LightMix® Kit human MPV

Cat.-No. 40-0184-16

Kit with reagents for the quantitative detection of the *human MPV* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 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 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 Instrument see pages 6-7

1. Introduction

The *human Metapneumovirus* (*MPV*) from the family *Paramyxoviridae* is a minus strand ssRNA virus. Sequence alignments suggest that this virus may has its natural pool in bats (personal communication Dr. C. Drosten, Bonn). MPV is besides the *respiratory syncytial virus* (RSV) the second most common respiratory virus worldwide and appears seasonal like influenza. Patients have acute lower respiratory tract infections and show symptoms ranging from wheeze to bronchiolitis; in fatal cases they even may require assisted ventilation. Up today there is no therapy established.

Routine diagnosis uses immune fluorescence detection of the viral antigens, cell culture or RT-PCR. Preferred targets for Real-Time-PCR detection are the polymerase[1], the fusion gene[2] or the N[2] gene.

The LightMix® Kit for the detection of cDNA from *MPV* provides a fast, easy and accurate system to identify and quantify cDNA of *MPV* after reverse transcription of the *MPV* minus strand RNA genome.

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 II Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'. An 1-step RT PCR procedure has not been tested.

[1] Comparative evaluation of real-time PCR assays for detection of the human metapneumovirus. Cote S, Abed Y, Boivin G. JCM 41 (2003) 3631-3635

[2] A Sensitive, Specific, and Cost-Effective Multiplex Reverse Transcriptase-PCR Assay for the Detection of Seven Common Respiratory Viruses in Respiratory Samples. Syrmis et al., J. Mol. Diagnostics, Vol.6 , 125-131

[3] Molecular assays for detection of human metapneumovirus. Mackay IM, Jacob KC, Woolhouse D, Waller K, Syrmis MW, Whiley DM, Siebert DJ, Nissen M, Sloots TP. JCM 41 (2003) 100-105

2. Description

This LightMix® kit detects the N gene of *MPV* indicating the presence of *MPV* in a nucleic acid extract obtained from nasopharyngeal swabs. The kit includes a control of amplification (internal control, IC).

A PCR product of up to 300 bp lenght gene is amplified with N gene specific primers and detected with probes labeled with LightCycler® Red 640 (detected in channel 640). 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.

An additional PCR product of 278 bp is formed from the internal positive control DNA. This control will not interfere with the *MPV* specific reactions. The amplification of the control will usually fail in the presence of higher concentrated *MPV* samples (1,000 - 10,000 copies or higher) but it will display an amplification signal in negative and low-concentrated samples. The probes are labeled with the dye LC690. Detection is achieved in channel 705. The IC is supplied separately to allow running the assay with or without IC

The use of a color compensation file generated with the TIB Molbiol LightMix® Kit - Color Compensation 530/640/690' (or Roche Diagnostics 'LightCycler®-Color Compensation Set' or with the Roche Diagnostics 'LightCycler® Multicolor Demo Set') is a prerequisite to run the duplex reaction.

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 blue caps containing premixed lyophilized primers, and probes for 16 PCR reactions each of *human MPV*
6 Vials with white caps containing the internal control (IC)
1 Standard row with 6 lyophilized cloned plasmid standards of *human MPV* from 10^1 to 10^6 target equivalents per reaction
1 Sealing foil for the standard row

4. Additional reagents and items required

TIB MOLBIOL:

LightMix® Kit – Color Compensation 530/640/690	Cat.-No. 40-0318-00

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Multicolor Demo Set	Cat.-No. 03 624 854 001
or LightCycler® Color Compensation Set (LightCycler® 1.x Instrument)	Cat.-No. 12 158 850 001
High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
Transcriptor First Strand cDNA Synthesis Kit	Cat.-No. 04 379 012 001
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 Instrument.

Sensitivity
These reagents detect 10 copies of *human MPV* cDNA 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 *human MPV* 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 3 months after shipment when stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 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 LightCycler® Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure RNA Isolation Kit' combined with Roche Diagnostics 'Transcriptor First Strand cDNA Synthesis 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 **blue** cap contains primers, and probes to run 16 LightCycler® reactions for *human MPV*
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 to 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. 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).

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 FastStart 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

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 Instruments).

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 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 [℃]	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 [℃/s]	20	20	20	20	20	20	0.2	20
Sec Target [℃]	-	-	55	-	-	-	-	-
Step Size [℃]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

(Melting not relevant for detection)

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 Color Compensation kit.

For the LightCycler 1.x Instruments use the 'LightCycler® – Color Compensation Kit' or the 'LightCycler® Multicolor Demo Set (Roche).

For the LightCycler 2.0 Instrument use the LightMix® Kit –Color Compensation 530/640/690' kit. If using the standard Roche color compensation the LC640 derived signals from the viral amplification might be visible in the LC705 channel.

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 *MPV* 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. The negative control and the low-concentrated *MPV* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 27.

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 *MPV* should have CPs between cycles 18 and 37 (CPs calculated with Second Derivative Maximum method).

7.3. Sample Data – typical results

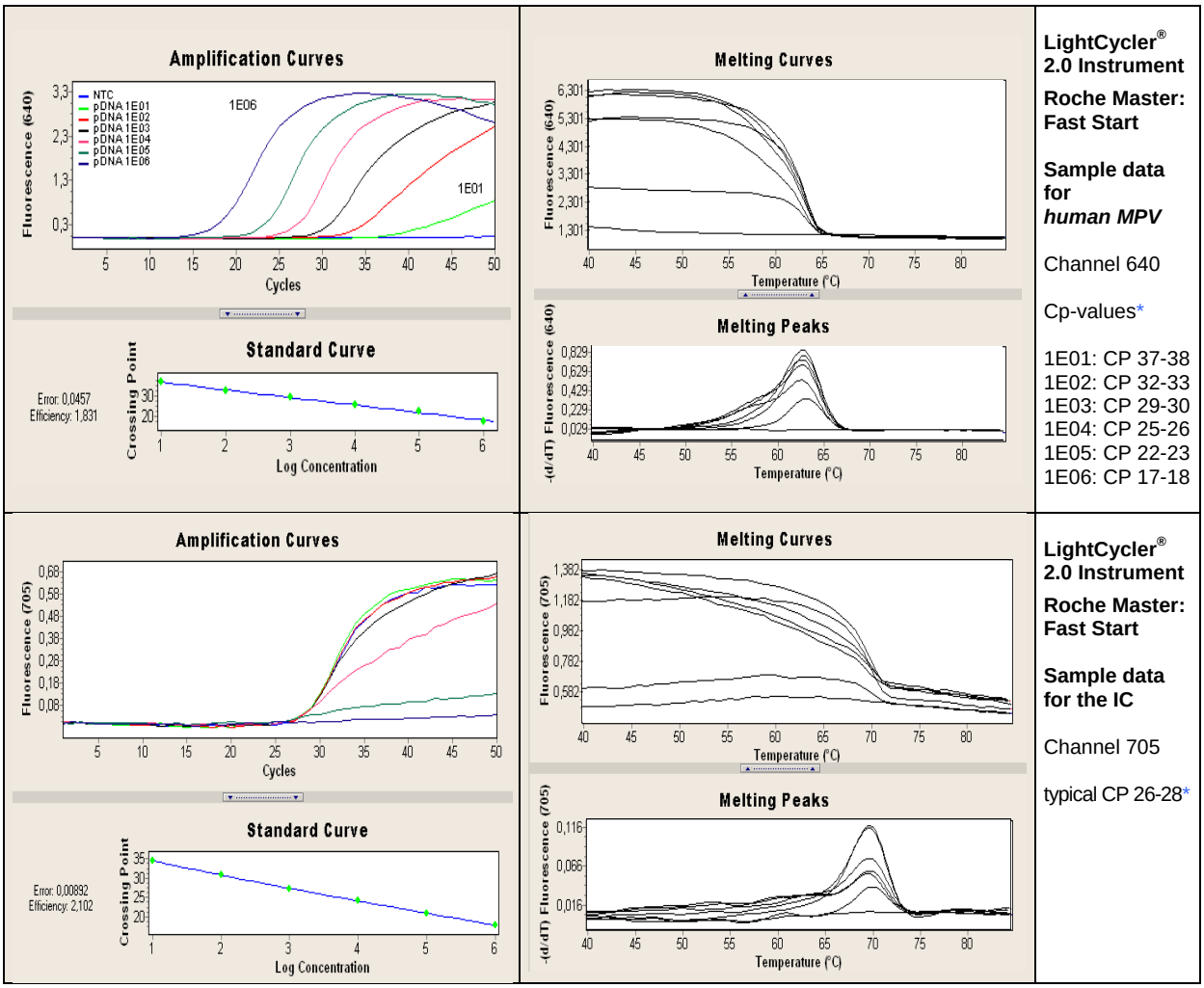

Fig.1. Sample data for *human MPV* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with standard curve for human MPV. Right panel channel 640 melting analysis for human MPV (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.

* **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

***Human MPV* (sample)**	**IC (sample)**	**PositiveControl**	**Negative Control (NTC)**	**Result (warnings)**
640 (F2)	**705 (F3)**	640 (F2)	640 (F2)	
no amplification	detectable	amplification	negative	**Negative (not detectable)**
amplification signal	not relevant	amplification	negative	**Positive**
no amplification	not detectable	amplification	not relevant	sample preparation 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

Tab. 3. Typical analysis results

8. LightCycler® 480 II Instrument

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

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	-

(Melting not relevant for detection)

8.2. 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 the Roche Diagnostics 'LightCycler® Multicolor Demo Set' or TIB MOLBIOL 'LightMix® Kit – Color Compensation 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 *MPV* 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 498-640, Quantification mode and the IC with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *MPV* 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 *MPV* should have CPs between cycles 18 and 35 (CPs calculated with Second Derivative Maximum method).

8.3. Sample Data – typical results

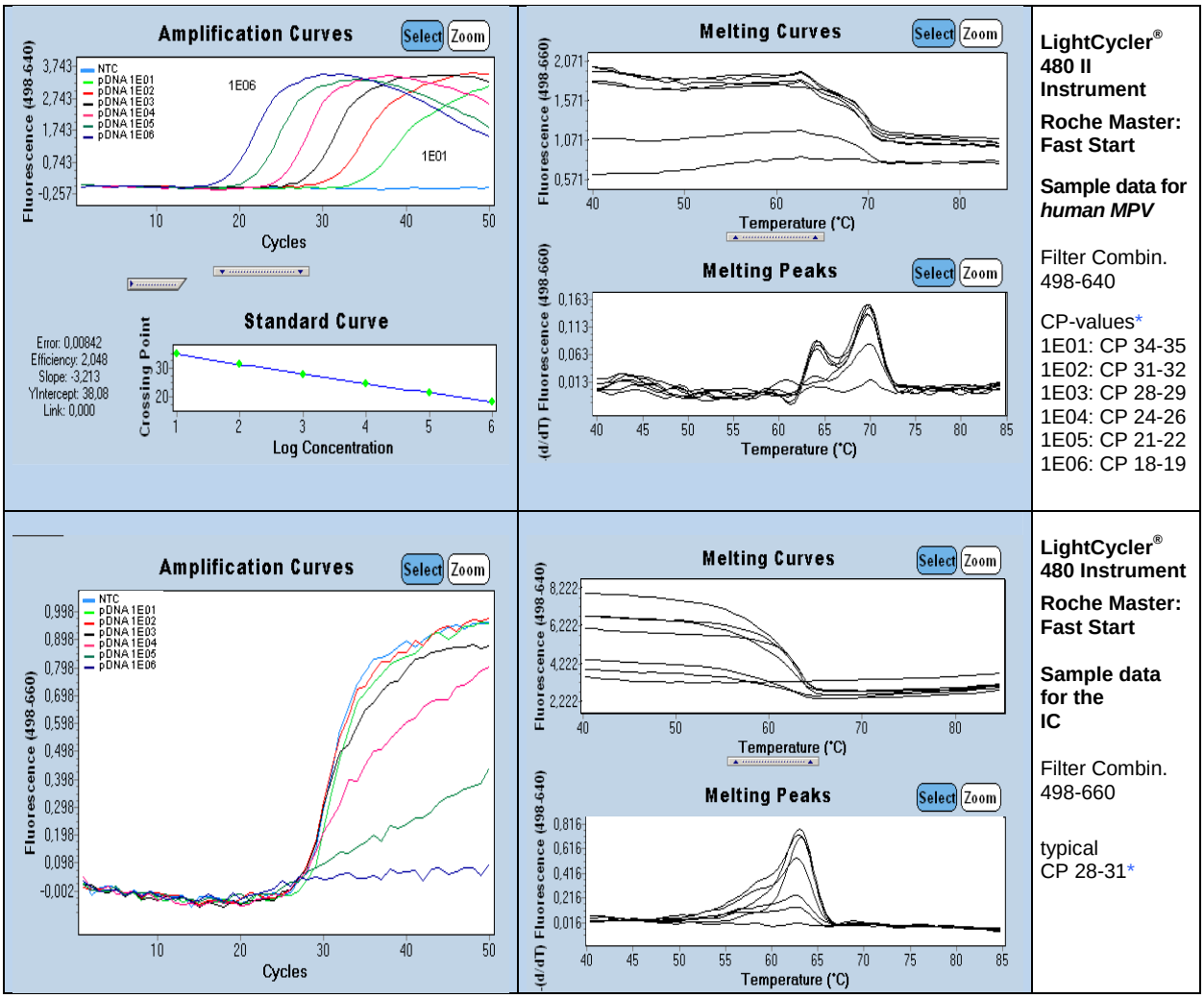

Fig.1. Sample data for the *human MPV* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with standard curve *human MPV*. Right panel Filter Combination 498-640 melting analysis for *human MPV* (not relevant for detection).
Lower panels: Data from LightCycler® 480 II Instrument. 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

***Human MPV* (sample)**	**IC (sample)**	**PositiveControl**	**Negative Control (NTC)**	**Result (warnings)**
498-640	**498-660**	498-640	498-640	
no amplification	detectable	amplification	negative	**Negative (not detectable)**
amplification signal	not relevant	amplification	negative	**Positive**
no amplification	not detectable	amplification	not relevant	sample preparation 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

Tab. 3. Typical analysis results

9.0 Version history

V-111117	**Introduction of version history**

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

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