



Real-time RT-PCR Protocol for the Detection of Avian Influenza A(H7N9) Virus

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The WHO Collaborating Center for Reference and Research on Influenza at the Chinese National Influenza Center, Beijing, China, has made available attached real-time RT-PCR protocol for the detection of avian influenza A(H7N9) virus.

It is strongly recommended that all unsubtypeable influenza A specimens should be immediately sent to one of the six WHO Collaborating Centres for Influenza in the Global Influenza Surveillance and Response System (GISRS)¹ for testing and analysis.

For further information please contact us at: gisrs-whohq@who.int

¹ http://www.who.int/influenza/gisrs_laboratory/collaborating_centres/list/en/

Real-time RT PCR (rRT-PCR) Protocol for the Detection of A(H7N9) Avian Influenza Virus (April 15, 2013)

The protocol is developed by and belongs to the WHO Collaborating Centre in Beijing. It is made available for emergency use as a service to the public health. It is not for commercial development or for profit.

1. Purpose

To specifically detect avian influenza A(H7N9) virus using real-time RT-PCR with specific primers and probes targeting the matrix, H7 and N9 genes.

2. Materials and equipments

2.1 Real-time fluorescence quantitative PCR analysis system

2.2 Bench top centrifuge for 1.5mL Eppendorf tubes

2.3 10, 200, 1000 μ L pipettors and plugged tips

2.4 Vortex

2.5 QIAGEN RNeasy Mini Kit

2.6 AgPath one-step RT-PCR kit

2.7 The specific primers and probes for the H7 and N9 genes are summarized in the table below. In addition, the use of a primer and probe targeted M gene and house-keeping gene such as RNP is recommended for typing all influenza A virus and internal control in the tests.

Table of PCR primers and probes

ID	Sequence	Note
H7		
CNIC-H7F	5'-AGAAATGAAATGGCTCCTGTCAA-3'	Primer
CNIC-H7R	5'-GGTTTTTCTTGTATTTTATATGACTTAG-3'	Primer
CNIC-H7P	5'FAM-AGATAATGCTGCATTCCCGCAGATG-BHQ1-3'	Probe
N9		
CNIC-N9	5' -TAGCAATGACACACACTAGTCAAT-3'	Primer
CNIC-N9R	5' -ATTACCTGGATAAGGGTCATTACACT-3'	Primer
CNIC-N9P	5'FAM- AGACAATCCCCGACCGAATGACCC -BHQ1-3'	Probe
FluA		
InfA Forward	5' GACCRATCCTGTACCTCTGA C 3'	Primer
InfA Reverse	5' AGGGCATTYTGGACAAKCGTCTA3'	Primer
InfA Probe1	5' FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1-3'	Probe
RnaseP		
RnaseP Forward	5' AGATTTGGACCTGCGAGCG 3'	Primer
RnaseP Reverse	5' GAGCGGCTGTCTCCACAA GT3'	Primer
RnaseP Probe1	5'FAM-TTCTGACCTGAA GGCTCTGCGCG-BHQ1-3'	Probe

Note: FluA and RNase primer/probe sets were from published WHO protocol provided by CDC, Atlanta.

2.8 Other materials: RNase-free 1.5mL eppendorf tubes, RNase-free 0.2mL PCR tubes, powder-free disposables latex glove, goggles, headgear, shoe cover, tips for pipettors, β - thioglycol, 70% alcohol.

3. Biosafety

The lysis of the specimen (500 μ L lysis buffer with 200 μ L clinical samples is recommended) should be to be carried out in a BSL-2 facility with BSL-3 level personal protection equipment. Subsequent procedures can be performed in a BSL-2 laboratory which has separate rooms including reagent preparation area, specimen preparation area and amplification/detection area. The DNA-free area is the clean area and the area of amplified DNA is the dirty area. The work flow is from clean to dirty areas.

4. Procedures

4.1 Nucleic acid extraction

The procedure is performed in a BSL-2 biohazard hood in the specimen preparation area according to the manufacturer. Elution of the RNA using a final volume of 50 μL H_2O is recommended.

4.2 Quality control parameters

Negative control: Sterile water is extracted as a negative control at the same time as the nucleic acid extraction of the other specimens.

Reagent blank control: RNase free H_2O .

Positive control: RNA of the A(H7N9) virus provided.

Internal positive control: ribonucleoprotein (RNP) is recommended.

4.3 The reaction system preparation

(1) Thaw the RT-PCR Master Mix, primers and probes at room temperature in the reagent preparation area of the BSL-2 facility.

(2) Prepare reaction mixture. Different primer pairs and probes should be prepared in different tubes respectively. For each reaction:

Components	volume (μL)
2x RT-PCR Master Mix	12.5
primer-forward (40 μM)	0.5
primer-reverse (40 μM)	0.5
Probe (20 μM)	0.5
25xRT-pcr enzymes mix	1
Template RNA	5.0
RNase Free H_2O	5
Total	25

4.4 Aliquot the reaction mixture into 0.2mL PCR tubes or a 96-well PCR plate as 20 μL per tube and label clearly.

4.5 Add five μL of the template RNA for the negative control, test specimens, or positive control into the separate tubes with the reaction mixture in a BSL-2 biohazard hood in the specimen preparation area.

4.6 Load the tubes in the PCR cycler for Real-time RT-PCR detection and use the following programme for cycling:

- (1) 45°C 10min
- (2) 95°C 10min
- (3) 95°C 15s
- (4) 60°C 45s

Return to the 3th step, and perform 40 cycles

4.7 Result analysis:

The results are determined if the quality controls work.

- (1) The specimen is negative if the value of Ct is undetectable,
- (2) The specimen is positive if Ct value is ≤ 38.0 .
- (3) It is suggested that specimens with a Ct higher than 38 are repeated.

The specimen can be considered positive if the repeat results are the same as before i.e. Ct is higher than 38. If the repeat Ct is undetectable, the specimen is considered negative.

4.8 Criteria for quality control:

- (1) The result of the negative control should be negative.
- (2) The Ct value of positive control should not be more than 28.0.
- (3) Otherwise, the test is invalid.

5. Troubleshooting

- 5.1 False positives may be due to environmental contamination if there is amplification detected in the negative control and reagent blank control. The unidirectional work flow must be strictly obeyed. The following measures should be taken should there be false positives: ventilate the labs, wash and clean the workbench, autoclave centrifuge tubes and tips, and use fresh reagents.
- 5.2 RNA degradation should be taken into consideration if the Ct value of the positive control is more than 30. All materials should be RNase-free.

6. Cautions

- 6.1 In order to avoiding nucleic acid cross-contamination, add the negative control to the reaction mixture first, then the specimen, followed by the positive control respectively.
- 6.2 Dedicated equipment for each area including lab coats, pipettors, plugged tips and powder-free disposal latex glove are required.
- 6.3 Follow the instructions for maintenance of the incubator, PCR cycler and pipettors. Calibration should be performed every 6 months.

7. Protocol Use Limitations

These protocols were optimized using the quantitative one-step probe RT-PCR (AgPath one-step RT-PCR kit) that have been shown to produce comparable results on 96-well format thermocycler systems such as Stratagene QPCR instruments (MX3000®or MX3005®).