

LightMix® Kit Avian Influenza A Virus (Subtype Asia) N1

Cat.-No. 40-0230-32

Change to 32 rxn/vial, adapted to include H5N1 clade 2.3.2.1

Real-Time-PCR Kit with reagents for the detection of the N1 gene from the *Avian Influenza A Virus (Subtype Asia) H5N1* from cDNA using the Roche Diagnostics LightCycler® 1.x / 2.0 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!

1. Introduction

Influenza A (InfA) are negative strand ssRNA viruses from the Orthomyxovirus family infecting birds and mammals. They are characterized by the Hemagglutinin (H or HA) and Neuraminidase (N or NA) genes. Avian Influenza is caused in particular by InfA types H5 (H1N1, H5N6, H5N8), and H7 (H7N9).

Infection results in a wide spectrum of symptoms, ranging from mild illness to a highly contagious and rapidly fatal disease, characterized by sudden onset, severe illness, and death (mortality up to 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.

2. Description

This kit detects the N1 gene, indicating the presence of an *Avian InfA Virus (Subtype Asia) H?N1* in a nucleic acid extract. [The kit has been improved to detect also the new clade 2.3.2.1 viruses described first in Vietnam in 2013.](#)

A 198 bp fragment of the viral N1 gene is amplified with specific primers and detected with probes labeled with LightCycler® Red 640.

The supplied standard row allows the absolute quantification of the 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.

3. Set Contents

- 3 Vials with **blue** cap containing lyophilized primers and probes for 32 PCR reactions
- 1 Standard row with 6 lyophilized plasmid standards from 10¹ to 10⁶ target equivalents per rxn
- 1 Sealing foil for the standard row

4. 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.

5. Additional Reagents and Items Required

LightCycler® FastStart DNA Master^{PLUS} HybProbe
 or LightCycler® FastStart DNA Master HybProbe
 High Pure Viral Nucleic Acid Kit
 Transcriptor First Strand cDNA Synthesis Kit

Roche Diagnostics
 Cat.-No. 03 515 575 001
 Cat.-No. 03 003 248 001
 Cat.-No. 11 858 874 001
 Cat.-No. 04 379 012 001

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.

6. Product Characteristics

PCR results are obtained within 1 hour.

Sensitivity

These reagents detect 10 copies of *InfA N1* cDNA (in an exemplary system, using a cloned target).

Measuring range

The linear measuring range of the assay is 10² to 10⁶ copies of *Avian InfA* cDNA.

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 on 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).

7. Programming

The protocol consists of four program steps

- 1: Denaturation: samples 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	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:05	00:00:20	00:00:30	00:00:00	00:00:30
Ramp Rate [°C/s]	20	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	None	Cont	None

(Melting not relevant for detection) Table 1

8. 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) N1* data in channel 640, Quantification mode.

The negative control (NTC) should show no signal.

9. 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' 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).

9.1 Preparation of Parameter-Specific Reagents (PSR):

One reagent vial with a **blue** clip contains all primers and probes to run **32 reactions**.

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

► **Use 2 µl reagent** for a 20 µl PCR reaction.

| This solution is stable for ten days if stored refrigerated at 4°C. Avoid prolonged exposure to light.

9.2. Preparation of the Standard Row

The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 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. After adding the target DNA to the reaction mix, use the provided sealing foil to close the vials in order to avoid contaminations.

9.3. 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 the Roche FastStart ^{PLUS} kit		For use with the Roche FastStart kit	
Single reaction	Component		Single reaction
9.0 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart or FastStart ^{PLUS} kit)		9.4 µl
--	Mg ²⁺ solution 25 mM (blue cap, provided with the Roche FastStart kit)		1.6 µl
2.0 µl	reagent mix (parameter specific reagents containing primers and probes, see A)		2.0 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

Table 2

Mix gently, spin down and transfer 15 µl each of the reaction mix to a LightCycler[®] capillary.

Add 5 µl of sample or standard to each capillary for a final reaction volume of 20 µl. Close the capillaries and spin down.

Start run.

10. Sample Data - Typical Results

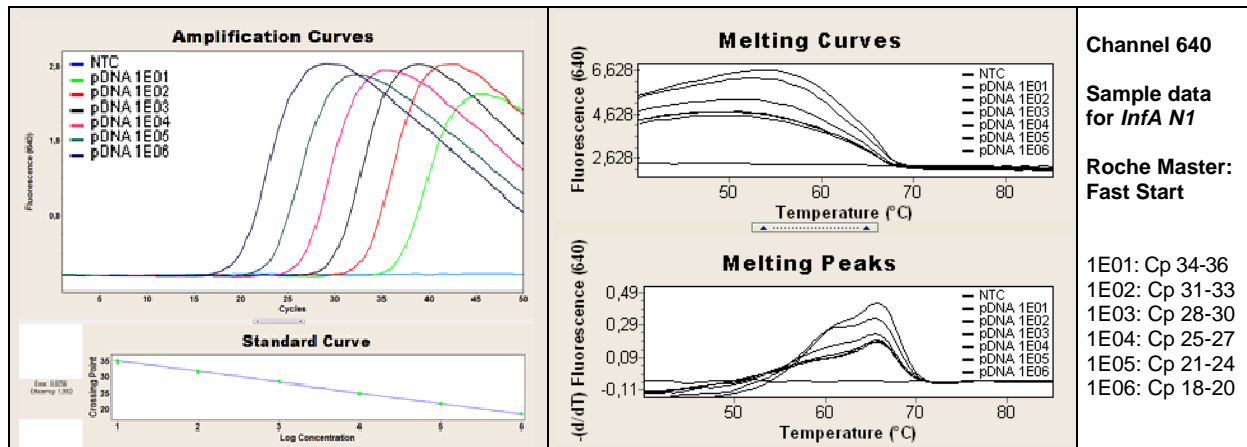


Fig.1. Sample data for the Avian Influenza A Virus (Subtype Asia) N1 detection system.

Data from channel 640. Left panel quantification (Second Derivative Maximum) with calibration curve. Right panel melting curves for the target (not relevant for detection, shape varies with concentration).

11. Interpretation of Data

Sample 640 N1	Channel 640 Positive Control	Channel 640 Neaqative Control	Result (warninas)
No amplification	amplification	negative	Negative (not detectable)
Cp < 37 ⁺	amplification	negative	Positive for Influenza A N1
not relevant	no amplification	not relevant	PCR failure, repeat experiment
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.

12. Version History

Events require changes in procedures red, mod. sequences blue

V051024
V100826
V150202

Release version
Editorial changes
Change to 3x32 rxns. FL probe and new primer added

Roche SAP order n° 05552397001



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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.