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

TIB MOLBIOL

LightMix® Kit *Influenza A Virus M2*

Cat.-No. 40-0234-16 **H3N2 (2015) adapted**

Real-Time-PCR Kit with reagents for the detection of *Influenza A* cDNA 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°C), 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

Influenza A is a negative strand ssRNA virus from the Orthomyxovirus family which infects birds and mammals. It is characterized by the **H**emagglutinin (H or HA) and **N**euraminidase (N or NA) genes.

Influenza A viruses lack mechanisms for proofreading and repair of errors occurring during replication; the genetic variability helps the virus to elude the host defense and explains why low pathogen viruses can occasionally mutate after a short time circulating in a host population into high pathogenic viruses. Furthermore, the virus genome consists of eight separate segments which can rearrange in case of a multiple infection of a host, giving rise to completely new virus variants.

An infection with Influenza (flu) generally affects the upper respiratory tract, causing a wide spectrum of symptoms from mild to severe illness with cough, high fever, fatigue and pneumonia, and can be fatal, particularly for elderly individuals. The virus is commonly transmitted through aerosols. Infections spread around the world in seasonal epidemics - typically during the winter season.

The Spanish flu 1918/19, a type H1N1 virus, was responsible for an estimated 10-50 million casualties worldwide. After 2004 an Asian H5N1 bird flu was accountable for many fatalities. In 2009/10 the world experienced a pandemic caused by the H1N1 sw virus. After a low 2011/12 Influenza season the numbers increased 2012/13, with remarkable more and also severe H3N2 infections. The 2014/15 circulating H3N2 virus is reported to have sequence changes in commonly targeted gene regions.

The genetically relative stable matrix protein gene 2 (M2 or MP) is the preferred target for RT-PCR based tests for Inf*A* [1-4]. This kit detects a part of the *M2* gene indicating the presence of the virus in a nucleic acid extract (obtained from viral RNA isolated from nasal swabs or other materials followed by reverse transcription). A control amplification reaction acts as internal control (IC).

In addition to this product we offer LightMix® kits for the specific detection of the N1 (H5N1) gene, H5 (Asia H5N1), and for the H1 (H1N1 sw) gene, which can be used for the identification of specific strains.

2. Description

This kit provides a fast and accurate system to detect *Influenza A* cDNA in a nucleic acid extract. A 113 bp long fragment of the *M2* gene is amplified with specific primers and detected with LightCycler® Red 640 labeled hybridization probes (channel 640).

The control reaction generates an additional product of 318 bases detected with LightCycler® Red 690 labeled hybridization probes. This second PCR has no visible impact on the *InfA* specific reaction and will even fail in the presence of higher amounts of *InfA* target (1,000 copies and more).

The use of a color compensation file generated with the ColorCompensation kit 40-0318 is a prerequisite to run the reaction *IfnA* and control. 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.

This manual describes the two-step RT PCR procedure only, starting with cDNA.
Performance testing has been made with the 'FastStart DNA Master HybProbe' using cDNA only.

This kit has been evaluated to detect the 2009 H1N1 pandemic virus. According to BLAST analysis the kit will detect the 2012/13 as well as the 2014/15 seasonal H3N2 virus.

3. Set Contents

6 Vials with **blue** cap containing premixed lyophilized primers and probes for 16 reactions
6 Vials with **white** cap containing lyophilized primers and probes for 16 control reactions (IC)

1 Standard row with 6 lyophilized standards of *InfA* from 10^1 to 10^6 target equivalents / rxn
1 Sealing foil for the standard row

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 for detection. We recommend upgrading to SW version 4.1.

4.1. Optional Additional Reagents

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® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001

5. Product Characteristics

PCR results (activation, 50 cycles and melting curve) are obtained within 50 minutes with the capillary based LightCycler® 1.x / 2.0 Instruments and within 80 minutes with '480' plate based Instruments.

Sensitivity
These reagents detect 10 copies of *Influenza A c*DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' (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 *Influenza A* cDNA using the Roche 'LightCycler® FastStart DNA Master HybProbe'.

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 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°C).

6. Experimental Protocol

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 (PSR) and control reaction (IC):

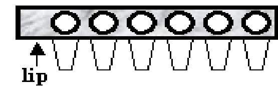
One reagent vial with a **blue** cap contains primers and probes to run 16 reactions *InfA*.
One reagent vial with a **white** cap contains primers and probes to run 16 reactions control reaction.

Check for the colored pellet, then **add 66 µl** PCR-grade water, mix (vortex) and spin down.

►**Use 4 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least ten days when stored refrigerated at 4℃. 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 ten times up and down.

►**Use 5 µl** standard for a 20 µl PCR reaction.

This dissolved standard row is not long-term stable and is intended for single use only. For further runs use the Positive Control.
After adding the target DNA to the reaction mix, use the provided sealing foil to close the vials in order to avoid contaminations.

6.3. 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 :

For use with: LightCycler® FastStart DNA Master HybProbe		**For use with: LightCycler® FastStart DNA Master PLUS HybProbe**	
Single reaction	**Component**	**Component**	**Single reaction**
2.6 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart kit)	water, PCR-grade (colorless cap, provided with the Roche FastStart PLUS kit)	3.0 µl
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.**)	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)	4.0 µl
4.0 µl	**IC** mix (IC reagents containing primers, probes and DNA, see **6.1.**)	**IC** mix (IC reagents containing primers, probes and DNA, see **6.1.**)	4.0 µl
2.0 µl	Roche Master (red cap, for preparation see Roche manual)	Roche Master (red cap, for preparation see Roche manual)	4.0 µl
15.0 µl	Volume of reaction mix	Volume of reaction mix	**15.0 µl**

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** 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 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 [℃]	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) 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 the 'LightMix® Kit – ColorCompensation HybProbes'.

Perform data analysis, as described in the 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 *InfA* data in channel 640, Quantification mode. The negative control must show no signal.

For the Control Reaction, view data in channel 705, Quantification mode. The negative control and the low-concentrated *InfA* cDNA samples (10 to 1,000 copies) should show an amplification curve for the IC/EC with a Cp value at approximately cycle 28-30.

The provided standard row with 10^6 copies/rxn to 10 copies/rxn of *InfA* should have Cp values between cycles 18 and 37 (Cp values calculated with Second Derivative Maximum method).

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

7.3. Sample Data – Typical Results

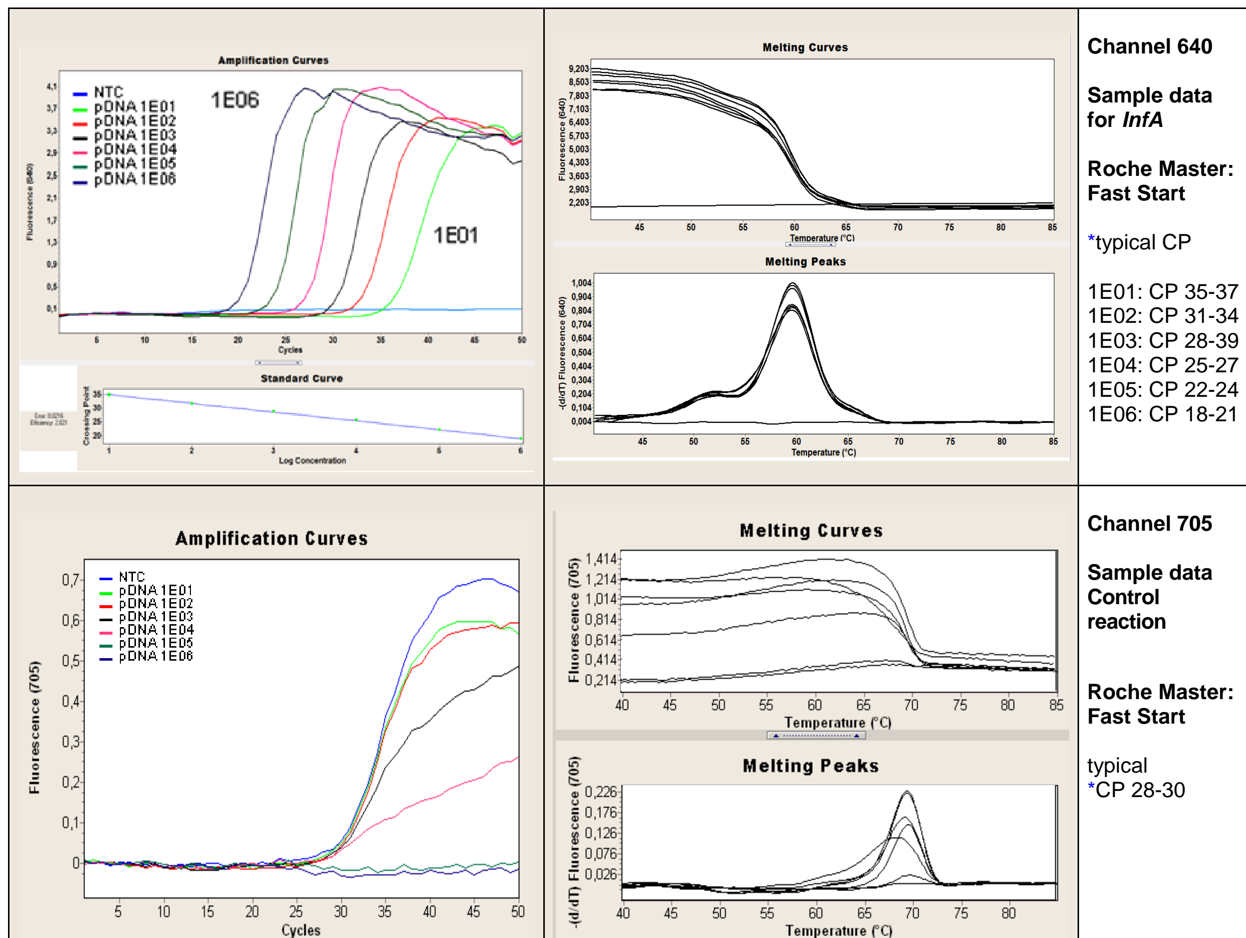


Fig.1. LightCycler® 2.0 sample data for the *Influenza A Virus M2* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) for *Influenza A Virus M2*.
Right panel channel 640 melting analysis *Influenza A Virus M2* (not relevant for detection).
Lower panels: 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.

Please note: The values of the respective melting temperatures (T_M) may vary over 3°C between different experiments, due to different virus strains or different viral titers.

7.4. Interpretation of Data

Sample 640 *InfA*	**Sample 705** *Control*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	Cp 28-33	amplification	negative	**Negative (not detectable)**
Cp < 40⁺	not relevant	amplification	negative	**Positive for *Influenza A***
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 with LightCycler® 2.0 Instrument

⁺ 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 for 10 copies corresponds to ~5 copies/reaction.

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:

TIB Molbiol 640-690 detection format as described in the Color Compensation manual
or
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 of TIB 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 *Influenza A Virus M2* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal.

View IC data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Influenza A Virus M2* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 28.

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 *Influenza A Virus M2* should have CP values between cycle 18 and 36.

8.3. Sample Data – Typical Results

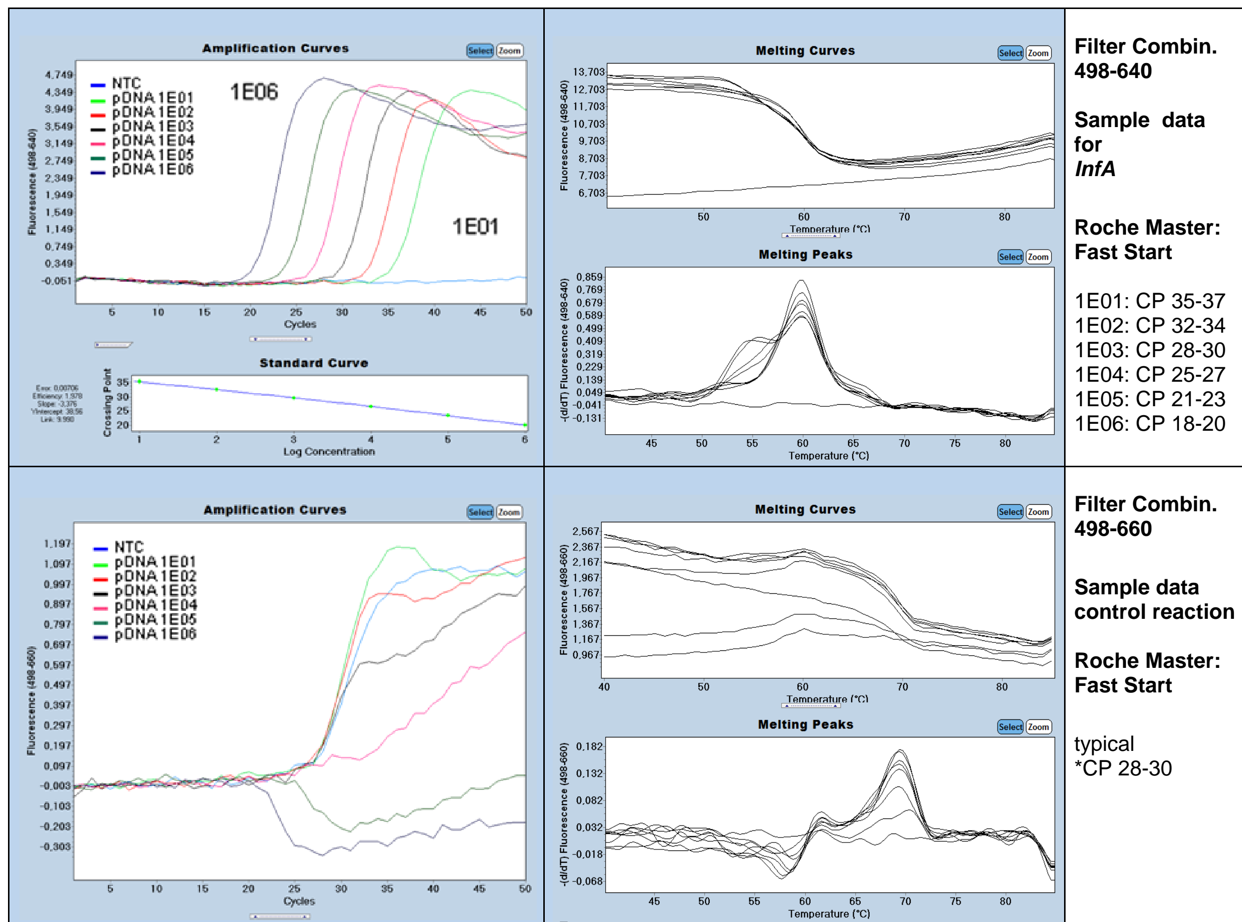


Fig.2. LightCycler® 480 II sample data for the *Influenza A Virus M2* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) for *Inf A Virus M2.* Right panel Filter Combination 498-640 melting analysis for *Influenza A Virus M2* (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 640 *InfA*	**Sample 660** *Control*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	Cp 28-33	amplification	negative	**Negative (not detectable)**
Cp < 38[+]	not relevant	amplification	negative	**Positive for *Influenza A***
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 4. Typical analysis results with LightCycler® 2.0 Instrument

[+] 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 for 10 copies corresponds to ~5 copies/reaction.

9. Influenza A Detection and Identification of the viral HA and NA Type

The common target for the detection of Influenza A (all types) is the matrix protein gene (M2) [1-4].

The Hemagglutinin (HA) and Neuraminidase (NA) types can be determined by specific PCR tests. The typical work flow will start with M2 gene testing and further analysis or typing of InfA positive samples. In pandemic situations one might change the procedure to test simultaneously for the HA type.

The NA gene is better conserved. LightMix® Kit N1 shall identify H1N1, H3N1, H5N1, H7N1 and H9N1 virus but is not suitable to identify one particular type. Due to some variability the test might fail to find one or the other N1 type isolate. In case of a pandemic situation we check the sequences and change the product (sequences) if needed.

The HA genes are more variable and PCR tests will hardly detect all members of one HA type. For the bird flu cases in 2007 we developed a LightMix® kit specifically for the H5 gene in H5N1 Asia. 2009 we launched a new LightMix® kit H1 sw for the specific detection of the 2009 InfA/H1N1 virus.

We recommend to run a 2-step RT-PCR procedure, starting with generation of cDNA using the Roche Diagnostics Transcriptor kit and run the PCR using the FastStart or the FastStart PLUS kit.

Order Information Influenza A detection with the LightCycler® Instruments :

LightMix® for the detection of *Influenza virus A M2*	40-0234-16 (this product)
LightMix® for the detection of *Influenza virus A H5 Asia*	40-0219-16
LightMix® for the detection of *Influenza virus A N1*	40-0230-16
LightMix® for the detection of *Influenza virus A H5N1 Asia*	40-0242-16
LightMix® for the detection of *Influenza virus A H9N2*	40-0299-16
LightMix® for the detection of *Influenza virus A H1 sw*	40-0580-32

10. References

[1] Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. Ward CL, Dempsey MH, Ring CJ, Kempson RE, Zhang L, Gor D, Snowden BW, Tisdale M. J Clin Virol. 2004 Mar;29(3):179-88

[2] Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. Schweiger B, Zadow I, Heckler R, Timm H, Pauli G. JCM 38 (2000) 1552-1558

[3] Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Spackman et al. JCM 40 (2002) 3256-3260

[4] Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM. JCM 39 (2001) 196-200

11. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU 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.

12. Version History

Events require changes in procedures red, mod. sequences blue

V080304	EAN number included
V090804	New reverse primer (LC 480 II performance and adaption to H1N1)
V130117	Introduction; FastStart PLUS included, Use of Color Compensation
V150202	HybProbe for detection of H3N2 (2015) types added

Notice to Purchaser

LightCycler® hybridization probes and kits produced under license agreements with Roche. The purchase price of this product includes a limited, nontransferable license under US patent claims and corresponding patent claims outside the United States, licensed from BioFire Defense, to use only this amount of the product for HybProbe assays and related processes described in said patents solely for the research and development activities of the purchaser. Diagnostic uses require a separate license from Roche.

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