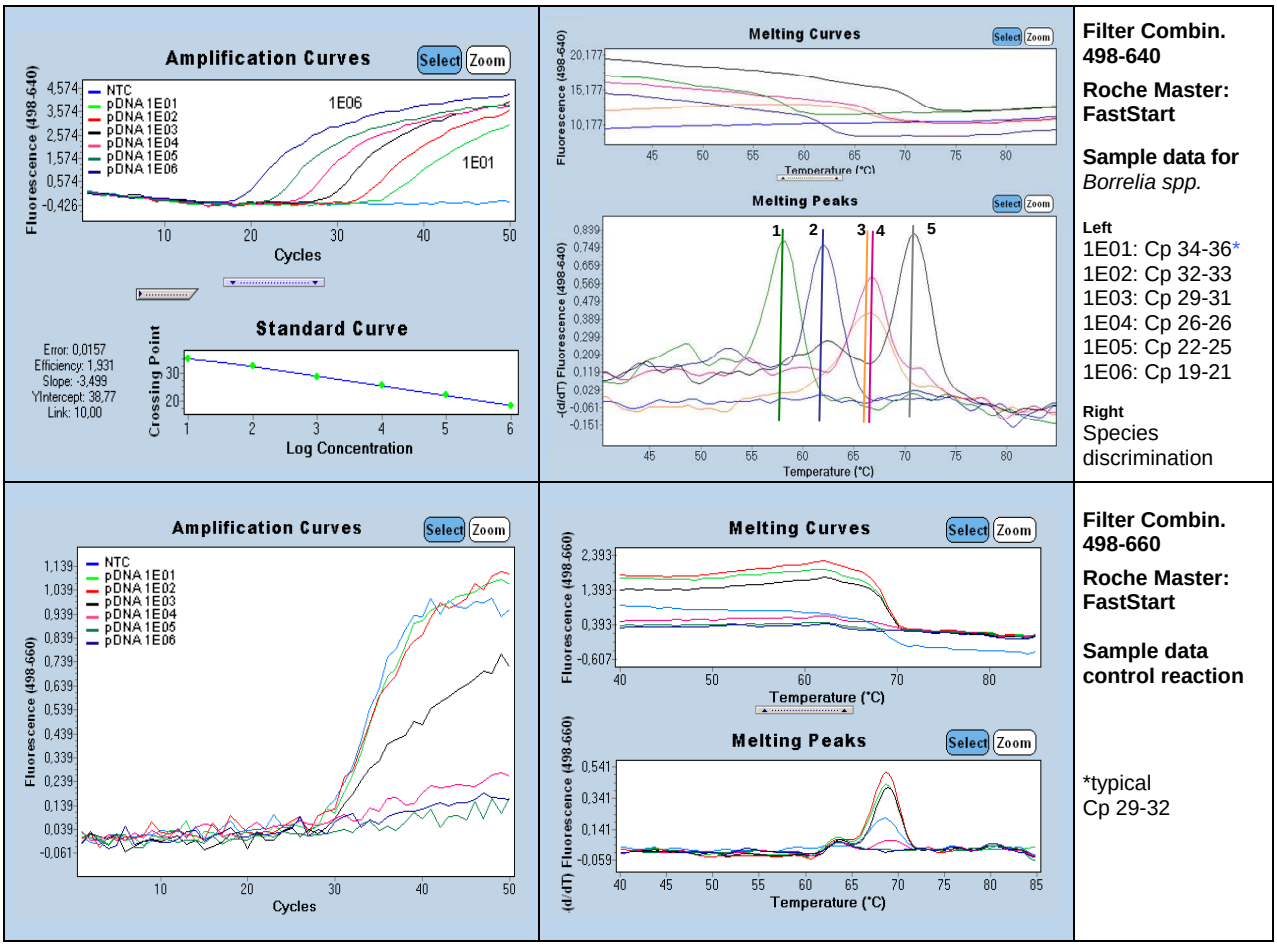


Fig.2. LightCycler® 480 II sample data for the *Borrelia spp.* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum). Right panel Filter Combination 498-640 species specific melting point (Tm): 62.1℃ for *Borrelia burgdorferi* sensu stricto (2), 70.8℃ for *Borrelia afzelii (5)*, 67.7℃ for *Borrelia garinii* (3) and *Borrelia lusitaniae (4), 57.0℃ for Borrelia spielm anii (1)*. The standard included with this kit corresponds to the sequence of *Borrelia burgdorferi* sensu stricto and yields a Tm of about 62℃.
Lower panels: Control Reaction. Left: Filter 498-660 quantification mode (Sec. Derivative Maximum). Right : melting analysis.

* **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 zig-zag 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

Sample 640 *Borrelia*	**Melting Tm**	**Sample 705** *Ctrl. Reaction*	**Channel 640** *Pos. Control*	Channel 640 *NTC*	*Result (warnings)*
no amplification	**-**	detectable	amplification	negative	**Negative (not detectable)**
Cp < 38⁺	≈ 70℃	not relevant	amplification	negative	***Borrelia* positive** Most likely *Borrelia afzelii*
Cp < 38⁺	< 70℃	not relevant	amplification	negative	**Pos. for other *Borrelia***
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

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

⁺ The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies.

9. Evaluation Study Results

Seven proficiency panels (INSTAND, Germany, 2004-2009) and DNA from 1,121 ticks were analyzed. 20.2% of the ticks revealed detectable *Borrelia* DNA, with *B. afzelii* the most prominent subspecies. *Borrelia burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. spielmanii*, and *B. valaisiana* were reliably detected, while the non pathogenic *B. lusitaniae* was missed. Melting curve analysis allowed for a reliable *Borrelia* genospecies differentiation. Dilution series of proficiency panel samples revealed a detection limit of approximately 10(1) genomes/mL. Based on Cp values the mean interassay coefficient of variation of the assay was 3.6%, while the intraassay coefficients of variation were 1.6% and 0.8% for a strong positive and a weak positive kit standard, respectively. [2]

Comment : This kit was 2012 improved to include *B. lusitaniae* (starting lot 17811401)

10. References

[1] Distribution of clinically relevant Borrelia genospecies in ticks assessed by a novel, single-run, real-time PCR. Rauter C, Oehme R, Diterich I, Engele M, Hartung T. JCM 40 (2002) 36-43

[2] Evaluation of a real-time PCR assay for the detection, genotyping, and quantification of Borrelia burgdorferi sensu lato in Ixodes ticks in a routine laboratory setting. Wolff and Gerritzen Clin Lab. 2011;57(1-2):67-73.

12. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU 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.

13. Version History

Red notes mark events require changed procedures, blue mod. sequences

V081002	Initial version for capillary based LightCycler® 1.x / 2.0 Instruments
V100819	IC changed to dye LC690 to work with LightCycler® 480 II systems also
V120203	Improved to detect also species *B. lusitaniae* and *B. spielmanii*
V140606	Editorial changes, MSDS included
V140909	IC changed to Extraction Control, control assay changed to PhHV Kit changed from 6 x 16 rxns to 3 x 32 rxns (remains 96 reactions total)
V150101	MagNA pure Compact may fail to recover the sEC extraction target Section 9 with Evaluation data inserted.Section 10 References added.
V150505	Universal Extraction Control target ^{n}ECT with Lambda and PhHV

Roche SAP order n° 05945275001

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