LightMix® Kit *Pneumocystis jiroveci*

Cat.-No. 40-0578-32

Kit with reagents for the detection of *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II / Cobas® Z480 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°C), do N OT 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® Z480 Instrument see pages 6-7

1. Introduction

*Pneumocystis jiroveci (*formerly *Pneumocystis carinii)* is an ascomycetous fungus that causes opportunistic infections, particularly pneumonia, in patients with impaired immunity. Pneumocystis pneumonia (PCP) is the most prevalent opportunistic infection in patients with AIDS. Likewise, patients receiving immunosuppressive medication, patients with haematological or solid malignancies, transplant recipients and those with an altered immune system are also at risk of contracting PCP. Symptoms of PCP range from dyspnoea and dry cough to acute respiratory failure. Increasing evidence of human to human transmission supports an early diagnosis of the disease to ensure treatment with appropriate antibiotics.

P. jirovecii can not be cultured. Classical diagnosis is based on the microscopic examination of respiratory specimens. PCR diagnosis is based on the detection of the rRNA genes [1], the heat-shock protein 70B/SSB1, the DHFR gene [2] or the multicopy surface glycoprotein (MSG) gene [2,3].

[1] Detection of Pneumocystis carinii with DNA amplification. Wakefield et al. Lancet 336 (1990) 451–453

[2] Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing Pneumocystis carinii pneumonia. Larsen et al. J Clin Microbiol. 40 (2002) 490-4

[3] Inter-laboratory comparison of three different real-time PCR assays for the detection of Pneumocystis jiroveci in bronchoalveolar lavage fluid samples. Linssen et al., J.Med. Micro (2006)

The LightMix® Kit *Pneumocystis jiroveci* is based on the LightCycler® MSG PCR from Larsen et al.[2] and contains additional probes enhancing the signal intensity as kindly communicated by U. Reischl. The kit provides a fast system to identify this target in a nucleic acid extract obtained from bronchoalveolar lavage (BAL), oropharyngeal wash or induced sputum. A control amplification reaction acts as internal control (IC).

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

2. Description

An about 244 bp long fragment from the multicopy MSG gene from *P. jirovecii* is amplified with specific primers. The resulting PCR fragment is analyzed with two pairs of 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 control. This control does not interfere with the *P. jirovecii* specific reactions. The amplification will usually fail in the presence of higher concentrated *P. jirovecii* DNA samples (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, LC 1.x /2.0, or filter 660).

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

The supplied standard row allows to determine the linear range of the test and to estimate the quantity of the target sequence in unknown samples; due to the variability of the different MSG gene copies there is no accurate correlation between the amount of pathogen and the Cp value.

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

3 Vials with **green** cap containing pre-mixed lyophilized primers and probes for 32 PCR reactions each for the detection of *Pneumocystis jiroveci*
3 Vials with white cap containing the internal control (IC) reaction mix and target, 32 reactions

1 Vial with colorless cap containing the positive control DNA with 10^5 MSG target equivalents
Note : *P. jiroveci* contains approximately 80 MSG copies per genome

1 Standard row with 6 lyophilized standards of *P jiroveci* 10^1 to 10^6 MSG copies per reaction
Note : Iintended for single use only
1 Sealing foil for the standard row

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

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 II Instrument.

Sensitivity
These reagents detect 10 copies of *Pneumocystis jiroveci* 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 *Pneumocystis jiroveci* 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 o n 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

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 Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. 'High Pure PCR Template Prep. 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 (32 reactions):

One reagent vial with a **green** cap contains primers and probes to run 32 reactions for *Pneumocystis*.
One reagent vial with a **white** cap contains primers, probes and DNA to run 32 reactions for the IC.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (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 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 DNA 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. After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

Control DNA
Add 40µl PCR-grade water to each vial (8 x 10^5 target molecules) with a colorless cap. Mix the target DNA by pipetting the solution up and down 10 times (final concentration: 10^5 target molecules in 5 µl).

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage free ze at -20°C. Avoid repeated freezing thawing cycles. Please note that opening these vials may cause contaminations of the work-space (aerosol).

►**Use 5 µl** control DNA for a 20 µl PCR reaction

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.
Note : The sample volume can be increased by using respective less water in the master mix.

For use with the Roche FastStart Master	
Single reaction	**Component**
6.6 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)
2.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
2.0 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

Table 1

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 sample or standard/control DNA 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	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

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

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

If the internal control (IC) is used, view IC data in channel 705, Quantification mode. The negative control and the low-concentrated *Pneumocystis jiroveci* DNA samples (10 to 1,000 copies) should show an amplification curve for the 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 *Pneumocystis jiroveci* should have Cp values between cycles 17 and 37.

7.3. Sample Data – typical results

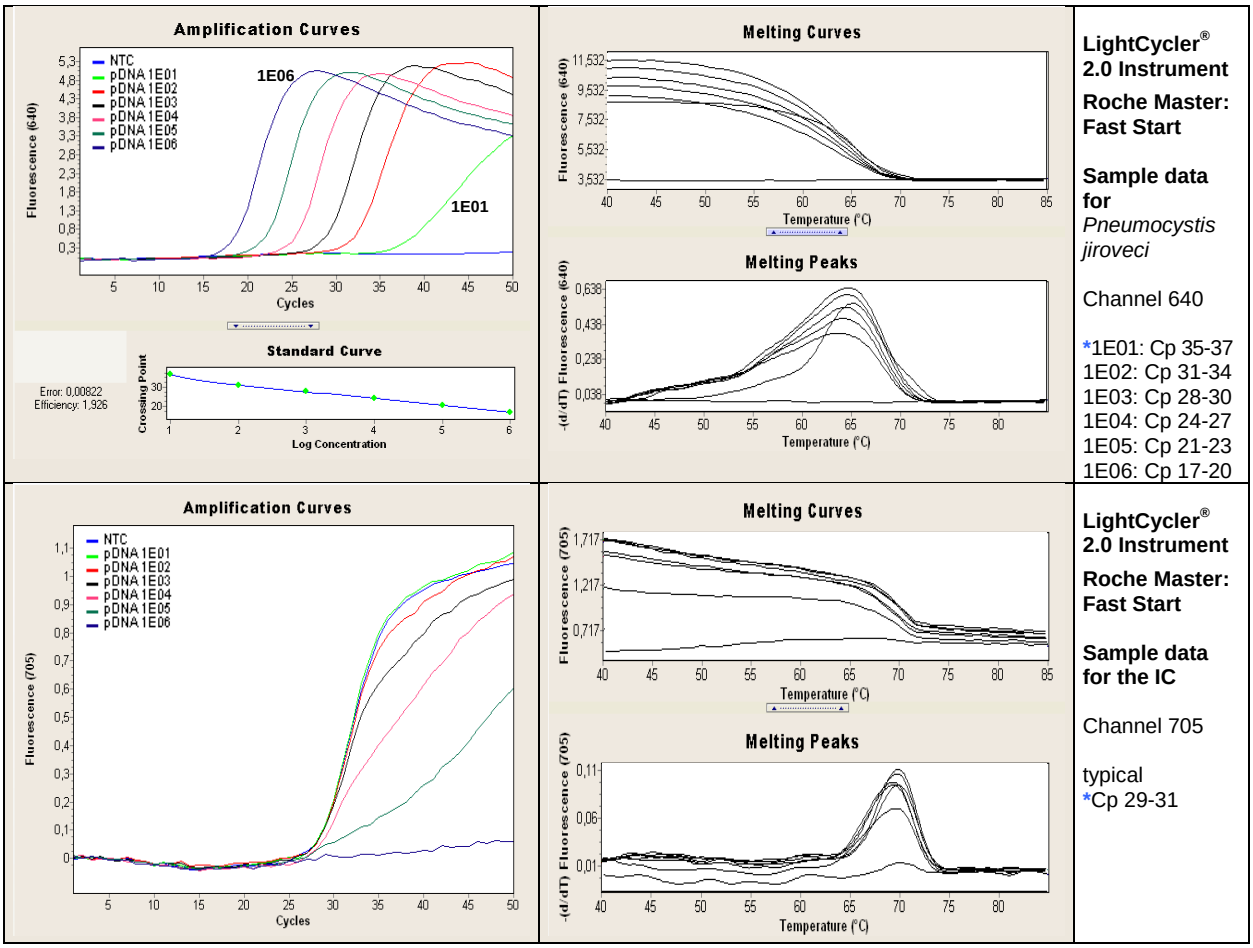

Fig.1. Sample data for the *Pneumocystis jiroveci* detection system.

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

Sample (640) ***Pneumocystis jiroveci***	**Sample (705)** **IC**	**Negative Copntrol** **NTC**	**Result** (Warning)
No amplification	Detectable	Negative	Negative (not detectable)
Cp < 38	Not relevant	Negative	*Pneumocystis* Positive
No amplification	Not detectable	Not relevant	PCR failure, repeat experiment
Amplification signal	Not relevant	Positive	Contamination, repeat experiment

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

8. LightCycler® 480 II / Cobas® Z 480 Instruments

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® Z480 Instrument: 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]	-	-	-	-	-	-	3*	-

(Melting not relevant for detection)

Table 4

8.2. Data Analysis

Note: Cobas® Z480 Instruments 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 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 *Pneumocystis jiroveci* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal.

If the internal control is used, view data with Filter Combination 498-660, Quantification mode and the IC with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Pneumocystis jiroveci* DNA samples (10 to 1,000 copies) should show an amplification curve for the 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 *Pneumocystis jiroveci* should have Cp values between cycles 17 and 35.

8.3. Sample Data – typical results

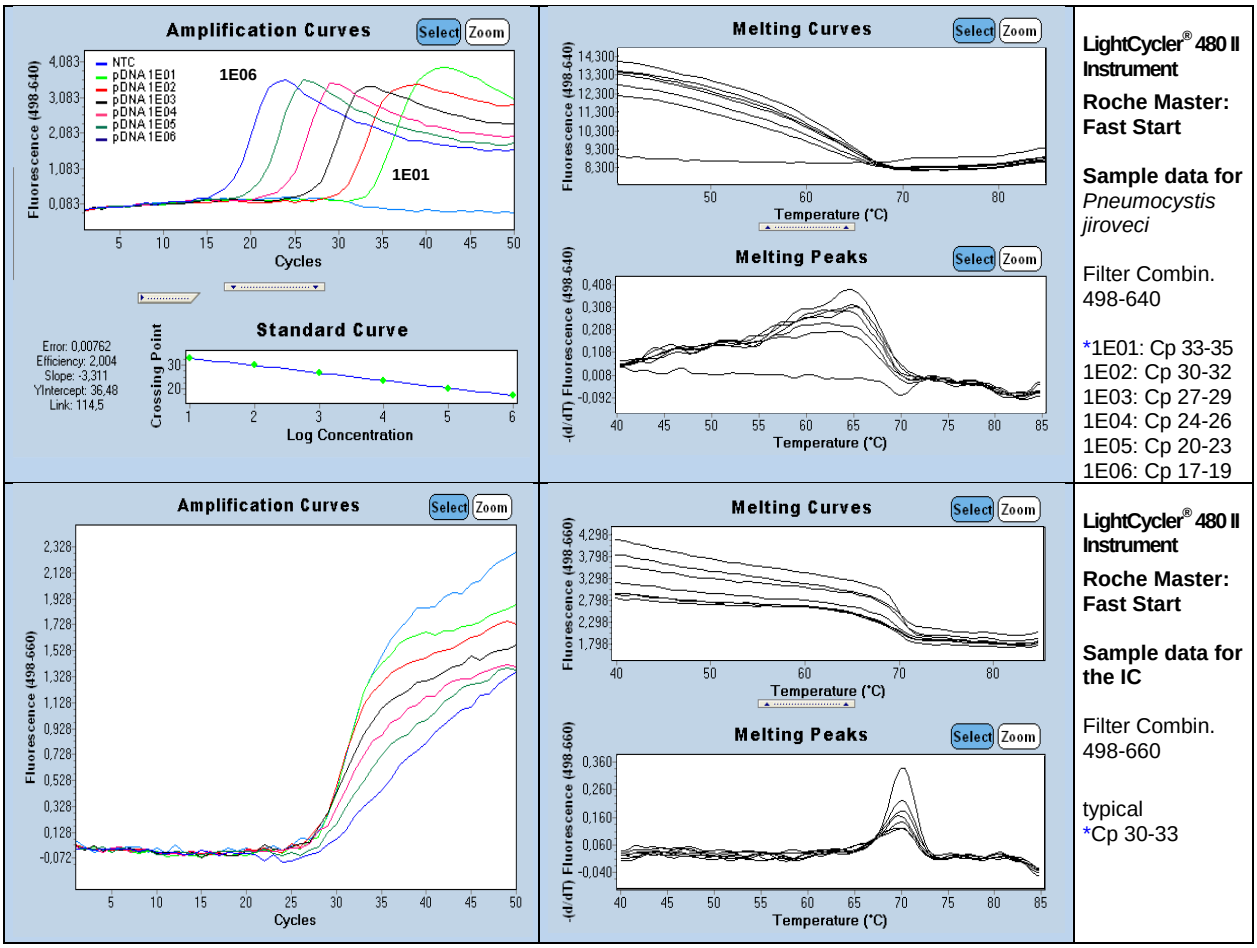

Fig.1. Sample data for the *Pneumocystis jiroveci* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *Pneumocystis jiroveci*. Right panel Filter Combination 498-640 melting analysis for *Pneumocystis jiroveci* (not relevant for detection).
Lower panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) with calibration curve/melting curves 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

Sample (640) ***Pneumocystis jiroveci***	**Sample (660)** **IC**	**Negative Copntrol** **NTC**	**Result** (Warning)
No amplification	Detectable	Negative	Negative (not detectable)
Cp < 37	Not relevant	Negative	*Pneumocystis* Positive
No amplification	Not detectable	Not relevant	PCR failure, repeat experiment
Amplification signal	Not relevant	Positive	Contamination, repeat experiment

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

9. Material Safety Data

According to OSHA 29CFR1910.1200, 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 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

Notes in red mark events require to change procedures

V100525	Release version
V100824	Editorial changes
V130813	Z480 included, Roche color compensation discontinued, MSDS included

Roche SAP order n° 06296840001

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