8. Sample data - typical results

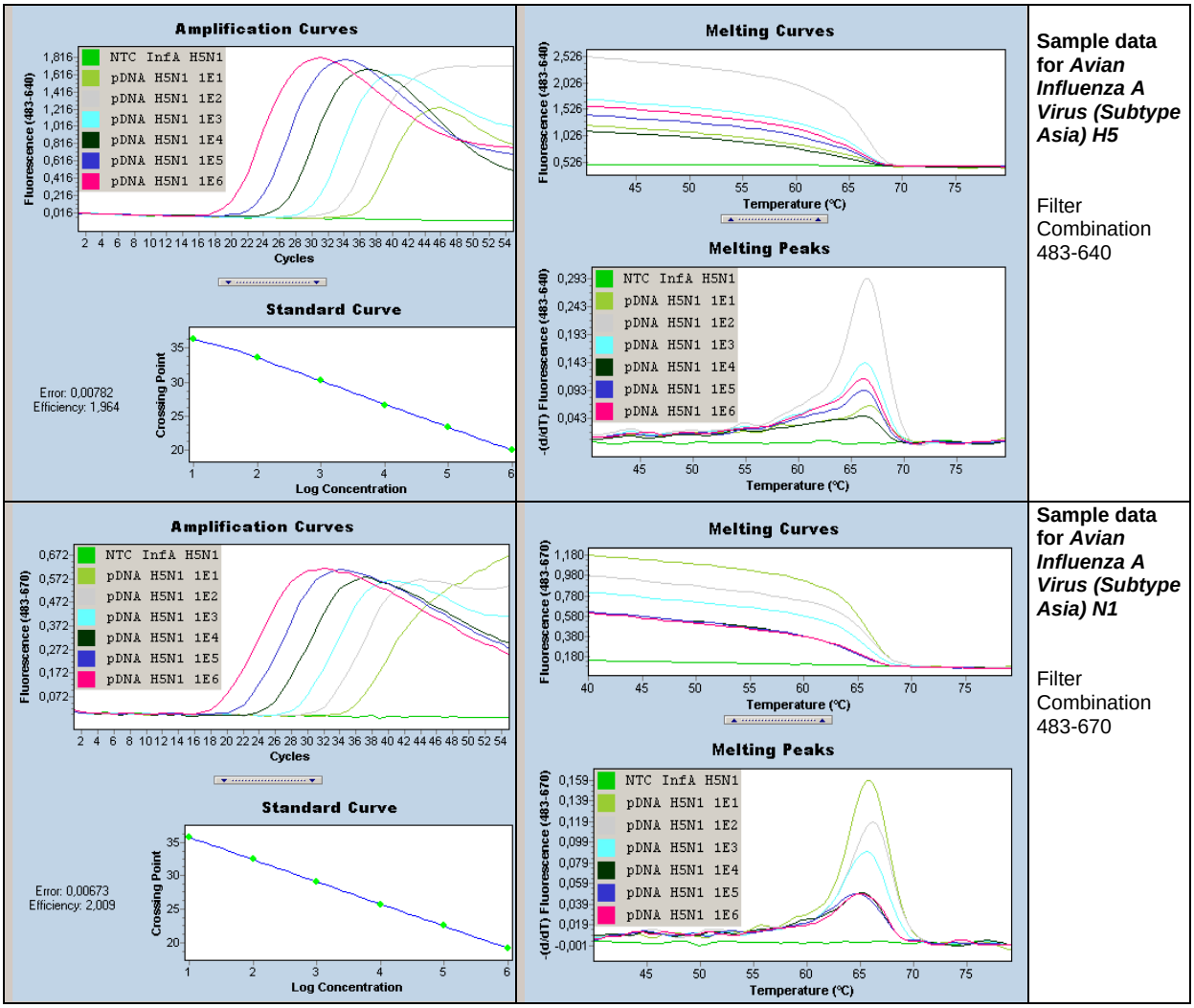

Fig.1. Sample data for the *Avian Influenza A Virus (Subtype Asia) H5N1* detection system.

Upper panels: Data from Filter Combination 483-640 Left panel quantification (Second Derivative Maximum) with calibration curve. Right panel melting curves for the target (H5).
Lower panels: Data from Filter Combination 483-670 Left panel quantification (Second Derivative Maximum) with calibration curve. Right panel melting curves for the target (N1).

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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.

LightMix® 480HT for the detection of *Avian Influenza A Virus (Subtype Asia) H5N1*

Cat.-No. 40-0311-96

Reagents for the quantitative detection of *Avian Influenza A Virus (Subtype Asia) H5N1 c*DNA using the LightCycler® 480 Instrument.

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 NO T freeze!**

Additional reagents required (Roche Diagnostics):

LightCycler® 480 Probes Master	Cat.-No. 04 707 494 001
High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
Transcriptor First Strand cDNA Synthesis Kit	Cat.-No. 04 379 012 001

1. Introduction

Avian Influenza is an infectious disease of birds caused by negative strand RNA viruses of the type A strain of the Influenza virus with several subtypes.
Infection results in a wide spectrum of symptoms, ranging from mild illness to a highly contagious and rapidly fatal disease.The latter is characterized by sudden onset, severe illness, and rapid death, with a mortality that can approach 100%.
All type A influenza viruses, including those that regularly cause seasonal epidemics of influenza in humans, are genetically labile and well adapted to elude host defenses. Influenza viruses lack mechanisms for the “proofreading” and repair of errors that occur during replication. As a result of these uncorrected errors, the genetic composition of the viruses changes as they replicate in humans and animals. This also results in the possibility that viruses of low pathogenicity can, after circulation for sometimes short periods in a host population, mutate into highly pathogenic viruses.

The LightMix® for the detection of cDNA from *Avian Influenza A Virus (Subtype Asia) H5N1* provides a fast, easy and accurate system to identify and quantify this virus.

This LightMix®-System is tested with the Roche Diagnostics “LightCycler® 480 Probes Master” ready–to-use reaction mix in the LightCycler® 480 Instrument.

2. Description

This LightMix® detects parts of the *Avian Influenza A Virus (Subtype Asia) H5N1* genes indicating the presence of *Avian Influenza A Virus (Subtype Asia) H5N1* cDNA in a nucleic acid extract.

A 161 bp fragment of the *Avian Influenza A Virus (Subtype Asia) H5* gene is amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (detected with Filter Combination 483-640).

A 198 bp fragment of the *Avian Influenza A Virus (Subtype Asia) N1* gene is amplified with specific primers and detected with probes labeled with LightCycler® Red 670 (detected with Filter Combination 483-670).

The supplied standard row allows the absolute quantification of the unknown samples.

3. Set contents

1 Vial containing premixed and lyophilized primers and hybridization probes for 96 reactions
1 Row with 6 lyophilized standards from 10^1 to 10^6 target equivalents per reaction of *Avian Influenza A Virus (Subtype Asia) H5N1* DNA
1 Sealing foil for the standard row

4. Programming (384 well plate)

The protocol consists of four program steps

- Program 1: Denaturation of sample and activation of the enzyme
- Program 2: PCR-amplification of the target DNA
- Program 3: Melting curve for identification of the *Avian Influenza A Virus (Subtype Asia) H5N1* cDNA derived PCR product
- Program 4: Cooling the instrument

Before starting the run choose detection format 483-533, 483-640, 483-670

Program:	**Denaturation**	**Cycling**			**Melting**			**Cooling**
Parameter								
Analysis Mode	None	Quantification			Melting Curves			None
Cycles	1	55			1			1
Segment	1	1	2	3	1	2	3	1
Target [℃]	95	95	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:15	00:00:10	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [℃/s]	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	Continu.	None
Acquisition							1	

5. 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 LightCycler® Instrument.

Perform data analysis, as described in the LightCycler® operator's manual. The cycle number of the Crossing Point (CP) of each sample is calculated automatically.

View *Avian Influenza A Virus (Subtype Asia) H5* data with Filter Combination 483-640, Quantification mode. The negative control (NTC) should show no signal.

View *Avian Influenza A Virus (Subtype Asia) N1* data with Filter Combination 483-670, Quantification mode. The negative control (NTC) should show no signal.

Typical results (Software Version 1.0)
The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn should have CPs between cycles 17 and 35.

6. Product characteristics

PCR results are obtained within 1.5 hours.

Sensitivity
These reagents detect 10 copies of *Avian Influenza A Virus (Subtype Asia) H5* DNA and *Avian Influenza A Virus (Subtype Asia) N1* DNA (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 *Avian Influenza A Virus (Subtype Asia) H5* DNA *and* 10^2 to 10^6 copies of *Avian Influenza A Virus (Subtype Asia) N1* DNA.

Storage and Stability
- Lyophilized reagents are stable for at least 3 months after shipment if stored protected from light at room temperature (18-25℃).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if stored protected from light and refrigerated (4℃).

7. Experimental protocol

The following procedure was developed for use with the LightCycler® 480 Instrument.
Start programming before preparing the solutions. See the LightCycler® operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. High Pure Viral Nucleic Acid Kit combined with Transcriptor First Strand cDNA Synthesis Kit).

Negative control: Always run at least one negative control - 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).

7.1 Preparation of parameter-specific reagents (96 reactions):

One reagent vial with a blue clip contains all primers and probes to run 96 LightCycler® reactions for *Influenza A Virus (Subtype Asia) H5N1*.

Add 100 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►Use 1 µl **reagent** for a 20 µl PCR reaction.

This solution is stable for three days or longer if stored refrigerated at 4°C. Avoid prolonged exposu re to light.

7.2 Preparation of the standard row (quantification)

The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 target molecules in 5 µl once resuspended. 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 loose 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 reopening of these vials may cause contaminations of the work-space (aerosol).

7.3 Preparation of the LightCycler® reaction mix

In a reaction tube cooled below 4°C, prepare the re action 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 480 Probes Master kit	
Component	**Single reaction**
water, PCR-grade (colorless cap, provided with the Roche 480 Probes Master kit)	4.0 µl
reagent mix (parameter specific reagents containing primers and probes, see **7.1**)	1.0 µl
LightCycler® 480 Probes Master (red cap)	10.0 µl
Volume of reaction mix	**15.0 µl**

Mix gently, spin down and transfer 15 µl each of the reaction mix to a plate well.
Add 5 µl of sample or standard (standard dilutions of control target, see instruction **7.2**) to each plate well to give a final reaction volume of **20 µl.**

Start run.