8. Sample data - typical results

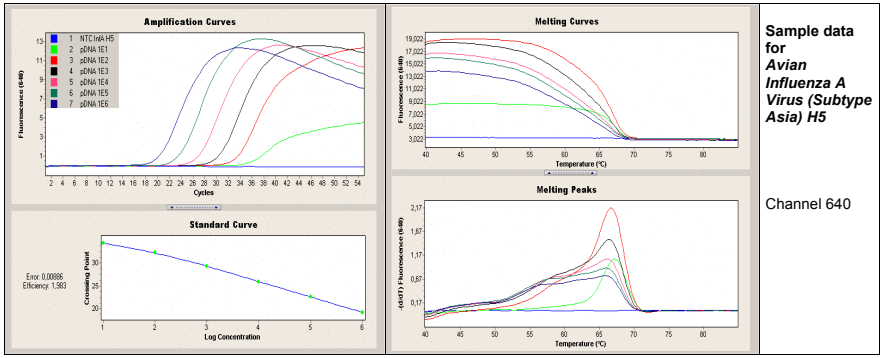

Fig.1. Sample data for the *Avian Influenza A Virus (Subtype Asia) H5* detection system.

Data from channel 640. Left panel quantification (Second Derivative Maximum) with calibration curve. Right panel melting curves for the target.

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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® for the detection of *Avian Influenza A Virus (Subtype Asia) H5*

Cat.-No. 40-0219-16

Reagents for the quantitative detection of *Avian Influenza A Virus (Subtype Asia) H5* cDNA using the LightCycler® Instrument 1.x / 2.0.

Lyophilized mix of primers and probes (6 tubes with 16 rxns each) 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!**

Additional reagents required (Roche Diagnostics):

LightCycler® FastStart^{PLUS} DNA Master Hybridization Probes	Cat.-No. 03 515 575 001
or LightCycler® FastStart DNA Master Hybridization Probes	Cat.-No. 03 003 248 001
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 positive 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%. To date, all outbreaks of this highly pathogenic form have been caused by Influenza viruses A of subtypes H5 and H7.
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.
Avian Influenza viruses do not normally infect species other than birds and pigs. The first documented infection of humans with an avian influenza virus occurred in Hong Kong in 1997, when the *Influenza virus H5*N1 strain caused severe respiratory disease in 18 humans, of whom 6 died.
Patients infected with *Avian Influenza A Virus (Subtype Asia) H5*N1develop symptoms of fever, sore throat, cough and, in several of the fatal cases, severe respiratory distress secondary to viral pneumonia.

The LightMix® for the detection of cDNA from *Avian Influenza A Virus (Subtype Asia) H5* provides a fast, easy and accurate system to identify and quantify this virus.

This LightMix®-System is tested with the Roche Diagnostics "LightCycler® FastStart DNA Master Hybridization Probes" ready–to-use reaction mix in the LightCycler® Instrument 2.0.

2. Description

This LightMix® detects a part of the Avian *Influenza A Virus (Subtype Asia) H5* gene indicating the presence of *Avian Influenza A Virus (Subtype Asia) H5* cDNA in a nucleic acid extract.

A 189 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 in channel 640).

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

For use in LightCycler® Instruments other than 2.0 use channel F2 instead of channel 640 for detection.

3. Set contents

6 Vials containing premixed and lyophilized primers and hybridization probes for 16 reactions each
1 Row with 6 lyophilized standards from 10^1 to 10^6 target equivalents per reaction of *Avian Influenza A Virus (Subtype Asia) H5* DNA
1 Sealing foil for the standard row

4. Programming

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) H5* cDNA derived PCR product
- Program 4: Cooling the instrument

When using the Roche FastStart reagents run an initial heating for 10 min at 95°C.

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 [°C]	95	95	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:15	00:00:08	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	Continu.	None

5. Data analysis

Perform data analysis, as described in the LightCycler® 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 user's influences.

View *Avian Influenza A Virus (Subtype Asia) H5* data in channel 640, Quantification mode. The negative control (NTC) should show no signal.

Typical results (Software Version 4.0)
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 18 and 35 (CPs calculated with Second Derivative Maximum method).

6. Product characteristics

PCR results are obtained within 1 hour.

Sensitivity
These reagents detect 10 copies of *Avian Influenza A Virus (Subtype Asia) H5* DNA (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^2 to 10^7 copies of *Avian Influenza A Virus (Subtype Asia) H5* DNA.

Storage
Store the reagents protected from light at room temperature (18-25°C). **Do not freeze** lyophilized reagents.
Store dissolved reagents refrigerated (4°C).

7. Experimental protocol

The following procedure was developed for use with the LightCycler® Instrument 1.x / 2.0.
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 use always a fresh solution prepared from the provided standard row.

(A) Preparation of parameter-specific reagents (16 reactions):

One reagent vial (labeled transparent vial with a blue pellet) contains all primers and probes to run 16 LightCycler® reactions.

Add 66 µl PCR-grade water to the non-colored reagent vial containing the blue pellet, mix the solution (vortex) and spin down.

►Use 4 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 exposure to light.

(B) Preparation of the standard row (quantification)

The target DNA is provided in 6 different concentrations from 10^1 to 10^6 target molecules/reaction. 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).

(C) Preparation of the LightCycler® reaction mix

In a reaction tube cooled below 4°C, 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^{PLUS} kit		For use with the Roche FastStart kit
Single reaction	**Component**	**Single reaction**
7.0 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart or FastStart^{PLUS} kit)	7.4 µl
--	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	1.6 µl
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **A**)	4.0 µl
4.0 µl	FastStart mix (vial 1 (red cap), combined from vials 1a and 1b, see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

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

Start run.