LightMix® Kit for the detection of *Yersinia pestis*

Cat.-No. 40-0251-16

Kit with reagents for the detection of *Yersinia pestis* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II / cobas z 480 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 / z 480 Instruments see pages 6-7

1. Introduction

Plague is a zoonotic infection caused by *Yersinia pestis* (*Y. pestis)*, a pleomorphic, and gram-negative nonspore-forming coccobacillus. It is usually transmitted by bites of the rat flea or through aerosols leading to outbreaks of highly infectious pneumonic plague with case fatality rates of 50-100 %.

Y. pestis has the potential of being used as a biological warfare agent.

The genus includes eleven species but only *Y. pest*is, Y. *pseudotuberculosis* and Y. *enterocolitica* are human pathogens. Most *Y. pestis* strains contain the three virulence plasmids pPla, pMT1 and pCD1.

The pPla plasmid encodes the plasminogen activator/coagulase (pla), a proteinase being important for the transmission through bites. The pMT1 plasmid encodes for the capsular antigen caf1, which is used for its detection, the murine toxin (mt), which is crucial for arthropod-borne transmission and for the highly immunogenic fraction capsule antigen F1; *Y. pestis* strains without F1 are very rare.
The pCD1 plasmid contains the genes for the *Yersinia* outer proteins (yop's) which are required for full virulence as tested in the mouse, as well as the virulence antigen lcrV, which is used for its detection. All human pathogenic members of *Yersinia* share the pCD1 plasmid.

The16S RNA does not allow differentiate between all species from the genus *Yersinia*. We use a parallel detection of 16S RNA as genomic marker and two of three plasmids as published elsewehere[1].

The LightMix® Kit *Yersinia pestis* detects the 16S RNA and at least one of two plasmid encoded targets enabling to identify *Y. pestis* in a nucleic acid extract. The kit contains an internal control (IC).

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

[1] Rapid detection of Yersinia pestis with multiplex real-time PCR assays using fluorescent hybridisation probes. Tomaso H, Reisinger EC, Al Dahouk S, Frangoulidis D, Rakin A, Landt O, Neubauer H. FEMS Immunol Med Microbiol. 38 (2003) 117

2. Description

A 304 bp fragment (16S rRNA), a 240 bp fragment (caf1) and a 287 bp fragment (lcrV) of the *Y. pestis* genome are amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (detected in channel 640). The PCR products are identified by running a melting curve with specific melting points (Tm) of 69°C (16S rRNA) and 61°C (caf1 and lcrV) in channel 640.

The PCR reaction is monitored by an additional PCR product of 278 bp, formed from the internal control (IC). The IC does not interfere with the *Y. pestis* specific reactions. The IC amplification will usually fail in the presence of higher concentrated *Y. pestis* DNA samples (1,000 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). The IC is supplied separately to allow running the assay in the presence or absence of the IC.

The use of a color compensation file generated with the 'LightMix® Kit - Color Compensation HybProbe´ 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.
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

6 Vials with **green** cap containing premixed primers and probes for each 16 PCR reactions
6 Vials with **white** cap containing the internal control (IC) for each 16 PCR reactions

1 Standard row with 6 lyophilized plasmid standards *Y. pestis* 10^1 to 10^6 target equivalents / rxn
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 Instruments)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instruments)	Cat.-No. 04 729 692 001

5. Product Characteristics

PCR results are obtained within 50 minutes (55 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (55 cycles and melting curve) with the LightCycler® 480 II Instrument.

Sensitivity
These reagents detect 10 copies of *Yersinia pestis* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments.

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Yersinia pestis* 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°C). 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°C).

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 and reagents for the IC (16 reactions):

One reagent vial with a **green** cap contains primers and probes to run 16 reactions for *Y. pestis.*
One reagent vial with a **white** cap contains primers, probes and DNA to run 16 reactions for the IC.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**Use 4 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 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. Older solutions can be used as a qualitative standard (positive control). Please note that opening these vials may cause contaminations of the work-space (aerosol).

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.

For use with the Roche FastStart Master	
Single reaction	**Component**
2.6 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)
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
4.0 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

To include the internal control **add 4 µl** of the IC reagent per reaction to the reaction mix.
To run the assay without the internal control substitute the 4 µl of IC with 4 µl PCR-grade water.

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 of sample or standard to each capillary or well for a final reaction volume of 20 µl.

Start run.

7. LightCycler® 1.x / 2.0 Instrument

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	55			1			1
Target [°C]	95	95	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:08	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
Acquisition Mode	None	None	Single	None	None	None	Continuous	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 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 *Yersinia pestis* data in channel 640 Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Yersinia pestis* data in channel 640 Melting Curves mode.

If the internal control (IC) is used view IC data in channel 705 Quantification mode. The negative control and the low-concentrated *Yersinia pestis* 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 and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Yersinia pestis* should have Cp values between cycles 18 and 35.

7.3. Sample Data – Typical Results

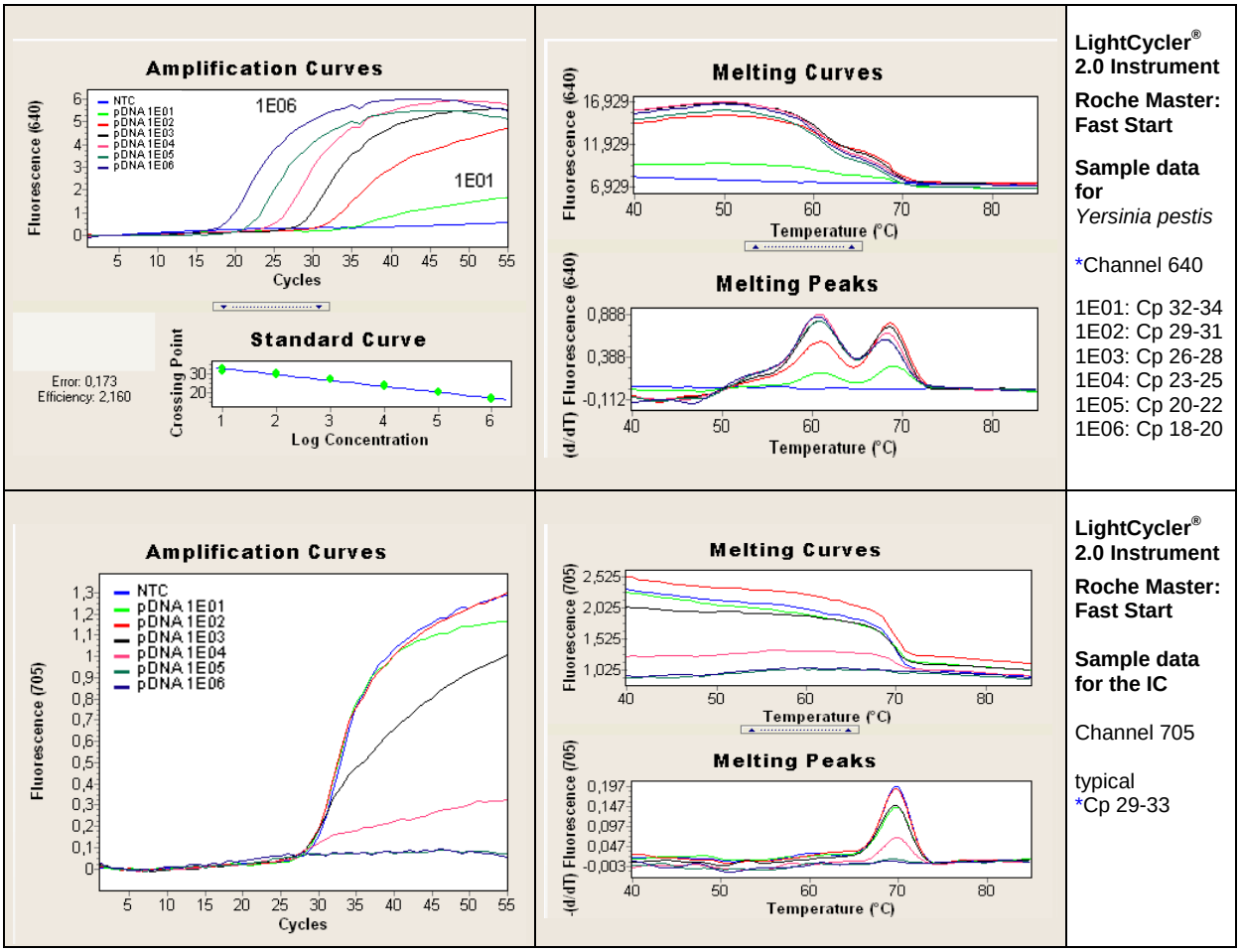

Fig.1. LightCycler® 2.0 sample data for the *Yersinia pestis* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *Y. pestis.* Right panel channel 640 melting analysis for *Y. pestis*. (not relevant for detection).
Lower panels: Left panel channel 705 quantification mode (Second Derivative Maximum) for the IC. Right panel channel 705 melting analysis/peaks for the IC.

* **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 **Quantification 640**	***Sample*** **Melting Analysis**	**Internal Control (sample) Quantification 705**	**NTC (control sample) Quantification 640**	**Result**
No amplification	no melting peak	detectable	negative	**Negative (not detectable)**
Cp < 36	2 peaks 61℃ +	not relevant	negative	**Positive for *Y. pestis***
Cp < 36	1 peak at 69℃ only	not relevant	negative	**Positive for *Yersinia***
No amplification	no melting peak	not detectable	not relevant	**PCR failure, repeat**
amplification signal	2 peaks 61°C &	not relevant	positive	**Contamination, repeat**

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 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	55			1			1
Target [°C]	95	95	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:08	00:00:15	00:00:30	00:2:00	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
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 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 *Y. pestis* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Y. pestis* data with Filter Combination 498-640, Melting Curves mode.

If the internal control is used, view data with Filter Combination 498-640, Quantification mode, and the IC with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Y. pestis* 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 *Y. pestis* should have Cp values between cycles 18 and 35.

8.3. Sample Data – Typical Results

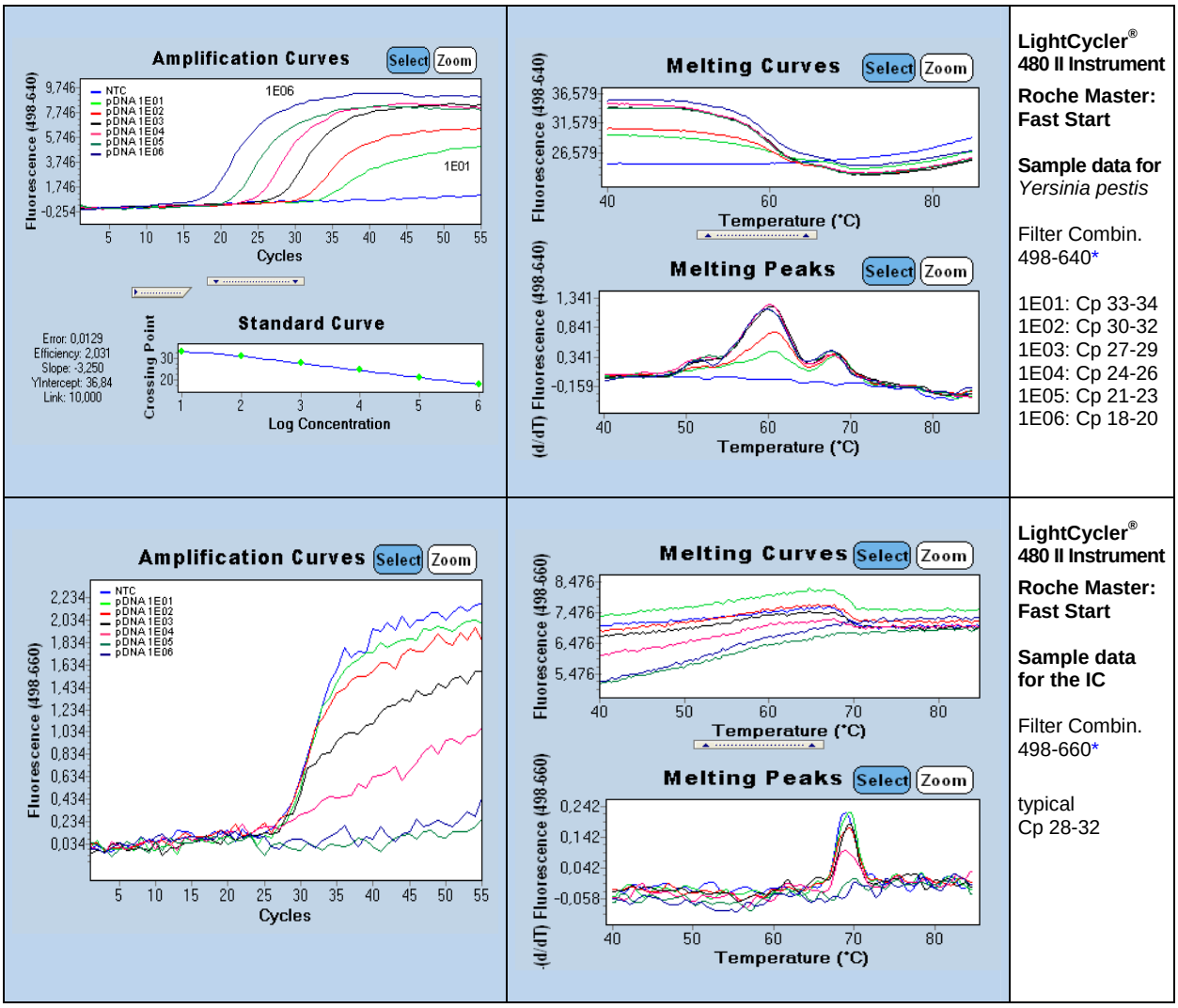

Fig.2. LightCycler® 480 II sample data for the *Y.pestis* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *Y. pestis.* Right panel Filter Combination 498-640 melting analysis for *Y. pestis* (not relevant for detection).
Lower panels: Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) 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 **Quantification 640**	***Sample*** **Melting Analysis**	**Internal Control (sample) Quantification 705**	**NTC (control sample) Quantification 640**	**Result**
No amplification	no melting peak	detectable	negative	**Negative (not detectable)**
Cp < 36	2 peaks 61℃ +	not relevant	negative	**Positive for *Y. pestis***
Cp < 36	1 peak at 69℃ only	not relevant	negative	**Positive for *Yersinia***
No amplification	no melting peak	not detectable	not relevant	**PCR failure, repeat**
amplification signal	2 peaks 61°C &	not relevant	positive	**Contamination, repeat**

Table 3. Typical analysis results (LightCycler® 480 II Instrument, Roche Master: Fast Start)

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.

Export: This product is under export control (Dual Use, Biological and Toxin Weapons Convention).

10. Version History

Notes in red mark events require to change procedures

V060719	Release version
V100908	Editorial changes
V130813	MSDS included, z 480 included, Roche color compensation removed

Roche SAP order n° 05997828001

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