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

LightMix® Kit *Chlamydia trachomatis (EC)*

Cat.-No. 40-0098-32

Kit with reagents for the detection of *Chlamydia trachomatis* 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.
Shipping at ambient temperature. Store protected from light at 4°C to 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 and cobas z 480 Analyzer see pages 6-7

1. Introduction

Chlamydia trachomatis is one of the most prevalent sexually transmitted pathogens. The organism is an obligate intracellular parasite that exclusively infects humans. 75% of women and 25% of men with Chlamydia show no symptoms at all. In women, symptoms include increased vaginal discharge, burning during urination, or bleeding after sexual intercourse. In men, non-gonococcal urethritis is the main symptom. Contact of infected secretions from the genitals to the hands and eventually to the eyes can cause trachoma which can lead to blindness.

Chlamydia trachomatis persists at body sites that are inaccessible to phagocytes, T-cells, and B-cells. The surface of *C. trachomatis* does not contain proteins that are distinctive enough to induce a full immune response. Treatment of *C. trachomatis* is accomplished with various antibiotics.

2. Description

This kit provides a fast and accurate system to detect *C. trachomatis* DNA in a nucleic acid extract. A 136 bp long fragment of the *C. trachomatis* MOMP gene[1] is amplified with specific primers and detected with LightCycler® Red 640 labeled probes. The PCR product can be identified by running a melting curve with a melting point of 66°C or occasionally 59°C.

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 *C. trachomatis* 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 HybProbe 40-0318 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.

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 *C. trach.*
3 Vials with **white** cap containing premixed primers and probes for each 32 control reactions

1 Standard row with 6 lyophilized plasmid standards from 10^1 to 10^6 target equivalents per rxn
1 Sealing foil for the standard row

1 Vial with **white** cap with the universal 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
LightMix® Kit ColorCompensation HybProbe 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 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 target genomic DNA.

Storage and Stability
- Lyophilized reagents stable for 12 months after production. See expiry date on outer product label.
- Store protected from light at 4°C to 25°C. **Do not freeze** lyophilized reagents.
- Dissolved reagents stable for at least 10 days when stored protected from light and cooled (4°C to 10°C).
- Dissolved reagents can be long-term stored frozen at -15 to -25°C. Minimize multiple thaw-freeze cycles.

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 for *C. trachomatis.*
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 five 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 10^1 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** 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	IC Procedure
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, primers and probes, see **6.1.**)	2.0 µl
2.0 µl	**Primers and probe mix for the IC/EC**	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

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

For the identification of the PCR product view *Chlamydia trachomatis* data in channel 640, Melting Curves mode.

For the Control Reaction, view data in channel 705, Quantification mode. The negative control and the low-concentrated *Chlamydia trachomatis* DNA samples (10 to 1,000 copies) should show an amplification curve for the EC/IC with a Cp at approximately cycle 30.

The provided standard row of cloned DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Chlamydia trachomatis* should have Cp values between cycles 17 and 34.

7.3. Sample Data – Typical Results

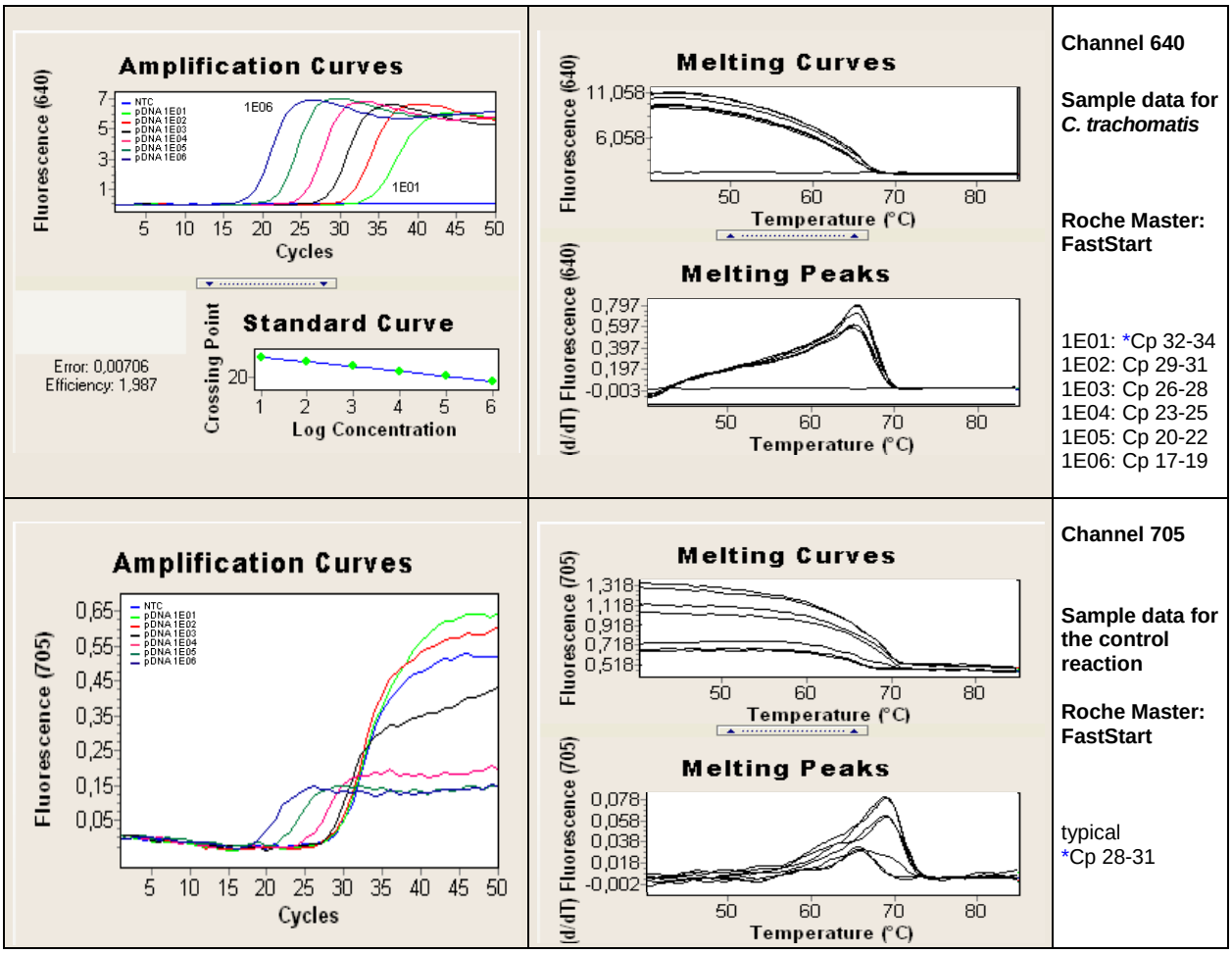

Fig.1. LightCycler® 2.0 sample data for the *C. trachomatis* detection system.

Upper panels: Left panel channel 640 quantification mode (Sec. Der. Maximum) with amplification curves for *C. trachomatis*. Right panel channel 640 melting analysis for *C. trachomatis* yields a melting peak at 66°C or 59°C (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 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

Sample 640 *C. trachomatis*	Sample 705 Control reaction	PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 36[+]	not relevant	amplification	negative	**Positive for *C. trachomatis***
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)

[+] 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: 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	Continuous	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. The Fit Points method is more-error prone due to the user's influence.

View *Chlamydia trachomatis* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Chlamydia trachomatis* data with Filter Combination 498-640, Melting Curves mode.

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

The provided standard row of cloned DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Chlamydia trachomatis* should have Cp values between cycles 18 and 35.

8.3. Sample Data – Typical Results

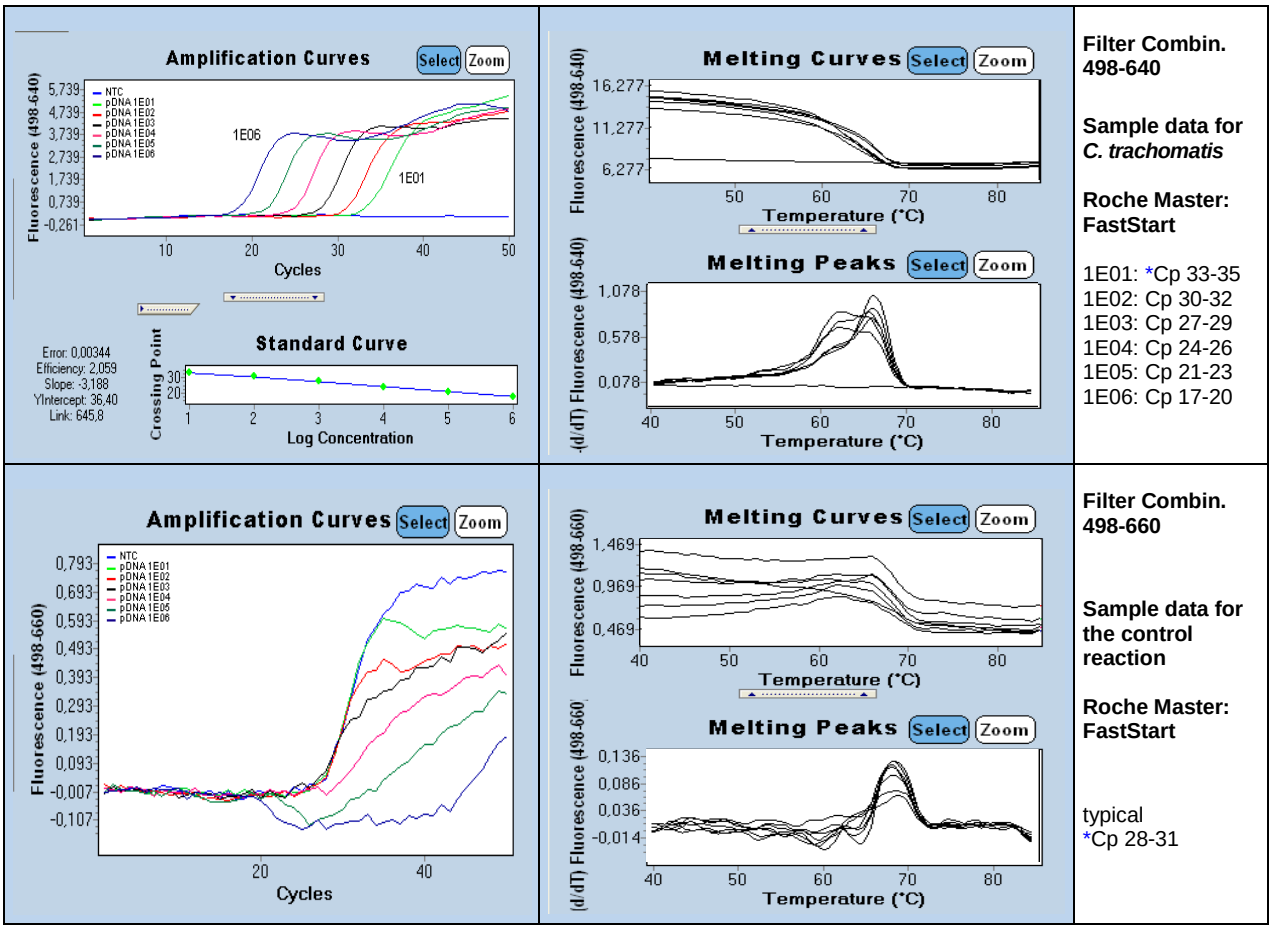

Fig.2. LightCycler® 480 II sample data for the *C. trachomatis* detection system.

Upper panels: Left: Filter Comb. 498-640 quantification mode (Sec. der. Maximum) with amplification curves for *C. trachomatis*. Right: Filter Comb. 498-640 melting analysis for *C. trachomatis* yields a melting peak at 66°C or 59°C (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 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

Sample 640 *C. trachomatis*	Sample 660 Ctrl. Reaction	PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 37[+]	not relevant	amplification	negative	**Positive for *C. trachomatis***
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 with LightCycler® 480 II Instrument

[+] 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 Data

One study in 2004 included 50 positive and 50 negative samples and found a diagnostic sensitivity of 95.7 % and a specifity of 100%, resulting in 100% for the PPV and 96.4% for the NPV values [2].

Whiley et al., 2005 [1] reported a sensitivity of 97.7% for this *C. trachomatis* MOMP assay.

Van der Pol et al, 2006 [3] reported 99% concordance for 822 samples obtained from swabs and urines, using the Roche Amplicor CT test as reference test.

In the external evaluation there was a 100% agreement for > 100 positive and > 20 negative samples.

Unemo et al., 2007 [4] confirmed to detect all 73 deletion mutant samples tested.

INSTAND RV 531 June 2017 100% (18/18) positive, 100% (6/6) negative.

10. References

[1] Comparison of three in-house multiplex PCR assays for the detection of Neisseria gonorrhoeae and Chlamydia trachomatis using real-time and conventional detection methodologies. Whiley DM, Sloots TP. *Pathology* **37(5)**:364-70 (2005).

[2] Evaluation of a LightCycler confirmatory assay for Chlamydia trachomatis. Bennett L., Landt O., Jones M. and Bromhead C. Poster Graz (2004).

[3] Distribution of Chlamydia trachomatis organism load in urogenital specimens de-termined using quantitative PCR. Van Der Pol B., Reischl U., Alberdi M.B., Landt O. and Peeling R.W. Poster WHO (2006).

[4] Experiences with the new genetic variant of Chlamydia trachomatis in Örebro county, Sweden – proportion, characteristics and effective diagnostic solution in an emergent situ. Unemo, Olcén, Agné-Stadling, Feldt, et al.. Euro Surveill 2007;12

[5] Update on the new variant of C. trachomatis; Prevalance and Diagnostics. Herrmann (2008)

11. Contents, Material Safety Data, Certificate of Origin

Product contains : 99.8% Synthetic oligonucleotides (< 100 microgram)
0.1% CAS 77-86-1 Tris (hydroxymethyl) aminomethane
0.1% CAS 60-00-4 Ethylenediamine tetraacetic acid (EDTA)

Product not hazardous, not toxic, not IATA-restricted. HS code 29349990.

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 from human, animal or plant origin. Country of Origin: Germany.

12. Version History

Events requiring changes in procedures red, mod. sequences blue

V081001	Universal PCR program
V091005	Working on LightCycler® 1.x/2.0/480 II Instruments
V100816	32 rxn per vial
V111105	Introduction of version history
V130801	Editorial changes. MSDS, z 480 included
V140414	Melting peak 59°C due to C>T variation (M36533) - not interfering
V150505	Internal Ctrl. changed to Extraction Control, target changed to PhHV Universal Extraction Control target ^{n}ECT with Lambda and PhHV
V170818	11. Contents. Patent Disclaimer removed

Roche SAP order n° 06295037001

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Notice to Purchaser

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany (www.tib-molbiol.com)
Customs Tarif no.(HS code) 2934 9990 Weight: 34 gram.