TIANLONG

Influenza Virus A (H1N1) RNA Detection Kit (Fluorescence PCR Method)

User Guide

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FOR ANIMAL USE ONLY!

Version 1.0

In-Vitro Diagnostics / For use with Real-time PCR Instruments compatible with Influenza Virus A (H1N1) RNA Detection Kit (Fluorescence PCR Method)

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Suzhou TianLong Biotechnology Co., Ltd.

NW-07-501 & NW-06-504 Nanopolis, No.99 Jinjihu Road,
215123 Suzhou, Jiangsu Province, P.R. China.

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Introduction

Influenza viruses are divided into type A (A), type B (B) and type C (C), the first two viruses most infected by humans. Influenza A viruses are the most common influenza viruses in humans. They usually cause seasonal influenza and may also cause influenza pandemics. Influenza A viruses can also infect animals, such as birds, pigs and horses. Influenza B virus infection is basically limited to humans and rarely causes epidemics. Influenza A viruses can be further divided into different subtypes based on their two surface proteins-hemagglutinin (H) and neuraminidase (N).

In March and April 2009, a novel swine-origin pandemic H1N1 influenza virus appeared and spread worldwide. The H1N1 (2009) influenza virus pandemic poses a public health threat. The H1N1 virus strain causing the current pandemic is a novel influenza virus that has never been detected previously in either humans or animals. Most human infections with H1N1 influenza viruses seem to be mild and many of the reported deaths have been associated with chronic diseases. However, the H1N1 virus has been found to cause severe pathological lesions in the lungs of infected mice, ferrets and non-human primates.

Therefore, rapid and accurate diagnosis of the influenza virus A (H1N1) is very important, the ***Influenza Virus A (H1N1) RNA Detection Kit*** developed by TianLong Biotechnology assist in the diagnosis of the influenza virus A (H1N1) and public healthcare management.

Intended Use

The TianLong ***Influenza Virus A (H1N1) RNA Detection Kit*** is intended for the qualitative detection of the influenza virus A (H1N1) nucleic acid by real-time reverse transcription polymerase chain reaction (Real-time RT-PCR) method.

This kit is used for qualitative detection of the influenza virus A (H1N1) RNA in animals' throat swabs/cloacal swabs/blood/poultry meat/innards and other samples.

Positive test results are indicative of the presence of the influenza virus A (H1N1) RNA, whereas clinical correlation with medical history of the affected animals and other diagnostic information is necessary for the determination of the infection status of the affected animals. Positive results from this test do not rule out bacterial infection or co-infection with other viruses.

Negative test results from the test do not completely preclude influenza virus A (H1N1) infection and should not be used as the sole basis for management decisions. Negative results must be used in combination with clinical observations, the affected animals' life history and epidemiological information for a medical decision.

The TianLong ***Influenza Virus A (H1N1) RNA Detection Kit*** is intended for use by qualified laboratory professionals who have received training in the techniques of RT-PCR. The TianLong ***Influenza Virus A (H1N1) RNA Detection Kit*** is intended for use in qualified laboratories in accordance with applicable professional organization and government administration guidelines and regulations.

The TianLong ***Influenza Virus A (H1N1) RNA Detection Kit*** is designed for use with RT-PCR instruments with 3 or more fluorescence detection channels, on which the kit test performance has been validated. These RT-PCR thermal cyclers include appropriate fluorescence reading channels for FAM, HEX/VIC and Cy5 channels, such as Applied Biosystems™ 7500 Real-Time PCR Systems and Tianlong Gentier Real-time PCR Systems.

Principles of the Assay

The highly conserved sequences in the HA gene of influenza virus A (H1) and the NA gene of influenza virus A (N1) were selected as target regions, and specific primers and TaqMan fluorescent probes were designed. The probes can specifically bind to a nucleic acid template in the middle of the primer amplification region. In the process of PCR extension reaction, the exonuclease activity of Taq enzyme will cut off the 5'-end fluorescent group from the probe, freeing it in the reaction system, thus breaking away from the shielding of the 3'-end fluorescence quenching group, which can receive light stimulation and emit fluorescence that can be detected by the instrument, so as to achieve the automatic detection of the influenza virus A (H1N1) nucleic acid in closed reaction system.

The internal control of the kit was a pair of housekeeping gene-specific primers combined with specific probes. The

probe can specifically bind to a nucleic acid template in the middle of the primer amplification region, and the internal standard adopts the Cy5 channel to realize the monitoring of the detection process and the occurrence of false negative in the closed reaction system.

Reagent Kit

Reagent Kit Components

Reagents for 25/50 tests (Real-time RT-PCR reactions) are contained in one reagent kit box.

Real-time RT-PCR Reagents	Volume (25 T/50 T)	In Tube (25 T/50 T)
H1N1 Reaction Mix	350 µL/700 µL	1 tube/1 tube
H1N1 Enzyme Mix	25 µL/50 µL	1 tube/1 tube
Controls		
H1N1 Positive Control	80 µL/80 µL	1 tube/1 tube
Negative Control	80 µL/80 µL	1 tube/1 tube
Internal Control	250 µL/500 µL	1 tube/1 tube

Note: Mix matching and use of the reagent components from different reagent lots should be avoided unless be specifically instructed to do so. The negative control could also be referred to as a "No Target Control (NTC)".

Reagent Storage, Shipment and Handling

All reagents should be stored at the temperature between -25°C to -15°C in a non-frost-free freezer and should be used before the expiry date. Freeze and thaw more than three times should be avoided during the kit's usage period. The reagents should be shipped at the temperature between -25°C to 8°C.

Assay Procedures

Before Starting

- Check reagent components and supplies to ensure there are enough materials ready for planned work.
- Check to ensure equipment and instruments are ready for work.
- Follow the up-to-date instructions for use.
- Complete appropriate planning and calculations for coming testing.

Sample Requirements

The ***Influenza Virus A (H1N1) RNA Detection Kit*** is designed to detect the influenza virus A (H1N1) RNA in animals' throat swabs/cloacal swabs/blood/poultry meat/innards and other samples.

Utilize the specimen collection, transportation and storage medium specified by the reagent manufacturer. Extraction of nucleic acid should be carried out according to the reagent manufacturer's instructions.

Equipment and Instruments Required but Not Provided

- Micropipette dedicated for assay setup (1-10 or 1-20 µL; 20-200 µL; 1000 µL).
- Refrigerated benchtop centrifuge with rotor for 0.5 mL/1.5 mL reaction tubes (capable of attaining 10,000 rpm)
- Benchtop vortex mixer.

- It is recommended to use a detection kit with RT-PCR thermal cyclers with appropriate fluorescence reading channels for FAM, HEX/VIC and Cy5 dyes, such as Applied Biosystems™ 7500 Real-Time PCR Systems and Tianlong Gentier Real-time PCR systems.

Note:
Equipment and instruments should be maintained and calibrated according to the manufacturer's recommendations. Refer to manufacturer's manuals for operation procedures.

Nucleic Acid Extraction

The TianLong ***Influenza Virus A (H1N1) RNA Detection Kit*** is compatible with nucleic acid of adequate quality prepared from intended samples using common nucleic acid extraction kits/methods. The prepared nucleic acids can be used directly as sample nucleic acid material, moved forward to the Real-time RT-PCR setup step.
Add 10 µL of Internal Control to each 400 µL samples and extract together when extracting nucleic acid of samples (For Tianlong Animal Virus DNA and RNA Extraction Kit Series).
If under certain circumstances, prepared nucleic acid needs to be stored in the frozen state before testing, storage in a freezer of -70°C or lower is recommended whenever possible to prevent from nucleic acid degradation during storage.
Repeated freeze and thaw of prepared sample nucleic acid should be avoided whenever possible.

Real-time RT-PCR Reaction Setup

1. Thaw the following reagents on ice: ***H1N1 Reaction Mix*** and ***H1N1 Enzyme Mix***. Gently invert to mix each individual reagent, then briefly centrifuge (2000 rpm, 10 s) to let solutions be settled to the bottom of tubes before proceeding to the next step.
2. Prepare Premix solution based on the planned number of samples to be tested.
   To calculate the volume of each reagent component required for Premix preparation, it needs to cover all the samples and controls to be tested in the assigned assay run with reasonable extra set aside for operational tolerance.
   In many cases, preparing Premix with 10-20% extra volume is a good practice.
   A demo calculation worksheet for Premix preparation is listed below for reference:

Table 1 Calculation of Premix Preparation – A Demo Worksheet

Component Reagents	For 1 Test	For 10 Tests	For 40 Tests	For 80 Tests	For 100 Tests
H1N1 Reaction Mix	14 µL	140 µL	560 µL	1120 µL	1400 µL
H1N1 Enzyme Mix	1 µL	10µL	40 µL	80 µL	100 µL
Total Volume	**15 µL**	**150 µL**	**600 µL**	**1200 µL**	**1500 µL**

3. 96-well PCR reaction plates or PCR reaction tube stripes could be used for reaction setup. Evenly aliquot 15 µL of the prepared Premix into each PCR tube. Add 10 µL of each extracted RNA/nucleic acid solution to the designated PCR tube. Add 10 µL of H1N1 Positive Control and Negative Control (without extraction) to the respectively assigned tubes.
   At the end of setup, each PCR tube shall have a total volume of 25 µL.
   Then immediately close/cover the tubes and transfer the reaction setup tube stripes/plate into a RT-PCR cycler for amplification reactions.
   Positive Control and Negative Control must be run in each assay run.

Thermal Cycler Settings

Real-time RT-PCR cycling program:

Table 2 RT-PCR Cycling program

Stage	Number of cycles	Temperature (°C)	Heating rate*	Time (min:sec)
1	1	50	6°C/S	10:00
2	1	95	6°C/S	00:20
3	45	95	6°C/S	00:02
		60	6°C/S	00:20** (collect fluorescence)

* It is recommended to use the Tianlong Gentier series real-time fluorescent PCR instrument with a heating rate of 6°C/S or select other instruments according to the specific performance of the instrument.

** For other instruments such as ABI7500, the collection fluorescence is set to 31 s, which has no effect on the results.

For Group A:

- FAM channel for IFVA H1
- HEX/VIC channel for IFVA N1
- Cy5 channel for Internal Control

Result Analysis and Interpretation

For data analysis, a Fluorescence Threshold Setting needs to be assigned.

- Auto Setting: the instrument automatically sets the threshold value. Auto setting is recommended for routine operations and data analysis.
- Manual Setting: in case the manual setting is desired under certain circumstances, the threshold value could be set just above the fluorescence baseline of the normal negative control.

Run Validity Check

All tests performed on one batch setup through the whole course of Real-time RT-PCR are considered in one run. Only results from valid test runs are moved forward for analysis and interpretation.

Test run is valid when

1) In the run, there is no Ct generated for FAM, HEX/VIC and Cy5 (IC) channels from the negative control.

2) Ct value for FAM, HEX/VIC channels from the positive control is less than 30, and no Ct generated for Cy5 (IC) channel.

Results from valid test runs could be further analyzed for reports.

If the results of the controls do not meet the validity criteria outlined in 1) and 2), the test run is usually considered invalid. All samples involved in the test run need to be retested for reportable results.

In uncommon cases of extremely high target viral (IFVA) load in samples, the efficiency of RT-PCR reaction for internal control could be negatively influenced. This may result in delayed IC Ct in companion with a highly advanced target Cts. In this case, the validity of the test run, as well as the interpretation of positive test results could be confirmed with no need for a further retest of samples.

If the target Ct values for FAM and/or HEX/VIC Channels generated from negative control reactions repeatedly reach 37 or below, it is implicated that amplicon contamination may be present in the working environment. Replacement of opened reagent components, and comprehensive working area cleaning and troubleshooting for contamination should be performed.

The analysis and interpretation of test results:

Target Gene		Internal Control (CY5)	Interpretation of Results
Influenza A virus H1 subtype (FAM)	Ct ≤ 37	Ct < 45	Positive influenza A virus H1 subtype
	37 < Ct < 45	Ct < 45	retest***
	No Ct value or Ct value = 45	Ct < 45	Negative influenza A virus H1 subtype
	No Ct value or Ct value = 45	No Ct value or Ct	invalid****
Influenza A virus N1 subtype (HEX/VIC)	Ct ≤ 37	Ct < 45	Positive influenza A virus N1 subtype
	37 < Ct < 45	Ct < 45	recheck***
	No Ct value or Ct value = 45	Ct < 45	Negative influenza A virus N1 subtype
	No Ct value or Ct value = 45	No Ct value or Ct	invalid****

*** The test result of the sample to be tested is 37 < Ct < 45. At this time, the sample should be tested again. If the Ct value of the repeated test result is less than 45, the curve is S-shaped and there is an obvious exponential growth period, it is judged as positive, otherwise it is negative.

**** This test is invalid and needs to be checked and re-tested.

Assay Performance Characteristics

The following performance characteristics of the TianLong ***Influenza Virus A (H1N1) RNA Detection Kit*** have been established as described below.

Non-Clinical Studies

- Limit of detection: 500 copies/mL
- Specificity:
  There was no cross-reaction between this kit and influenza virus B, adenovirus type 3/7, respiratory syncytial virus type B, parainfluenza type 1/2/3, the intestinal virus, enterovirus 71, coxsackie virus 16, *Mycoplasma pneumonia, Chlamydia pneumonia*, rhinovirus, human cytomegalovirus, human metapneumovirus, human coronavirus OC43/229E, human coronavirus NL63/HKU1, EB virus, measles virus, *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Streptococcus salivarius*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Bordetella pertussis* and other relevant pathogens (above the viral concentration of 10^5 pfu/ml and bacteria of 10^6 cfu/ml).
- Precision:
  In a precision study with reference specimens of high and low concentrations, multiple operators and instruments were utilized. A total of over 600 tests were completed.
  The within run and between run Precision of test results, as represented by the Ct value of CV%, are all less than 5%.
- Disruptor:
  0.2 mg/L beclomethasone, 0.15 mg/L dexamethasone, 12 mg/L triamcinolone, 0.4 mg/L budesonide, 0.05 mg/L mometasone, 0.5 mg/L fluticasone, 75 mg/L benzocaine, 5 mg/L zanamivir, 75 mg/L tobramycin, 75 mg/L sulfur, 150 mg/L genistein, 0.125 mg/L epinephrine, 500 mg/L flunisolide, 500 mg/L mupirocin and 10 g/L mucin have no influence on the detection results on the test result of this kit.

Quality Control

In accordance with the ISO 13485:2016 Medical Devices—Quality management systems and TianLong ***Influenza Virus A (H1N1) RNA Detection Kit*** Quality Control Program, each batch of the ***Influenza Virus A (H1N1) RNA Detection Kit*** is tested against predetermined specifications to ensure consistent product quality.

Limits and Precautions

Limits

- All reagents in the kit are intended for *in vitro* diagnostic use as indicated.
  The test should be carried out by professionals adequately trained in IVD lab practices. It is the user's responsibility to verify/validate the testing system performance in their respective laboratory settings. Expired reagents should not be used.
- Strict compliance with the IFU is required for optimal results. Deviation from standard procedures during sample collection, preservation, transportation, processing and testing could lead to false negative or false positive testing results.
- Theoretically, variations in the target sequences of the influenza A virus (H1N1) arise from natural mutations that could potentially influence testing performance and result in false testing results. Until today, results from bioinformatics analysis and comprehensive laboratory studies indicate that, partially due to the emphasis on mutation tolerance concept during assay design and development, this kit could tolerate currently known influenza A virus (H1N1) mutations without obvious compromise on assay performance.
- Test results should be used in combination with clinical and epidemic information for medical decisions.

Laboratory Precautions

Laboratories using the assay should be ISO 15189 qualified and/or in compliance with local regulations.

Use extreme caution to prevent:

- **RNase contamination that may cause degradation of the template RNA.**
- **Amplicon contamination that may result in false positive test results.**

The following are recommended for desirable test performance:

- Use DNase/RNase-free disposable pipette tips, tubes, and supplies as appropriate.
- A standard PCR lab suite under workflow and air pressure control would be desirable for testing use. If not available, separated/segregated working areas could be used with precaution for contamination control:
  1) Reagent preparation area: preparing the reagents for amplification.
  2) Sample preparation area: extraction and separation of the RNA/nucleic acids from samples and controls, and
  3) Amplification area: amplification and detection of the nucleic acid target.
- Perform regular decontamination practice and cleaning of working areas, equipment and instruments. Commercially available cleaning products containing sodium hypochlorite, 75% alcohol and ultraviolet light could be applied for the purpose of cleaning and decontamination.
- Nucleic acid samples should be stored at -70℃ or lower for long-term storage.
- Equipment, such as micropipettes, needs to be calibrated per the manufacturer's recommendation.
- The Real-time RT-PCR instrument needs calibration per manufacturer's schedule.
- The handling and management of samples and lab wastes should follow relevant guidelines recommended by professional organizations and regulations imposed by authorities.

Symbols

The following table describes the symbols that may appear on the labeling or in this document.

	Catalog number
	Batch code
	Contains reagents sufficient for <N> tests
	Use-by date
	Caution
	Temperature limit
	Manufacturer
	Consult instructions for use
	Keep away from sunlight
	Fragile handle with care
	Recycling symbol PAP21: non corrugated cardboard

References

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Contact Information

For technical assistance and more information, please contact with our Technical Support Center at

+86-029-82682132 (Tel), inquiry@medtl.com (Mail), www.medtl.net (Website).

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