New Influenza A Virus Real Time RT-PCR Kit
User Manual

LT028310RRFY

For use with ABIPrism® 7000/7300/7500/7900/Step One Plus, iCycler iQ™4/iQ™5; Smart Cycler II, Bio-Rad CFX 96; Rotor Gene ™6000; Mx3000P/3005P; MJ-Option2/Chromo4; LightCycler® 480 Instrument

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1. Intended Use

New Influenza A virus real time RT-PCR Panel is used for the detection of universal influenza A virus, universal swine Influenza A virus, human seasonal influenza A virus and new reassortment Influenza A virus (H1N1) by using real time PCR systems.

2. Principle of Real-Time RT-PCR

RT-PCR (Reverse Transcription-Polymerase Chain Reaction) is a technique in which an RNA strand is "reverses" transcribed into its DNA complement, followed by amplification of the resulting DNA using a polymerase chain reaction (PCR). RT-PCR can be used to examine gene expression level in cells and tissues, clone the specific gene of cDNA sequences and test RNA viruses. One Step RT-PCR Kit adopts one tube system. Because operator doesn't need to open the lid during the reaction process, this user-friendly improved version avoids cross contamination.

3. Product Description

Influenza A virus subtype H1N1 (A/H1N1), is a subtype of influenza A virus .It is the most common cause of influenza (flu) in humans. Some strains of H1N1 are endemic in humans, including the strain(s) responsible for the 1918 flu pandemic which killed 50–100 million people worldwide. Less virulent H1N1 strains still exist in the wild today, worldwide, causing a small fraction of all influenza-like illness and a large fraction of all seasonal influenza. In March and April 2009, hundreds of laboratory-confirmed infections and a number of deaths were caused by an outbreak of a new strain of H1N1.

The New Influenza A virus real time RT-PCR Panel contains a specific ready-to-use system for the detection of the New Influenza A virus (H1N1) using RT-PCR in the real-time PCR system. The kit contains 4 types of Super Mix for the specific amplification of the virus RNA. The reaction is done in one step real time RT-PCR. The first step is a reverse transcription (RT), during which the virus RNA is transcribed into cDNA. Afterwards, a thermostable DNA polymerase is used to amplify the specific gene fragments by means of PCR (polymerase chain reaction). Fluorescence is emitted and measured by the real time systems´ optical unit during the PCR. The detection of amplified virus DNA fragment is performed in fluorimeter channel FAM with the fluorescent quencher BHQ1. An external positive control is supplied which allow the determination of the gene load.

Internal control special for Super Mix A only is available in the kit. You may also use GAPDH (Human) Real Time RT-PCR Kit (Cat.No. QR-0132-02) instead of internal control which is only suitable for detection of human specimen. It can be used for monitoring the yield of the nucleic acid extraction and whether there existing inhibition in the sample or not.

4. Kit Contents

Ref.	Type of reagent	Presentation 25rxns
1	Super Mix A[1]	1 vial, 500µl
2	Super Mix B[2]	1 vial, 500µl
3	Super Mix C[3]	1 vial, 500µl
4	Super Mix D[4]	1 vial, 500µl
5	RT-PCR Enzyme Mix	1 vial, 110µl
6	Molecular Grade Water	1 vial, 400µl
7	Positive Control [5]	1 vial, 90µl
8	Internal Control (for Super Mix A only)[5][6]	1 vial, 30µl

[1]. Detection of MP gene of universal influenza A virus among human,swine,avian and so on.
[2]. Detection of H3HA gene of human seasonal influenza A virus.
[3]. Detection of H1HA gene of human seasonal influenza A virus.
[4]. Detection of H1HA gene of the new influenza A virus (H1N1) with new reassortment

genome.
[5] Because of transportation with carbon dioxide ice, there may be white precipitate in tubes of internal control and positive control ,but it will disappear in a few minutes when it is incubated at room temperature. Besides, the white precipitate have no effection on the detection result.
[6] It is necessary to dilute the internal control supplied in the kit by 10 times with molecular grade water before detection, and close the tube immediately then vortex for 10 seconds.

Analysis sensitivity: 5×10^3copies/ml.
Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors .If you use the RNA extraction kits recommended, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much.

5. Storage

- All reagents should be stored at -20°C. Storage at +4°C is not recommended.
- All reagents can be used until the expiration date indicated on the kit label.
- Repeated thawing and freezing (> 3x) should be avoided, as this may reduce the sensitivity of the assay.
- Cool all reagents during the working steps.
- Super Mix should be stored in the dark.

6. Additionally Required Materials and Devices

- Biological cabinet
- Real time PCR system
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- Vortex mixer
- RNA extraction kit
- Real time PCR reaction tubes/plates
- Cryo-container
- Pipets (0.5 µl – 1000 µl)
- Sterile filter tips for micro pipets
- Sterile microtubes
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator and freezer
- Tube racks

7. ⚠ Warnings and Precaution

Carefully read this instruction before starting the procedure.

- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.

• Prepare quickly the Reaction mix on ice or in the cooling block.
• Set up two separate working areas: 1) Isolation of the RNA/ DNA and 2) Amplification/ detection of amplification products.
• Pipets, vials and other working materials should not circulate among working units.
• Use always sterile pipette tips with filters.
• Wear separate coats and gloves in each area.
• Do not pipette by mouth. Do not eat, drink, smoke in laboratory.
• Avoid aerosols

8. Sample Collection, Storage and transport

• Collected samples in sterile tubes;
• Specimens can be extracted immediately or frozen at -20°C to -80°C.
• Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents

9. Procedure

9.1 RNA-Extraction

9.1.1 Type of specimens

A variety of specimens are suitable for the diagnosis of virus infections of the upper respiratory tract:

◆ Nasal swab	◆ Nasopharyngeal aspirate
◆ Nasopharyngeal swab	◆ Throat swab

In addition to swabs from the upper respiratory tract, invasive procedures can be performed for the diagnosis of virus infections of the lower respiratory tract where clinically indicated:

◆ Transtracheal aspirate	◆ Lung biopsy
◆ Bronchoalveoar lavage	◆ Post-mortem lung or tracheal tissue

Specimens for the laboratory diagnosis of avian influenza A should be collected in the following order of priority:

◆ Nasopharyngeal aspirate
◆ Acute serum
◆ Convalescent serum

9.1.2 Procedure for specimen collection

◆ Tongue depressor	◆ Specimen collection cup or Petri dishes
◆ 15-ml conical centrifuge tubes	◆ Transfer pipettes

Respiratory specimens should be collected and transported in virus transport media.

Virus transport medium

(A) Virus transportation medium use in collecting throat and nasal swabs

1) Add 10g veal infusion broth and 2g bovine albumin fraction V to sterile distilled water (to 200ml).
2) Add 0.8ml gentamicin sulfate solution(50mg/ml) and 3.2ml amphotericin B(250μg/ml).
3) Sterilize by filtration.

B） Nasal wash medium

Sterile saline(0.85% NaCl)

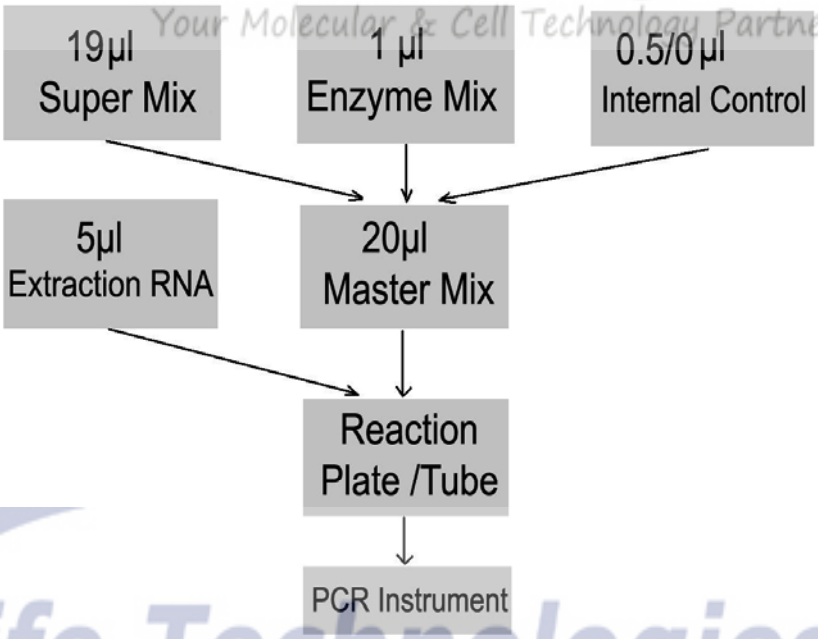
9.1.3 RNA extraction kits

Different brand RNA extraction kits are available. You may use your own extraction systems or the commercial kit based on the yield. For the RNA extraction, please comply with the manufacturer's instructions. The recommended Extraction kit is as follows:

Nucleic Acid Isolation Kit	Cat. Number	Manufacturer
RNA Isolation Kit	GEN 52-904 LT	Life Technologies

9.2 RT-PCR Protocol

The Master Mix volume for each reaction should be pipetted as follows:



1) The volumes of Super Mix and Enzyme Mix per reaction multiply with the number of reaction, which includes the number of controls, standards, and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unprecise pipetting, always add an extra virtual sample (n: the number of reaction). Mix completely then spin down briefly in a centrifuge.

Reaction Volume	Master Mix A Volume	Master Mix B/C/D Volume
Super Mix	19µl × (n+1)	19µl × (n+1)
Enzyme Mix	1µl × (n+1)	1µl × (n+1)
Internal control (IC)	0.5µl × (n+1)	—

2) Pipet **20µl** Master Mix with micropipets of sterile filter tips to each of the *Real time* PCR reaction plate/tubes. Separately add **5µl** RNA sample supernatantor positive and negative controls to different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination.
3) Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.
4) Perform the following protocol in the instrument:

45°C for **10 min, 1 cycle; 95°C** for **15 min, 1 cycle;**
95°C for **15 sec, 60°C** for **60sec, 45 cycles.**
Fluorescence is measured at 60°C;
Channel FAM and HEX/VIC/JOE should be chosen.

5) ⚠If you use ABI Prism® system, please choose **"none"** as **passive reference** and **quencher.**

10. Baseline setting: just above the maximum level of molecular grade water.

11.Quality control: The Ct value of molecular grade water and positive control in FAM

channel shows UNDET and ≤35 respectively; the Ct value of internal control in HEX/VIC/JOE channel of Super Mix A shows 25~35,otherwise the result is invalid.

12. Data Analysis and Interpretation

1) The Ct value in channel FAM shows ≤43. **The result is positive；**

2) The Ct value in channel FAM shows 43~45, please repeat again. **If the result still shows 43~45,it can be considered negative；**

3) In channel FAM no signal is detected**. It can be considered negative.**

4) Neither in channel FAM nor in channel HEX/VIC/JOE of Super Mix A is a signal detected. A diagnostic statement can not be made. **Inhibition of the RT-PCR reaction.**

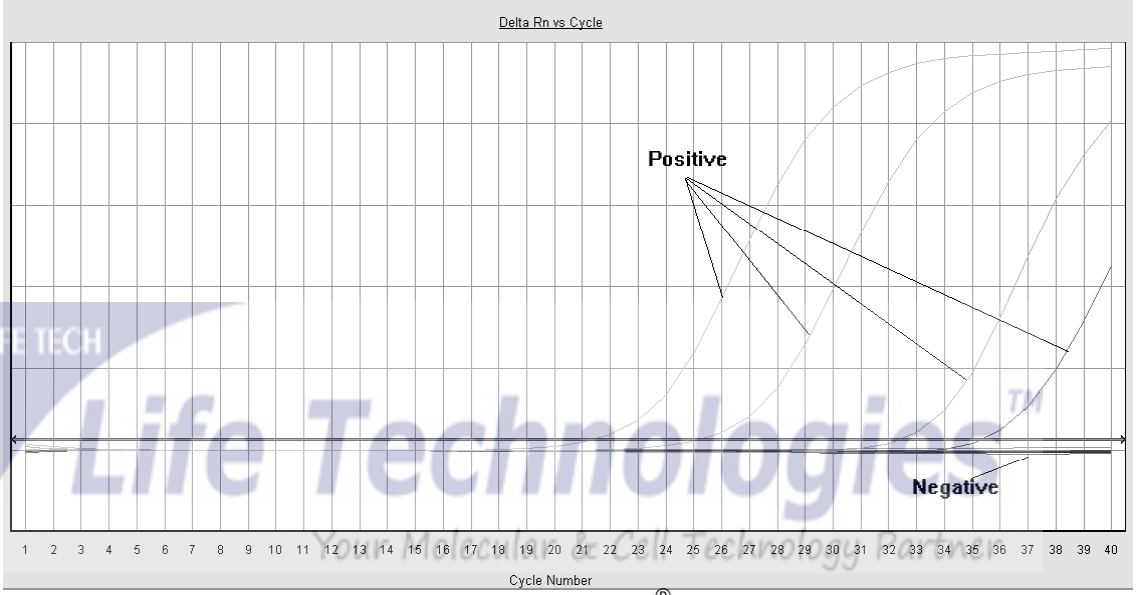


Fig.1 Date analysis (ABI Prism®7000 Instrument)

The following results are possible:

Result	Super Mix				Report
	A	B	C	D	
1	+	-	-	+	New Influenza A virus H1 subtype's genome bears strong resemblance to the genome of strain A/California/04/2009. If the results are obtained, it is likely that a new reassortment of the New Influenza A virus has emerged.
2	+	-	+	-	Human seasonal influenza A virus H1 subtype.
3	+	+	-	-	Human seasonal influenza A virus H3 subtype.
4	+	-	-	-	Influenza A virus
5	-	-	-	-	Influenza A virus Negative