Molecular Diagnostic Kit for Avian

Anigen

H5/N1 AIV Real-Time Detection Kit®

■Introduction

Highly pathogenic avian influenza (HPAI) viruses have emerged in poultry and wildlife worldwide, causing sporadic but serious and devastating outbreaks. These viruses have been restricted to hemagglutinin (HA) subtypes H5 and H7, although not all viruses of these subtypes are highly pathogenic. An outbreak of H5N1 HPAI in the live bird markets of Hong Kong in 1997 resulted in 18 human infections, 6 of them fatal. Similar H5N1 HPAI viruses have reemerged in several countries in Asia since 2001 and have continued to spread through Asia and into the Middle East and Eastern Europe. In addition to their geographic spread, H5N1 HPAI viruses were found in multiple animal species, such as poultry, wild birds, tigers, and leopards.

■Principles

H5/N1 AIV Real-Time Detection Kit® from BIONOTE, Inc. is useful for detecting the infection of subtype H5/N1 avian influenza. The kit can be exactly performed to detect hemagglutinin and nuraminidase gene of subtype H5/N1 avian influenza, so it can be used for both qualitative and quantitative analysis. It works most of Real-Time PCR apparatuses of block and capillary type.

■Materials provided (96Tests/Kit) Cat No. PD65-51

No.	Product	Amount
1	Standard 1 ($1x10^4$ copies/μl)	200μl x 1
2	Standard 2 ($1x10^5$ copies/μl)	200μl x 1
3	Standard 3 ($1x10^6$ copies/μl)	200μl x 1
4	Standard 4 ($1x10^7$ copies/μl)	200μl x 1
5	Detection Solution*1	460μl x 1
6	Nuclease Free Water	1500μl x 1
7	2X Enzyme buffer	1000μl x 1
8	Enzyme Mix	40μl x 1
9	Rox Reference Dye *2	40μl x 1

*1: Probe is labeled at the 5'-end of H5 with the reporter molecule 6-carboxy- fluorescein(**FAM**) and at the 5'-end of N1 with the reporter molecule 6-carboxy- fluorescein(**JOE**)

*2: The kits is provided extra Rox dye for ABI real-time PCR instruments (7000/7300/7700/ 7900).

■Precautions

1. For research use only.
2. Perform the reaction setup in an area separate from nucleic acid preparation or PCR product analysis. It is` generally recommended that the reaction setup is performed in clean bench.
3. Pipette with sterile filter tips.
4. The test tube should be force the solution to the bottom of tubes and remove any possible bubbles.
5. Minimize the exposure of Detection solution to light.
6. Do not use reagents beyond the stated expiration date marked on the package label.
7. Read the result as the infection of subtype H5/N1 avian influenza by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the quarantine center or any other epidemic control center when the results are doubted.

■Storage and Stability

This kit is shipped at +2 to +15℃. Store the kit after arrival at -20℃or less in the dark. The test kit is stable through the expiration date marked on the package label.

■Procedure of the test

1. Sample materials
   Use any virus template RNA suitable for RT-PCR. Template RNA can easily be prepared using kits such as RNeasy Mini Kit from Qiagen (Valencia, CA, USA) and Trizol from Life Technologies (Invitron, USA). But it is strongly recommended that the Viral RNA be extracted from specimen by using **Trizol solution**.
2. Negative Control
   To detect a potential contamination, run a negative control every time the kit is used. **Nuclease Free Water**⑥ should be used instead of template RNA.
3. Positive Control
   For qualitative analysis
   Use **Standard 3 ($1x10^6$ copies/μl)**③
   For quantitative analysis
   Use from **Standard 1 ($1x10^4$ copies/μl)**① to **Standard 4 ($1x10^7$ copies/μl)**④
4. Prepare a master mix by serially dispensing components to each tube in the following manner ;

Reagents	Volume per reaction
Detection Solution ⑤	N x 4.6μl
2X Enzyme buffer ⑦	N x 10μl
Enzyme Mix ⑧	N x 0.4μl
Rox Reference Dye*(Optional) ⑨	N x (0.4μl)
Total volume	15μl

Set up reaction in strip tubes or 96-well plates by combining 15μl of the master mix and 5μl of negative control, positive control and samples.

*Optional
When you use ABI real-time PCR instruments only(7000/7300/7700/ 7900), Rox dye should be added in master mix in each run.

5. Perform the real time RT-PCR reaction under the below condition.

Cycles	Reaction	Temp. (℃)	Time
1	Reverse transcription	50℃	30 min.
1	Activation	94℃	2 min.
40	Amplification	94℃	15 sec.
		50℃	35 sec.

Fluorescence data(FAM or JOE) collection during 50℃ extension step

■Analysis of the test

1. Ct value(Threshold cycle) is fluorescence growth curves that cross the threshold line.
2. If the test reactions should not exhibit fluorescence growth curves that cross the threshold line, Ct value is 40.
3. The Ct value of negative control reactions should be more than 37. If a false positive occurs, sample contamination may have occurred. Invalidate the run and repeat the assay with stricter adherence to the procedure guidelines
4. When you read the test results, the noise encountered in the initial cycle may indicate an incorrect result. In this case, the noise is removed by setting the threshold higher than the noise and then read the result.
5. The positive control reactions should produce Ct value less than negative control. If expected positive reactivity is not achieved, invalidate the run and repeat the assay with stricter adherence to procedure guidelines. Determine the cause of failed PTC reactivity, implement corrective actions, and document results of the investigation and corrective actions.

■Interpretation of the test

For qualitative assay
Calculate the Ct value of each sample. Based on the criteria of the test, the samples are classed as follows:

Ct value	Virus status
> negative control	Negative
≤ negative control	Positive

For example

- Ct value of negative control : 38
- Ct value of sample : 35

This sample is classified as **positive**.

For quantitative assay

1. Make a plot of the threshold cycle (Ct) against standards (Standard curve), and then calculate the Ct value of each sample. The quantity of sample can be estimated by comparing the Ct value of samples with standard curve.
2. Test validation

The Ct value of each standard should be as follow

Standard 1 > Standard 2 > Standard 3 > Standard 4

The R-value of standard curve should be within 0.9 ~0.99.

The result of standards should be all in positive.

■Limitations of the test

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. **H5/N1 AIV Real-Time Detection Kit®** can be detected subtype H5/N1 avian influenza RNA with high sensitivity, but it may be caused of false negative result due to RNA mis-extraction, operating error from unskilled researcher, denaturation of the kit, and any other unknown various reason.

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