LightMix® Kit *Ureaplasma urealyticum/parvum* EC

Cat.-No. 40-0152-32 Internal Control (IC) changed to spiked Extraction Control (sEC)

Kit with reagents for the detection of *Ureaplasma urealyticum/parvum* 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 Analyzer see pages 6-7

1. Introduction

Ureaplasma are bacteria of the family Mycoplasmataceae. The two biovars, *Ureaplasma urealyticum* and *U. parvum* are today designated as two separate species. *U. parvum* is the more common species while *U. urealyticum* is apparently more pathogenic in conditions such as male urethritis. Differentiation can be made only using molecular techniques such as PCR.

Ureaplasma are part of the normal genital flora of sexually active individuals and they are transmitted horizontally but also vertically from mother to offspring. *Ureaplasma is* associated with a number of diseases, including nonspecific urethritis, infertility, premature birth, chorioamnionitis and stillbirth. Infected newborns can develop pneumonia, meningitis and branchopulmonary dysplasia. Due to the lack of a cell wall penicillin is ineffective and typical treatment is based on tetracycline or doxycycline.

Similar to *Mycoplasma* they are difficult to culture, thus primary methods for detection are based on molecular techniques such as PCR. Preferred specimens are genital swabs; urine has been reported to be insufficient.

2. Description

This kit provides a fast and accurate system to detect but not differentiate *Ureaplasma urealyticum* and *U. parvum* DNA in a nucleic acid extract. A 187 bp long fragment of *16S RNA* gene is amplified with specific primers and detected with LightCycler® Red 640 labeled probes.

The control reaction generates a 125 bp fragment from the PhHV target, detected with LightCycler® Red 690 labeled hybridization probes. This PCR has no visible impact on the *Ureaplasma* specific reaction and will even fail in the presence of higher amounts of target (1,000 copies and more).

The former internal control (IC) has been changed to a spiked extraction control (sEC) to monitor a successful extraction and demonstrate the ability to run an amplification reaction (no PCR 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 amount of ECT might has to be adapted to the extraction method.

The use of a color compensation file generated with the ColorCompensation kit 40-0318 is a prerequisite to run is a prerequisite to detect the control 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.

The kit must be used with 'LightCycler® FastStart DNA Master HybProbe' only (capillary and plate based LightCycler® Instruments).

3. Set Contents

3	Vials with **green** cap containing premixed primers and probes for each 32 reactions of *U. urea.*
3	Vials with **white** cap containing premixed primers and probes for each 32 control reactions
1	Standard row with 6 lyophilized standards *U. urea.* 10^1 to 10^6 target equivalents per rxn
1	Sealing foil for the standard row
1	Vial with **white** cap containing Extraction Control Target (^{n}**ECT**): 4.8 x 10^6 copies (total)
1	Vial with **black** cap containing the stabilizer for the No Template Control (NTC)
1	Certicate of Analysis (CoA) with lot-specific data

4. Additional Reagents and items required

	Roche Diagnostics
ColorCompensation HybProbe order n°40-0318-00	Cat. -No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
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 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 640 and F3 instead of channel 705. We recommend to upgrade to software version 4.10 or higher.

4.1. Optional Additional Reagents

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

5. Product Characteristics

PCR results (activation, 50 cycles and melting curve) are obtained within 50 minutes with the capillary based LightCycler® 1.x / 2.0 Instruments and within 80 minutes with '480' plate based Instruments.

Sensitivity
These reagents detect 10 copies of positive control target DNA using FastStart DNA Master HybProbe.

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Ureaplasma urealyticum* DNA.

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 and reagents for the EC (32 reactions):

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

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 Extraction (NTC):

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 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** 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** for a 20 µl PCR reaction

This standard solution is not long-term stable. Use only fresh prepared solutions only. After adding the target DNA to the 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**	**Optional: IC**
Single reaction	**Component**	**Single reaction**
6.6 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.1 µl
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl
2.0 µl	**PSR** mix (parameter specific reagents, see 6.1 for *Ureaplasma*)	2.0 µl
2.0 µl	Primers and probe mix for the **EC/IC**	2.0 µl
-	**ECT** (white cap, DNA control target, see 6.2.)	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**

Table1

To run the assay without the control reaction substitute ECT with 0.5 µl PCR-grade water.

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

(Melting not relevant for detection) 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 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 *Ureaplasma* data in channel 640, Quantification mode. The negative control (NTC) must show no signal.

If the control reaction is used, view data in channel 705, Quantification mode. The negative control and the low-concentrated *Ureaplasma* DNA samples (10 to 1,000 copies) should show an amplification curve for the EC/IC with a Cp at approximately cycle 29-33.

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 *Ureaplasma* should have Cp values between cycles 20 and 39.

7.3. Sample Data – Typical Results

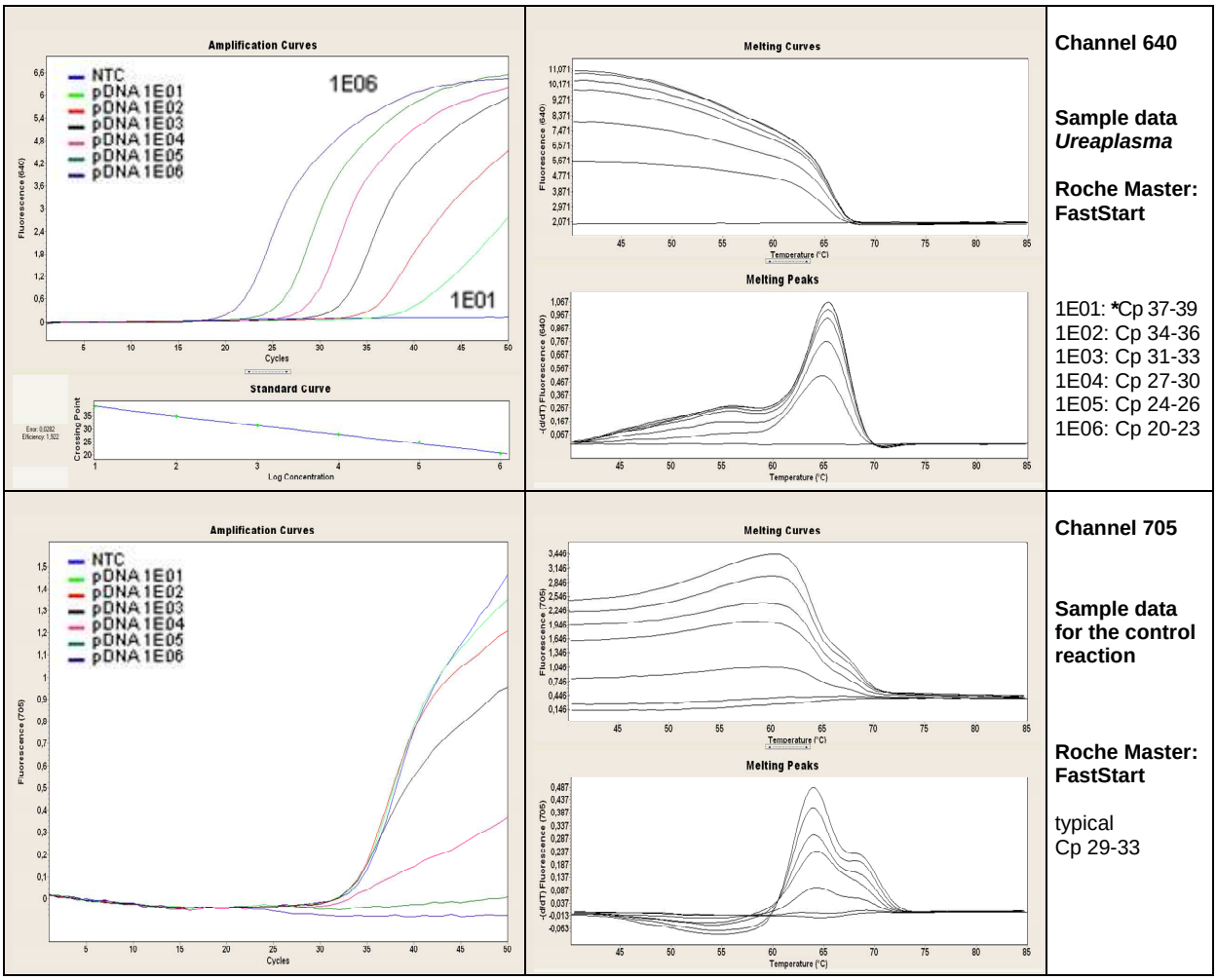

Fig.1. LightCycler® 2.0 sample data for the *Ureaplasma* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *Ureaplasma.* Right panel channel 640 melting analysis for *Ureaplasma* (not relevant for detection).
Lower panels: Left panel channel 705 quantification mode (Second Derivative Maximum) for the control reaction . Right panel channel 705 melting analysis for the control reaction (not relevant for detection).

* **Note:** Fluorescence levels may vary also depending on instrument settings. 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 relatively 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 horizontally shifted.

7.4. Interpretation of Data

Sample 640 *Ureaplasma*	Sample 705 *Control Reaction*	Channel 640 *Positive Control*	Channel 640 *Negative Control*	*Results(warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 40	not relevant	amplification	negative	**Positive for *Ureaplasma***
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

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

8. LightCycler® 480 II Instruments 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: 465-510, 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	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	Continuou	None
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**

(Melting not relevant for detection) 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 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.

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

If the control reaction is used, view data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Ureaplasma* DNA samples (10 to 1,000 copies) should show an amplification curve for the EC/IC with a Cp at approximately cycle 29-32.

The provided standard row of cloned and purified DNA with concentrations in the range from10^6 copies/rxn to 10^1 copies/rxn of *Ureaplasma* should have Cp values between cycles 19 and 36.

8.3. Sample Data – Typical Results

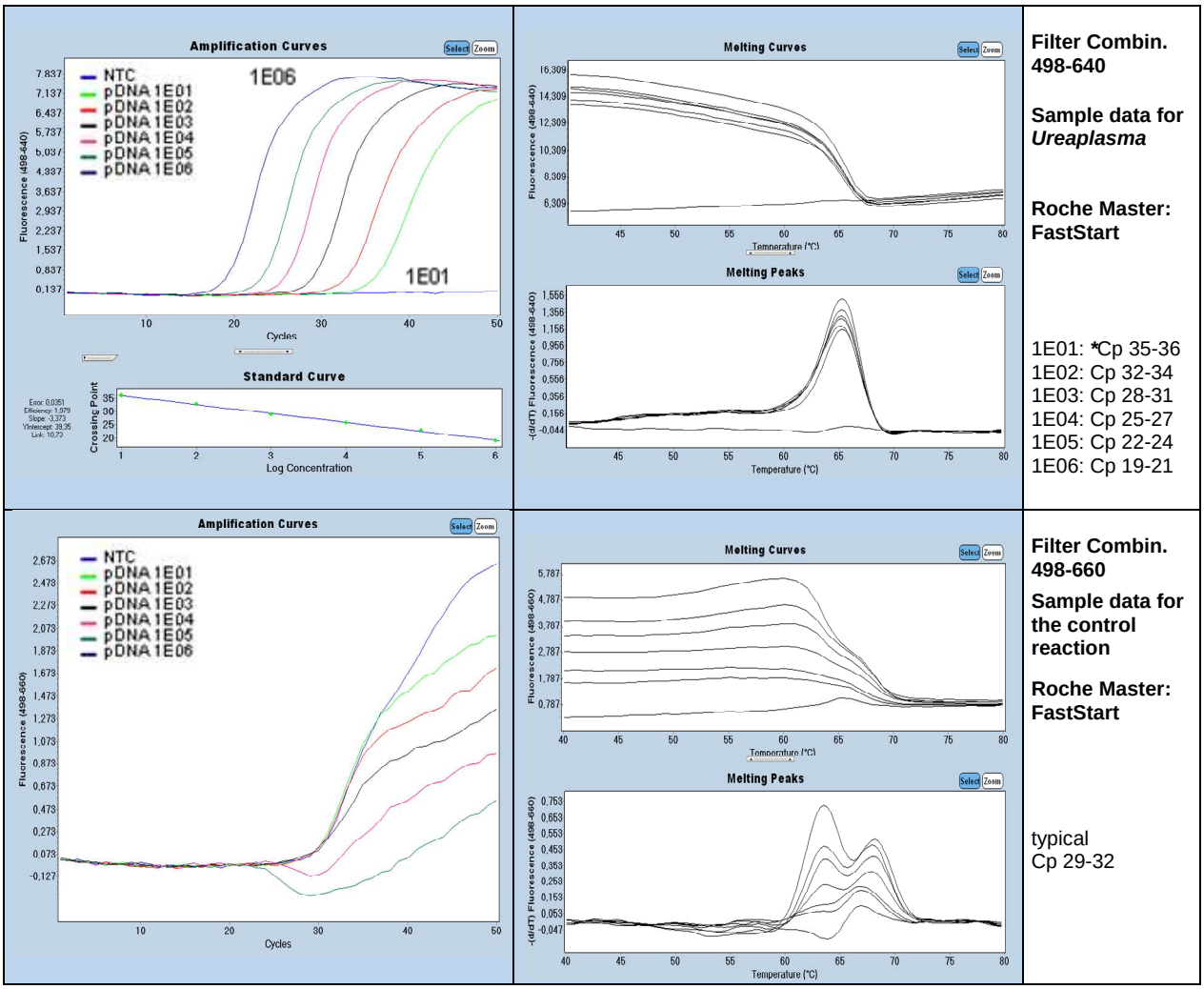

Fig.2. LightCycler® 480 II sample data for the *Ureaplasma* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *Ureaplasma*. Right panel Filter Combination 498-640 melting analysis *Ureaplasma* (not relevant for detection).
Lower panels: Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) for the control reaction. Right panel Filter Combination 498-660 melting analysis for the control reaction (not relevant for detection).

* **Note:** Fluorescence levels may vary also depending on instrument settings. 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 relatively 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 horizontally shifted.

8.4. Interpretation of Data

Sample 640 *Ureaplasma*	Sample 660 *Control Reaction*	Channel 640 *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 38	not relevant	amplification	negative	**Positive for *Ureaplasma***
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

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

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

Events requiring changes in procedures red, mod. sequences blue

V120606	Release version
V130128	Correction in 6.2. Preparation of the standard row
V130308	cobas z 480 Instruments. Correction length of IC PCR product
V130718	Correction of Cp values for LC480 II
V130813	Editorial changes, reference to kit 40-0138 removed
V150505	Change Internal Control (IC) to Extraction Control (EC) Universal ^{n}ECT target containing Lambda and PhHV DNA

Roche SAP order n° 05552354001

Notice to Purchaser

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

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