Molecular Diagnostic Kit

Influenza A (H1N1) Real-Time Detection Kit®

■Introduction

2009 H1N1 (sometimes called "swine flu") is a new influenza virus causing illness in people. This new virus was first detected in people in the United States in April 2009. This virus is spreading from person-to-person worldwide, probably in much the same way that regular seasonal influenza viruses spread. On June 11, 2009, the World Health Organization (WHO) signaled that a pandemic of 2009 H1N1 flu was underway.

■Principles

Influenza A (H1N1) Real-Time Detection Kit® from BIONOTE, Inc. is useful for detecting the infection of novel influenza (H1N1) virus. The kit can be exactly performed to detect RNA of novel influenza (H1N1) virus, so it can be used for qualitative analysis. It works most of real-time PCR apparatuses of block and capillary type.

■Materials provided (96Reactions/Kit)

No.	Product	Volume
1	Positive template Control (Red cap)	200µl x 1
2	Detection Solution Inf A*1 & RNaseP*2 (Brown cap)	460µl x 1
3	Detection Solution Novel InF A/H1*1 (Yellow cap)	460µl x 1
4	Nuclease Free Water (White cap)	1500µl x 2
5	2X Enzyme Buffer (Blue cap)	1000µl x 2
6	Enzyme Mix (Green cap)	80µl x 1
7	Rox Reference Dye *3(Pink cap)	80µl x 1

*1: Probe is labeled at the 5'-end with the reporter molecule 6-carboxy-fluorescein(**FAM**).

*2: Probe is labeled at the 5'-end with the reporter molecule 6-carboxy-fluorescein(**JOE**).

*3: The kits is provided extra Rox reference 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. Pipet 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 infection of novel influenza (H1N1) virus by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at 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 RNAEasy from Qiagen (Valencia, CA, USA) and Trizol from Life Technologies (Invitron, USA.
2. Negative Control
   To detect a potential contamination, run a negative control every time the kit is used. **Nuclease Free Water**④ should be used instead of template RNA.
3. Prepare a master mix by serially dispensing components to each tube in the following manner ;

Cautions: *Heat Detection Solution for 5 min at +85 ℃, and then chill on ice immediately. Spin briefly before use.*

Reagents	Volume per reaction
Each Detection Solution ②,③	N x 4.6µl
2X Enzyme Buffer ⑤	N x 10µl
Enzyme Mix ⑥	N x 0.4µl
Rox Reference Dye*(Optional) ⑦	N x (0.4µl)
Total volume	15µl

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

4. Set up reaction in strip tubes or 96-well plates and dispense 15µl of the master mix into each well going across the row as shown below:

Example Test Setup

	1	2	----	----	11	12
A	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
B	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1
C	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
D	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1
E	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
F	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1
G	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
H	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1

	1	2	----	----	11	12
A	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
B	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1
C	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
D	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1
E	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
F	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1
G	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP	Inf A&RP
H	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1	Inf A H1

Inf A : Influenza M2 gene
Inf A/H1 : New Influenza HA gene
RP : Internal control.(RNaseP)

Example Sample Setup

	1	2	----	----	11	12
A	NTC	S4	----	----	S40	S44
B	NTC	S4	----	----	S40	S44
C	S1	S5	----	----	S41	S45
D	S1	S5	----	----	S41	S45
E	S2	S6	----	----	S42	S46
F	S2	S6	----	----	S42	S46
G	S3	S7	----	----	S43	PTC
H	S3	S7	----	----	S43	PTC

	1	2	----	----	11	12
A	NTC	S50	----	----	S86	S90
B	NTC	S50	----	----	S86	S90
C	S47	S51	----	----	S87	S91
D	S47	S51	----	----	S87	S91
E	S48	S52	----	----	S88	S92
F	S48	S52	----	----	S88	S92
G	S49	S53	----	----	S89	PTC
H	S49	S53	----	----	S89	PTC

NTC : negative template control
S : samples
PTC : positive template control

5. Pipette 5μl of Nuclease Free Water into the NC wells. Cap NC well.
6. Cover the reaction plate and move the reaction plate to the nucleic acid handling area.
7. Vortex the tubes containing the samples for 5 sec. Centrifuge tubes for 5 sec.
8. Set up the extracted nucleic acid samples in the cold rack.
9. As shown above, samples can be added by column. Pipette 5 μl of the first sample into all the wells labeled for that sample (for example, Sample "S1" as shown above). Change tips after each addition.
10. Cap the column to which the sample has been added. This will help to prevent sample cross contamination and enable you to keep track of where you are on the plate.
11. Change gloves when necessary to avoid contamination.
12. Repeat steps 9. through 11. for the remaining samples.
13. Finally, pipette 5 μl of positive template control RNA into all PTC wells. Cap PC wells.
14. If using 8-tube strips, label the TAB of each strip to indicate sample position (DO NOT LABEL THE TOPS OF THE REACTION TUBES). Briefly centrifuge tube strips for 10-15seconds. Return strip tubes to cold rack.
    If using plates, centrifuge at 500 x g for 30 seconds at 4°C. Return to cold rack.
15. Perform the real time RT-PCR reaction under the below condition.

Cycles	Reaction	Temp. (℃)	Time
1	Reverse transcription	50℃	30 min.
1	Activation	95℃	2 min.
40	Amplification	95℃	15 sec.
		55℃	30 sec.

Fluorescence data(FAM or Joe) collection during 55℃ 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 45.
3. The Ct value of negative control reactions should be between 42 and 45. 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 threshould higher than the noise and then read the result.
5. When Inf A Ct value in Inf A & RP tube is less than 25 , it sometimes happens RNaseP Ct value to be no signal. In this case, you should read the result as Inf A positive..
6. 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.

■Calculation of the test

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 template control	Negative
≤ negative template control	Positive

For example
- Ct value of negative template control : 37
- Ct value of sample : 35

This sample is classified as **positive**.

■Interpretation of the test

Inf A	Inf A H1	RP	Result
Positive	Positive	Positive	**Influenza A** positive, **Novel influenza A (H1N1)** positive
Positive	Negative	Positive	**Influenza A** positive, Novel influenza A (H1N1) negative
Positive	Negative	Negative	**Influenza A** positive, Novel influenza A (H1N1) negative
Negative	Negative	Positive	Influenza A negative, Novel influenza A (H1N1) negative
Negative	Positive	Positive	Invalid*
Negative	Positive	Negative	Invalid*
Negative	Negative	Negative	Invalid*

*For invalid specimen result, the including specimen preparation, must be repeated.

■Limitations of the test

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. **Influenza A (H1N1) Real-Time Detection Kit®** can be detected novel influenza (H1N1) 2009 RNA with high sensitivity, but it may be caused of false negative result due to RNA misextraction, operating error from unskilled researcher, denaturation of the kit, and any other unknown various reason.

■Evaluation of Influenza A(H1N1) Real-Time Detection Kit®

Comparison of commercial H1N1 real-time PCR kit (Company A)and BioNote H1N1 real-time Detection Kit on sequence confirmed H1N1 samples.

	Bionote		Company A	
	Matrix gene	**Hemagglutinin gene**	**Matrix gene**	**Hemagglutinin gene**
Sensitivity	**99.20% (370/373*1)**	**100% (368/368*3)**	98.39% (367/373*1)	100% (368/368*3)
Sensitivity confidence interval (95%)	97.66~ 99.73%	98.98~ 100%	96.54~ 99.26%	98.98~ 100%
Specificity	**98.95% (662/669*2)**	**100% (670/670*4)**	99.40% (665/669*2)	94.63% (634/670*4)
Specificity confidence interval (95%)	97.86~ 99.49%	99.43~100%	98.47~ 99.77%	92.65~ 96.09%

*1 : H1N1 Matrix gene DNA sequencing Positive
*2 : H1N1 Matrix gene DNA sequencing Negaitive
*3 : H1N1 Hemagglutinin gene DNA sequencing Positive
*4 : H1N1 Hemagglutinin gene DNA sequencing Negaitive

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