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

TIB MOLBIOL

LightMix® Kit *Bordetella pertussis and parapertussis*
Cat.-No. 40-0575-32

Kit with reagents for the detection of *Bordetella pertussis and B. parapertussis* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 and 480 II 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 NOT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-6
Instructions for use with the LightCycler® 480 II / Cobas® Z480 Instrument see pages 6-7

1. Introduction

Pertussis (whooping cough) is a fatal respiratory disease in children caused by a bacterial infection with *Bordetella pertussis* - the mortality rate is 0.5% in infants under six months [1]. A milder form of the disease is caused by *B. parapertussis* while *B. holmesii* and *B. bronchiseptica* are rare in humans. Adults are the primary reservoir, because atypical or mild pertussis often remains unrecognized or is assumed to be related to other respiratory infections, in the absence of the characteristic cough. Since vaccination protects against pertussis most fatal cases are non- or incomplete vaccinated infants.

Diagnosis with immune fluorescence is limited to the first three weeks of the infection while serology and PCR based methods are reported be useful for an extended period of time. Typical clinical samples are nasopharyngeal or throat swabs. Simultaneous detection of *B. pertussis* and *parapertussis* using insertion sequences (IS) and the LightCycler® Instrument has been published by Reischl et al.[2]. Another common gene for the detection of *B. pertussis* is the Toxin ptxA promoter [3].

[1] http://www.health.vic.gov.au/ideas/bluebook/pertussis

[2] Real-time PCR assay targeting IS481 of Bordetella pertussis and molecular basis for detecting Bordetella holmesii. Reischl U, Lehn N, Sanden GN, Loeffelholz MJ. J Clin Microbiol 39 (2001) 1963-1966

[3] Comparison of serological and real-time PCR assays to diagnose Bordetella pertussis infection in 2007. André P, Caro V, Njamkepo E, Wendelboe AM, Van Rie A, Guiso N. JCM 46(5):1672-7 (2008)

The LightMix® Kit *Bordetella pertussis and parapertussis* provides a fast, easy and accurate system to identify these targets in a nucleic acid extract. A control amplification reaction acts as internal control (IC).

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 II (96 well and 384 well formats) Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

2. Description

Detection of *Bordetalla pertussis* as well as the rare *B. holmesii* and *B. bronchiseptica* is achieved by amplification of a 130 bp fragment from the IS481 region using hybridization probes with LC640 label while *B. parapertussis* as well as *B. bronchiseptica* is detected using a 97 bp fragment from the IS1001 region using probes with a LC690 label. The amplification from the internal control (IC) target is detected with very short probes with LC640 label, resulting in a specific melting peak at 49.5°C without interering signals in the quantification analysis.

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 run the multiplex reaction.

The supplied standard rows allow to determine the linear range of the reaction and to estimate the quantity of the target sequences 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

3 Vials with green caps containing lyophilized primers and probes for 32 PCR reactions *Bordetella*
3 Vials with white caps containing the internal control (IC)

1 Standard row with 6 standards of *Bordetella pertussis* from 10^1 to 10^6 target equivalents per rxn
1 Standard row with 6 standards of *B. parapertussis* from 10^1 to 10^6 target equivalents per rxn
2 Sealing foils for the standard rows

1 Vial with colorless cap containing control DNA *Bordetella pertussis*, 10^5 copies per reaction
1 Vial with colorless cap containing control DNA *Bordetella parapertussis*, 10^5 copies per reaction

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

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 *Bordetella pertussis / Bordetella parapertussis* 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 *B. pertussis / parapertussis* DNA using the '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 Viral Nucleic Acid 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).

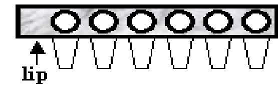
6.1. Preparation of parameter-specific reagents and reagents for the IC (32 reactions):

One reagent vial with a green cap contains primers and probes to run 32 reactions for *Bordetella.*
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.

6.2. Preparation of the standard row and the control DNA



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. 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℃, for long term storage free ze at -20℃. 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.

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

To include the internal control **add 2 µl** of the IC reagent per reaction to the reaction mix.
To run the assay without the internal control substitute the **2 µl** of IC with 2 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 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	Continuous	None

Table 2

7.2. Data Analysis

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).

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 *Bordetella pertussis* data in channel 640 and *Bordetella parapertussis* data in channel 705, Quantification mode. The negative control (NTC) must show no signal.

If the internal control (IC) is used view IC data in channel 640. Melting curve mode: no amplification signal will ever appear in Quantification mode. The negative control and the low-concentrated *Bordetella pertussis and Bordetella parapertussis* DNA samples (10 to 100 copies) should show a melting curve for the IC with a Tm of 49.5°C.

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 *Bordetella pertussis* and *Bordetella parapertussis* should have Cp values between cycles 18 and 35.

7.3. Sample Data – Typical Results

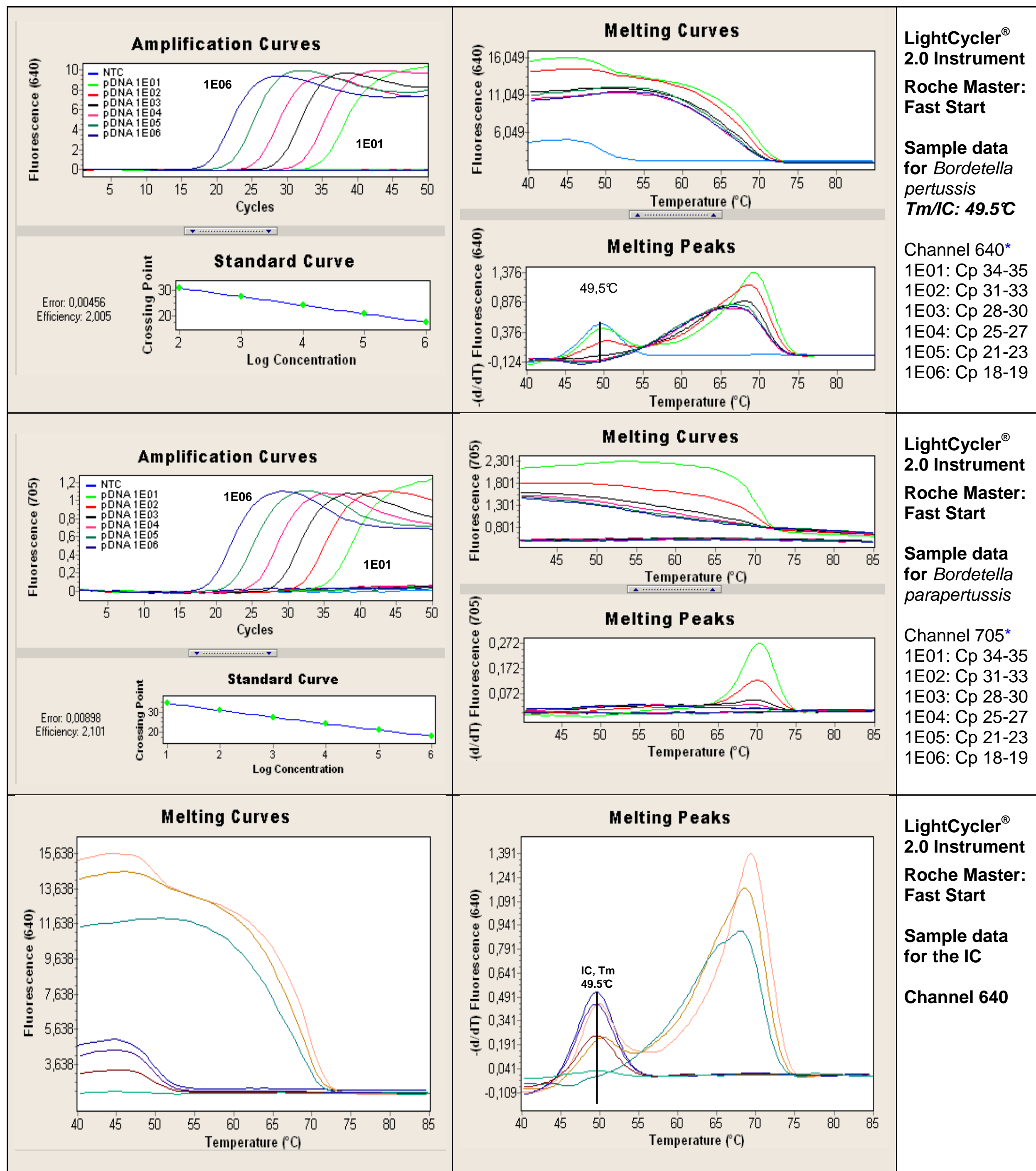


Fig.1. Sample data for the *Bordetella pertussis and parapertussis* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) for *Bordetella pertussis.* Right panel channel 640 melting analysis for *Bordetella pertussis.*
Central panels: Data from LightCycler® 2.0 Instrument. Left panel channel 705 quantification mode (Second Derivative Maximum) for *Bordetella parapertussis*.. Right panel channel 705 melting analysis for *Bordetella parapertussis.*
Lower panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 melting curves for *Bordetella pertussis* and IC. Right panel channel 640 melting peaks for the IC at 49.5°C.

* **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. 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	80	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:01:00	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**	**-**

Table 3

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. The Fit Points method is more-error prone due to the user's influence.

View *Bordetella pertussis* data with Filter Combination 498-640 and *Bordetella parapertussis* data with Filter Combination 498-660, Quantification mode / "Tm Calling" Analysis mode. The negative control (NTC) must show no signal.

If the internal control is used, view IC data with Filter Combination 498-640, melting curve. The negative control and the low-concentrated *Bordetella* DNA samples (10 to 100 copies) should show a melting curve for the IC at 49.5°C.

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 *B. pertussis and B. parapertussis* should have Cp values between cycles 18 and 35 (Cp values calculated with Second Derivative Maximum method).

8.4. Sample Data – Typical Results

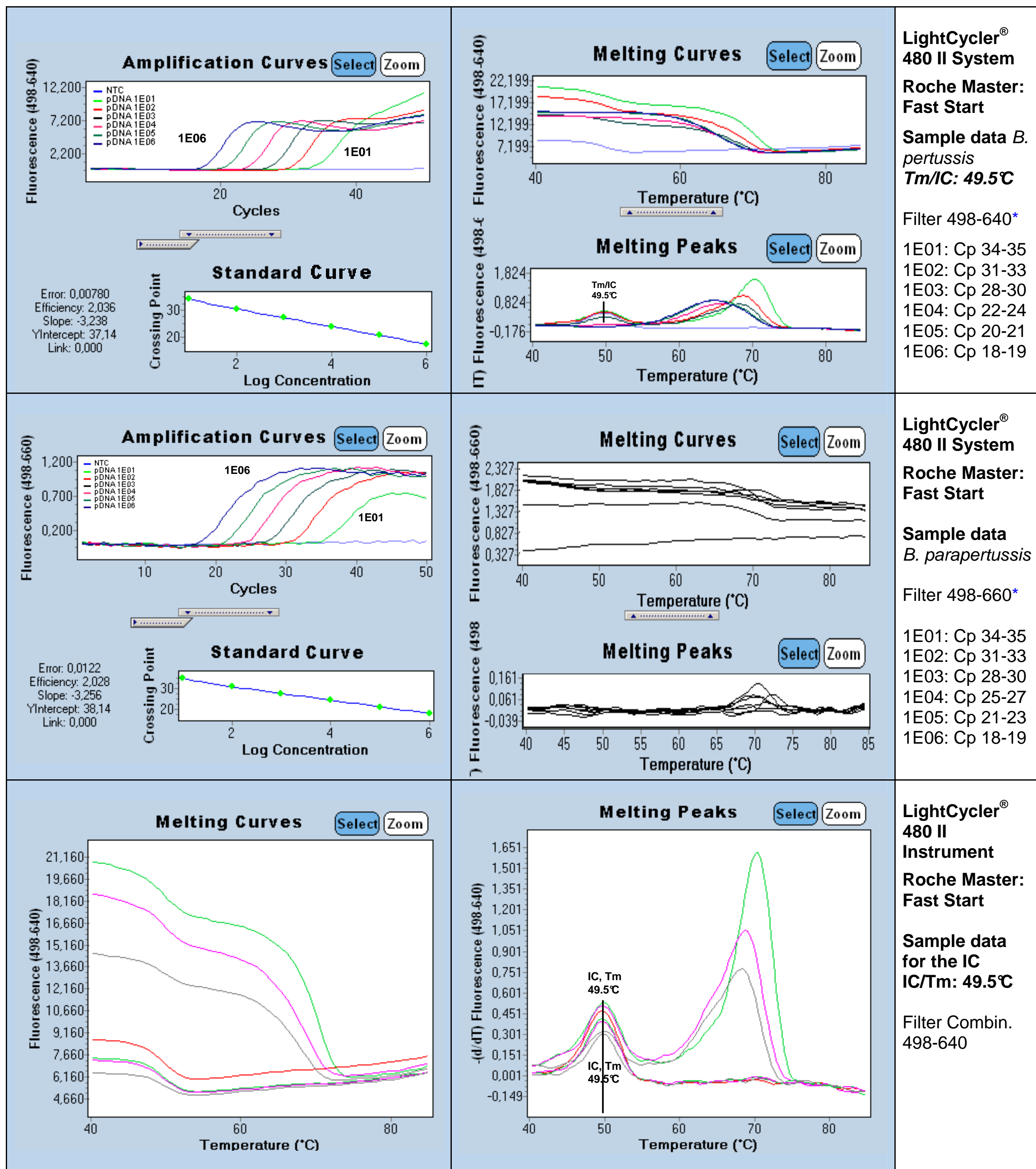


Fig.2. Sample data LC480 systems.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum for *Bordetella pertussis*. Right panel Filter Combination 498-640 melting analysis for *Bordetella pertussis.*
Central panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) for the *Bordetella parapertussis.* Right panel Filter Combination 498-660 melting analysis for the *Bordetella parapertussis* (not relevant for detection).
Lower panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 melting curves for *Bordetella pertussis* and the IC. Right panel Filter Combination 498-640 melting peaks for the IC at 49.5°C.

* **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.

9. Interpretation of Data

Read channels 640 and 705 (capillary) or 498-640 and 498-660 (LC480 II) in the Quantification mode. The positive controls must yield amplification signals while the negative control must stay negative. Analyze all unknowns to identify *Bordetella* positive and negative samples (**bold**).

Verify negative samples for the function of the Internal Control (IC): Read channel 640 (498-640), Melting Curve mode. Negative samples must show a melting peak for the internal control (green).

Sample testing Bordetella		*Sample IC*	Control Reaction: Positive Control		Negative Control (NTC)		**Sample Results (Warnings)**
640 (F2) 498-640	705 (F3) 498-660	640 (F2) 498-640	640 (F2) 498-640	705 (F3) 498-660	640 (F2) 198-640	705 (F3) 498-660	See also specificaion in Section 2 Description
no signal	**no signal**	**melting peak**	signal Cp 20-23	signal Cp 20-23	negative	negative	**Negative for *Bordetella* (*Bordetella* not detectable)**
positive	**no signal**	not relevant	signal Cp 20-23	signal Cp 20-23	negative	negative	**IS481 positive probably *B. pertussis***
no signal	**positive**	not relevant	signal Cp 20-23	signal Cp 20-23	negative	negative	**IS1001 positive probably *B. parapertussis***
positive	**positive**	not relevant	signal Cp 20-23	signal Cp 20-23	negative	negative	**Double infection or pos. *B. bronchiseptica***
no signal	**no signal**	**no peak**	signal Cp 20-23	signal Cp 20-23	not relevant	not relevant	**PCR failure**, repeat experiment/ sample preparation
not relevant	not relevant	not relevant	no signal	no signal	not relevant	not relevant	**PCR failure**, repeat experiment
not relevant	not relevant	not relevant	not relevant	not relevant	positive	positive	**Contamination**, repeat experiment

Table 4. Typical analysis results (Roche Diagnostics Master: FastStart)

10. 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.

11. Version History

Notes in red mark events require to change procedures

V100825 Kit released for LightCycler® 480 II instruments
V110616 Change to 32 rxn per vial
V130801 Editorial changes, correction data in 7.2 and 8.2 (melting curve IC), Interpretation

Roche SAP order n° 05945267001

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