**ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE**
**EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE**

# SOP VIR 1001
# DETECTION OF EURASIAN H7 AVIAN INFLUENZA VIRUS BY REAL-TIME RT-PCR
# (Van Borm et al., 2010 modified)

Standard operating procedure used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 07/04/2021.

# Contents


1. **Purpose and field of application** ... 3
2. **References** ... 3
3. **Safety** ... 3
4. **Materials** ... 3
4.1. **Reagents** ... 3
4.2. **Equipment** ... 4
5. **Procedure** ... 4
5.1. **Controls** ... 4
5.2. **Preparation of samples and storage** ... 4
5.3. **Isolation of nucleic acids** ... 4
5.4. **Real-time RT-PCR** ... 5
5.5. **Data analysis** ... 6
6. **Interpretation of results** ... 6
6.1. **Reliability of controls** ... 6
6.2. **Diagnostic samples** ... 7
7. **Characteristics of the method** ... 7

## 1. Purpose and field of application

This protocol describes the procedure to detect Eurasian H7 avian influenza virus (AIV) in biological samples by real-time reverse transcription polymerase chain reaction (RRT-PCR).

The protocol targets the segment 4 region encoding the HA2 subunit of the H7 subtype, based on the assay developed by Van Borm et al. (2010), adopting the probe as modified by Hoffmann et al. (2016). Modifications to the original method pertain: cycling conditions (i.e. annealing temperature) and real-time PCR apparatus.

The procedure can be applied to RNA purified from AIV isolates (normally allantoic fluid), tissue homogenates, stool, tracheal and cloacal swabs properly collected and preserved.

## 2. References


- S. Van Borm, D.L. Suarez, M. Boschmans, O. Ozhelvaci, S Marché, T.P. van den Berg. Rapid detection of Eurasian and American H7 subtype influenza A viruses using a single TaqManMGB real-time RT-PCR. Avian Dis 54(1 Suppl):632-8, 2010. doi: 10.1637/8734-032509-ResNote.1;
- B. Hoffmann, D. Hoffmann, D. Henritzi, M. Beer, T.C. Harder. Riems influenza A typing array (RITA): an RT-qPCR-based low density array for subtyping avian and mammalian influenza A viruses. Sci Rep 6:27211, 2016. doi: 10.1038/srep27211;
- Commission Decision 2006/437/EC approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC;
- OIE - World Organization for Animal Health, Terrestrial Manual, Chapter 3.3.4. Avian influenza (Version adopted in May 2015);
- SOP VIR 1000 - Sample preparation and nucleic acids isolation for the detection and typing of Avian influenza virus and Avian Orthoavulavirus type 1 by molecular methods.


## 3. Safety

Individual laboratories are responsible for ensuring that all the procedures described in this document are conducted under high safety standards, including awareness on chemical and biological risks. According to the risk hazard, either BSL2 or BSL3 facilities must be used. Safety rules at individual laboratories must be agreed with the biosecurity and biosafety officer and acknowledged by all the staff members involved.

## 4. Materials

### 4.1. Reagents

For commercial products, refer to the manufacturer's instructions for storage conditions.

- Nuclease-free water;
- Oligonucleotides resuspension buffer (e.g. TE pH 8.0);
- QuantiTect Probe RT-PCR Kit (Qiagen);
- Sense primer FeurH7 5'-GYA GYG GYT ACA AAG ATG TG-3';
- Antisense primer ReurH7 5'-GAA GAC AAG GCC CAT TGC AA-3';

- Probe IAV-HA7-CODA-FAM 5’-FAM-TGG TTT AGC TTC GGG GCA TCA TG-BHQ1-3’;
- RNase Inhibitor 40U/µl.

### 4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- CFX96 Touch Deep Well Real-time PCR Detection System (Biorad).

## 5. Procedure

### 5.1. Controls

To ensure test reliability, the controls listed in Table 1 must be included in each run.

| Control Type | Definition |
|---|---|
| Negative process control (NPC) | Sample containing no target organism that is processed along with the samples starting from the nucleic acids isolation phase |
| Positive template control (amplification control, PTC) | Sample containing a known amount of the target RNA of the AIV H7 subtype and stored at ≤ -70°C, that is processed along with the samples st arting from the RRT-PCR phase |
| Negative template control (NTC) | Sample containing all PCR reagents but no target RNA, that is processed along with the samples starting from the RRT-PCR phase |

**Table 1.** Controls to ensure test reliability

This procedure is applied as a downstream method to samples which have tested positive for AI by a different molecular diagnostic assay. In case this procedure is applied as a frontline method from a new sample aliquot, a positive process control (PPC) (i.e. sample containing the target organism that is processed along with the samples starting from the nucleic acids isolation phase) can be used to assess the reliability of the analytical process.

### 5.2. Preparation of samples and storage

For samples preparation (allantoic fluid, tissues, stool, tracheal and cloacal swabs), see SOP VIR 1000.
Clinical samples and viral isolates must be stored at refrigerated temperature (2-8°C) until the compl etion of the analysis. For long term conservation, store samples at ≤ -70°C.
Purified nucleic acids can be refrigerated for a few hours prior RRT-PCR, otherwise they must be stored at ≤ -70°C.

### 5.3. Isolation of nucleic acids

RNA can be isolated either by manual or automatic methods.
The IZSVe validated the following nucleic acids isolation systems:

- NucleoSpin RNA kit (Macherey-Nagel);
- QIAamp Viral RNA Mini Kit (Qiagen);
- QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen) on the QIAsymphony SP instrument (Qiagen);

- MagMAX Pathogen RNA/DNA Kit (Applied Biosystems) on the KingFisher Flex Magnetic Particle Processor (Thermofisher Scientific).

### 5.4. Real-time RT-PCR

The preparation of the master mix for the RRT-PCR reaction has to be carried out in a clean dedicated area, according to the specifications reported in Table 2. The reagents should be kept refrigerated throughout the entire preparation phase. For the whole procedure, the use of filter tips and nuclease-free plastics is recommended.

**Reaction mix preparation**

Prepare a master mix volume sufficient for the number of samples to be tested in each run. The volumes reported in Table 2 are per single reaction.

| Component | Initial concentration | Final concentration | µl per reaction |
|---|---|---|---|
| Nuclease-free water | - | - | 7.15 |
| 2x QuantiTect Probe RT-PCR Master Mix | 2X | 1X | 12.5 |
| Sense primer FeurH7 | 20 µM | 0.9 µM | 1.25 |
| Antisense primer ReurH7 | 20 µM | 0.9 µM | 1.25 |
| Probe IAV-HA7-CODA-FAM | 10 µM | 0.2 µM | 0.5 |
| QuantiTect RT Mix | - | - | 0.25 |
| RNase Inhibitor | 40 U/µl | 4 U | 0.1 |
| Master mix minus template | | | 23 |
| Template | | | 2 |
| Total reaction volume | | | 25 |

**Table 2.** Volume and concentration of the reaction mix components

The master mix should be mixed thoroughly and 23 µl solution per sample must be pipetted in real-time PCR strip/plate.

Template (2 µl) must be added in a separate room. To minimize the risk of cross-contamination, it is recommended to add controls and samples in the following order: NTC, NPC, nucleic acids isolated from diagnostic samples, PTC. In case of NTC, 2 µl of nuclease-free water should be added.

**Cycling conditions**

Place the strip/plate in the real-time PCR apparatus. Set up the loading scheme as well as the thermal profile as per Table 3, and select a FAM compatible detection channel for fluorescence acquisition.

| Step | Temperature and time | Repetitions |
|---|---|---|
| Reverse transcription | 50°C for 30 min | 1 |
| Initial PCR activation | 95°C for 10 min | 1 |

| | | |
|---|---|---|
| Denaturation | 95°C for 15 sec | 45 |
| Annealing (*) | 56°C for 33 sec | |
| Extension | 72°C for 30 sec | |

**Table 3.** Real-time RT-PCR thermal profile. (*) Fluorescence acquisition has to be done during the annealing phase

### 5.5. Data analysis

Upon completion of the amplification reaction, amplification plots must be critically assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise, across the exponential phase of all the amplification curves (corresponding to the early linear phase of the logarithmic view). The use of a PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

## 6. Interpretation of results

### 6.1. Reliability of controls

Test reliability is assured if the controls yield the expected results, as reported in Table 4. In case of invalid results, the causative reason must be investigated and proper actions have to be taken.

| Control | Expected Result | Action in case of invalid control |
|---|---|---|
| PPC (if used) | Positive, i.e. increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve at the expected Ct value | Repeat the analysis from the nucleic acids extraction and check the PPC stock |
| NPC | Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve | Repeat the analysis from the nucleic acids extraction and check nucleic acids isolation reagents |
| PTC | Positive, i.e. increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve at the expected Ct value | Repeat the analysis from the RRT-PCR and check the PTC stock |
| NTC | Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve | Repeat the analysis from the RRT-PCR and check RRT-PCR reagents |

**Table 4.** Assessment of test reliability. Ct = threshold cycle

### 6.2. Diagnostic samples

Criteria for data interpretation of diagnostic samples and subsequent actions are reported in Table 5.

| Result | Interpretation and action |
|---|---|
| Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct ≤ 39 | Positive. Proceed with downstream molecular and/or virological analyses for H7 AIV pathotyping |
| Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve | Negative. No action has to be taken |
| Weak increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct between 39 and 45 | Dubious. Repeat the analysis from the nucleic acids extraction starting from a new aliquot of the sample together with the original extract. Alternatively, another confirmatory method (e.g. conventional RT-PCR for AIV H7 subtype) can be applied to the nucleic acids extracts.<br>If PCR inhibition is suspected, dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the analysis from the RRT-PCR and/or repeat the analysis from the nucleic acids extraction, further diluting the original biological sample (SOP VIR 1000).<br>In case of recurrent dubious results, it is responsibility of the Head of the Laboratory to identify the actions to be taken |

**Table 5.** Interpretation of real-time RT-PCR diagnostic results

## 7. Characteristics of the method

The IZSVe assessed the performances of the method employing AIV samples and clinical specimens available at the IZSVe repository, as well as materials, equipment and procedures as described above.

For any technical query, please contact eurl.ai.nd.secretariat@izsvenezie.it