LightMix® Kit *Toxoplasma gondii* (EC)

Cat.-No. 40-0217-32 2014: Internal Ctrl changed to spiked Extraction Ctrl, 32 rxsn/vial

Kit with reagents for the detection of *Toxoplasma gondii* 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℃), 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 Instruments and cobas z 480 Analyzer see pages 6-7

1. Introduction

Toxoplasma gondii is an intracellular parasite which can infect a wide spectrum of warm-blooded animals (birds and mammals), causing toxoplasmosis. Typical path of transmission to humans is ingestion of *Toxoplasma* oocysts (prevalent in cat feces) or *Toxoplasma* bradyzoites (from raw or undercooked meat) but can be also transmitted transplacentally (Congenital Toxoplasmosis).

Infections with *Toxoplasma* are often asymptomatic, but in particular immune suppressed individuals (e.g., transplant and AIDS patients) can develop serious pathology, including hepatitis, pneumonia, blindness, and severe neurological disorders. During pregnancy the infection can cause spontaneous abortion or still born; infected newborns can have health problems affecting the eyes, nervous system, skin, or ears.

Sulfonamides and pyrimethamine (Daraprim) are two drugs used to treat toxoplasmosis in humans.

Possible targets for a NAT-based detection of *T. gondii* are the B1 gene[1], the surface antigen SAG1 gene [2], the 18S RNA [3], the 16S-RNA-like gene[4], the repeat element[5] or the P30 gene[6].

2. Description

This kit provides a fast and accurate system to detect *Toxoplasma gondii* in a nucleic acid extract. A 134 bp fragment from a repeat element of the *T. gondii* genome is amplified with specific primers. The resulting PCR fragment of *T. gondii* is analyzed with LightCycler® Red 640 labeled probes.

The control reaction generates an additional product of 300 bases from Lambda DNA, detected with LightCycler® Red 690 labeled hybridization probes. This second PCR has no visible impact on the *Toxoplasma* 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 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 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.

Performance testing has been made with the ‘FastStart DNA Master HybProbe’ only.

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.

3. Set Contents

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

1 Standard row with 6 lyophilized standards *T. gondii* from 10^1-10^6 target equivalents per reaction
1 Sealing foil for the standard row

1 Vial with **white** cap containing Extraction Control Target (ECT): 2.0 x 10^6 copies (total)
1 Vial with **black** cap containing the stabilizer for preparation of the 'No Template Control' (NTC)

1 Certificate 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
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 the LightCycler® 480 Instruments.

Sensitivity
These reagents detect 10 copies of *T. gondii* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments (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 of *T. gondii* 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 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℃).
- Dissolved reagents can be long-term stored frozen at -20℃. Avoid 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 (PSR) and Control Reaction :

One reagent vial with a **green** cap contains primers and probes to run 32 reactions *T. gondii.*
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:

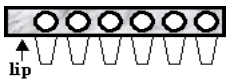
Before extraction add **10 µl** of ECT (vial **white** cap) to the sample; the amount may has to be adapted to the extraction method to get a Cp value in the range of 28-32. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

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**
6.2 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	5.7 µl
2.8 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.8 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

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 ColorCompensation Kit HybProbe 530/640/690.

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 *Toxoplasma gondii* data in channel 640 Quantification mode.
The negative control (NTC) must show no signal.

For the Control Reaction, view data in channel 705 Quantification mode. The negative control and the low-concentrated *Toxoplasma gondii* DNA samples (10 to 1,000 copies) should show an amplification curve with a Cp at approximately cycle 29.

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 *Toxoplasma gondii* should have Cp values between cycles 16 and 34.

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640 and channel F3 instead of channel 705 for detection (LightCycler software version 3.5.3).

7.3. Sample Data – Typical Results

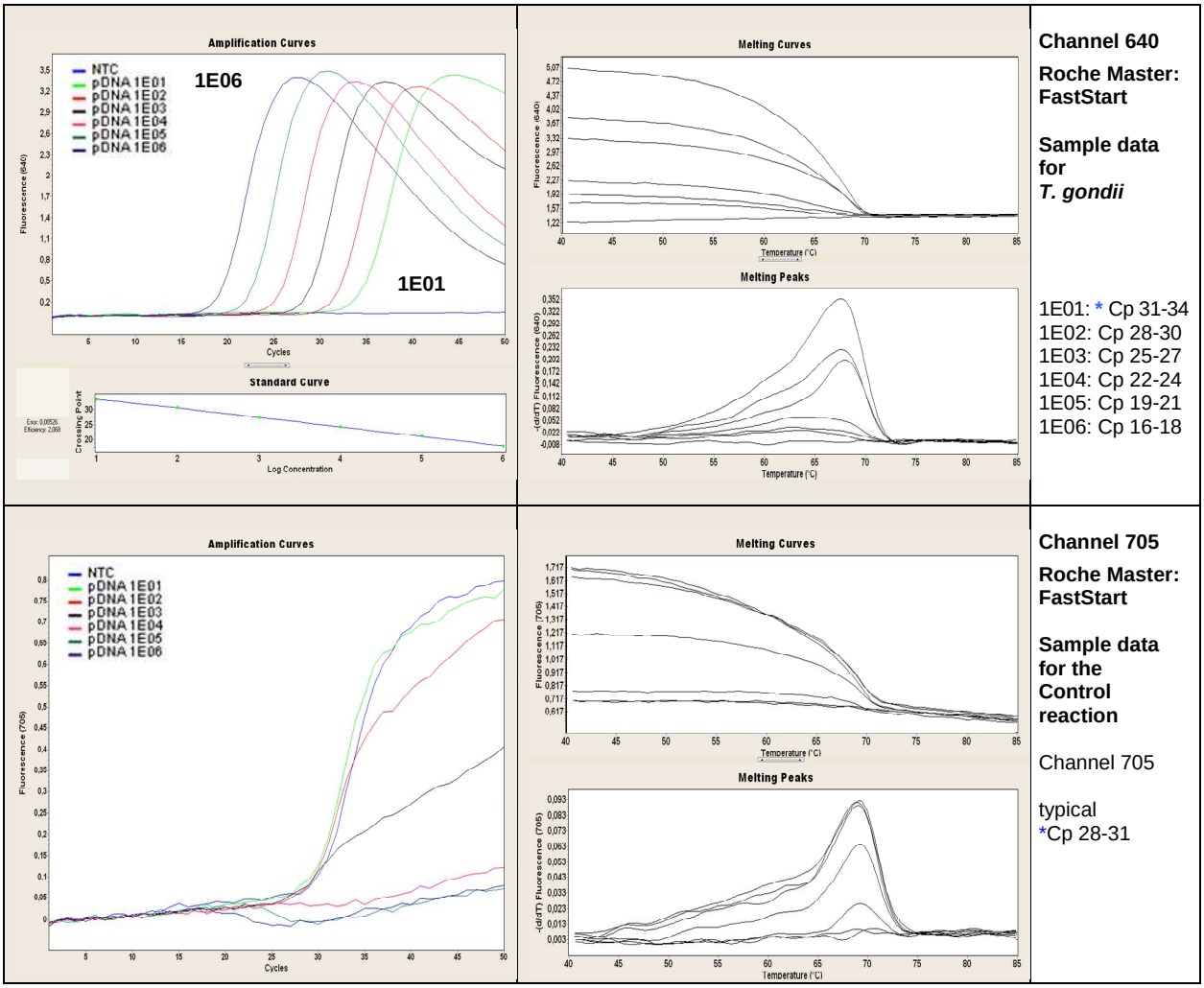


Fig.1. LightCycler® 2.0 sample data for the *Toxoplasma gondii* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *T. gondii*. Right panel channel 640 melting analysis for *Toxoplasma gondii* (not relevant for detection).
Lower panels: Left channel 705 quantification mode for the control reaction. Right 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 *Toxoplasma*	**Sample 705** *Ctrl. Reaction*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 36[+]	not relevant	amplification	negative	**Positive for *Toxoplasma***
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: 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: 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 'LightMix® Kit – ColorCompensation 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 *Toxoplasma gondii* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal.

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

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 *Toxoplasma gondii* should have Cp values between cycles 16 and 34.

8.3. Sample Data – Typical Results

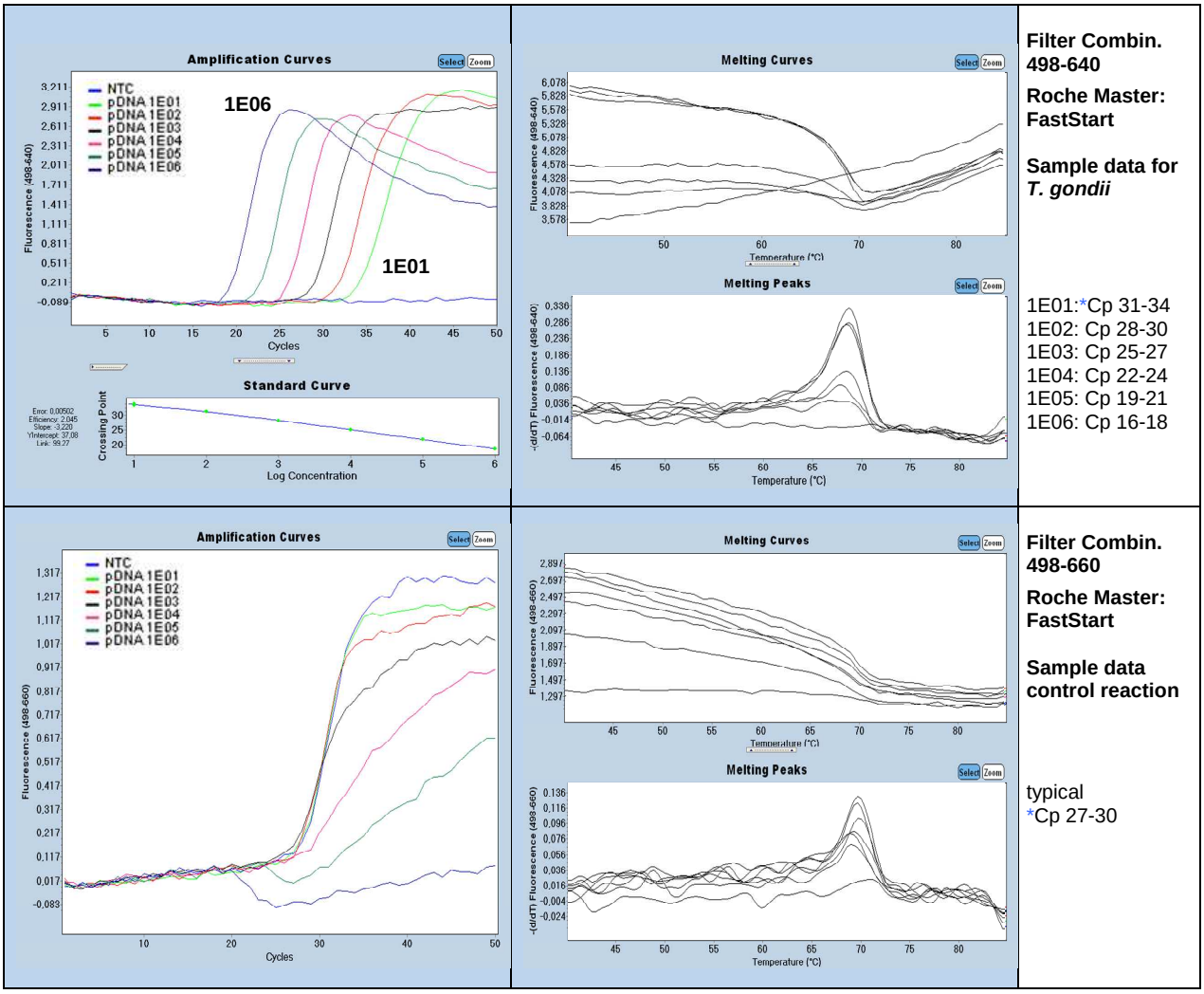


Fig.2. LightCycler® 480 II sample data for the *Toxoplasma gondii* detection system.

Upper panels: Left panel Filter Comb 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *T. gondii*. Right panel Filter Combination 498-640 melting analysis for *T. gondii* (not relevant for detection).
Lower panels:. Left panel Filter Comb 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 *Toxoplasma*	**Sample 660** *Ctrl. Reaction*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 36[+]	not relevant	amplification	negative	**Positive for *Toxoplasma***
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)

[+] 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. References

[1] Real-Time PCR for Diagnosis and Follow-Up of Toxoplasma Reactivation after Allogeneic Stem Cell Transplantation Using FRET Hybridization Probes. Costa, et al., JCM 38 (2000) 2929-2932

[2] Toxoplasma gondii Strains Defective in Oral Transmission Are Also Defective in Developmental Stage Differentiation. Fux et al., Infection and Immunity. 2007 May; 75(5): 2580–2590

[3] Quantitative detection of Toxoplasma gondii DNA in human body fluids by TaqMan polymerase chain reaction. Kupferschmidt et al., Clin Microbiol Infect 7 (2001) 120-124

[4] Development of a real-time PCR assay for detection of Toxoplasma gondii in pig and mouse tissues. Jauregui et al. JCM 39 (2001) 2065-2071

[5] Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. Reischl et al., BMC Infect Dis. 2003 May 2;3(1):7

[6] Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of T. gondii in aqueous humor. Jones et al., Invest Ophthalmol Vis Sci 2000 Mar;41(3):634-44

[7] Incidence and Diagnosis of Active Toxoplasma Infection Among Liver Transplant Recipients in Western Turkey. Caner et al., Liver Transplantation 14 (2008)

10. 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.

11. Version History

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

V120523	Release version replaced kit 40-0217-16 3 x 32 reactions; Internal Control included
V130813	MSDS included, Editorial changes
V150202	Internal Control changed to Extraction Control
V150505	Universal Extraction Control target nECT with Lambda and PhHV

Roche SAP order n° 05943663001

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Notice to Purchaser

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