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# SOP VIR 143
# DETECTION OF EURASIAN H5 AVIAN INFLUENZA VIRUS BY REAL-TIME RT-PCR
# (Slomka et al., 2007)

This protocol is a copy of the standard operating procedure used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 09/04/21.

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## 1. Purpose and field of application

This protocol describes the procedure to detect Eurasian H5 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 H5 subtype, based on the assay developed by Slomka et al. (2007). Modifications to the original method pertain: addition of an exogenous internal control 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

- M.J. Slomka, T. Pavlidis, J. Banks, W. Shell, A. McNally, S. Essen, I.H. Brown. Validated H5 Eurasian real-time reverse transcriptase-polymerase chain reaction and its application in H5N1 outbreaks in 2005-2006. Avian Dis 51(1 Suppl):373-7, 2007. doi: 10.1637/7664-060906R1.1;
- B. Hoffmann, K. Depner, H. Schirrmeier, M. Beer. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods 136(1-2):200-9, 2006. doi: 10.1016/j.jviromet.2006.05.020;
- 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;
- IZSVe PDP VIR 143.

## 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);
- Intype IC-RNA (Indical Bioscience) (required for the nucleic acids isolation phase);
- Qiagen OneStep RT-PCR Kit (Qiagen);

- $MgCl_2$ 25 mM;
- Sense primer H5LH1 5'-ACA TAT GAC TAC CCA CAR TAT TCA G-3';
- Antisense primer H5RH1 5'-AGA CCA GCT AYC ATG ATT GC-3';
- Probe H5PRO 5'-FAM- TCW ACA GTG GCG AGT TCC CTA GCA-TAMRA-3';
- Sense primer EGFP-11-F 5'-CAG CCA CAA CGT CTA TAT CAT G-3';
- Antisense primer EGFP-2-R 5'-GAA CTC CAG CAG GAC CAT G-3';
- Probe EGFP-Cy5 5'-Cy5-AGC ACC CAG TCC GCC CTG AGC A-BHQ2-3';
- RNase Inhibitor 40U/µl.

### 4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- CFX96 Touch Deep Well Real-time PCR Detection System (Biorad), RotorGene Q (Qiagen), RotorGene 6000 (Corbett) (or equivalent real-time PCR platform).

## 5. Procedure

### 5.1. Controls

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

| Control Type | Definition |
| --- | --- |
| Internal control (IC) | Non target RNA sequence contained in each sample, and subject to the whole analytical process, starting from the nucleic acids isolation phase |
| 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 H5 subtype and stored at ≤ -70℃, 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

The use of the internal control (IC) is usually recommended to prevent false negative results arising from PCR inhibition or failure in nucleic acids extraction. However, because this procedure is applied as a downstream method to samples which have tested positive for AI by a different molecular diagnostic assay, the use of the IC can be omitted to limit the unnecessary consumption of reagents. Contrarily, in case this procedure is applied as a frontline method from a new sample aliquot, the use of the IC is recommended. Whenever the internal control cannot be adopted, 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) should be used instead 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℃) until the compl etion of the analysis. For long term conservation, store samples at ≤ -70℃.

Purified nucleic acids can be refrigerated for a few hours prior RRT-PCR, otherwise they must be stored at ≤ -70℃.

### 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).

The intype IC-RNA must be added to each sample (including the NPC) during the lysis phase. The volume of intype IC-RNA to be spiked into each sample should be 1:10 of the elution volume, as recommended by the manufacturer. For additional details on the nucleic acids isolation procedures, see SOP VIR 1000.

### 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 Tables 2 and 3. 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.

**IC-RNA assay pre-mix preparation**

The IC-RNA oligonucleotides pre-mix can be prepared in advance and stored until use. The volumes reported in Table 2 are sufficient for approximately 100 RRT-PCR reactions.

| Component | Initial concentration | Final concentration | µl per reaction |
|---|---|---|---|
| TE pH 8.0 | - | - | 186.25 |
| Sense primer EGFP-11-F | 100 µM | 2.5 µM | 5 |
| Antisense primer EGFP-2-R | 100 µM | 2.5 µM | 5 |
| Probe EGFP-Cy5 | 100 µM | 1.8 µM | 3.75 |
| Total reaction volume | | | 200 |

**Table 2.** Volume and concentration of the IC-RNA pre-mix components

**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 3 are per single reaction.

| Component | Initial concentration | Final concentration | µl per reaction |
|---|---|---|---|
| RNase-free water | - | - | 11 |
| Qiagen OneStep RT-PCR Buffer | 5X | 1X | 5 |
| dNTP Mix | 10 mM | 0.4 mM | 1 |
| $MgCl_2$ | 25 mM | 1.25 mM | 1.25 |
| Sense Primer H5LH1 | 50 µM | 0.4 µM | 0.2 |
| Antisense Primer H5RH1 | 50 µM | 0.4 µM | 0.2 |
| Probe H5PRO | 6 µM | 0.3 µM | 1.25 |
| IC-RNA assay pre-mix | - | - | 2 |
| RNase Inhibitor | 40 U/µl | 4 U | 0.1 |
| Qiagen OneStep RT-PCR Enzyme Mix | - | - | 1 |
| Master mix minus template | | | 23 |
| Template | | | 2 |
| Total reaction volume | | | 25 |

**Table 3.** Volume and concentration of the reaction mix components. If the internal control is not used, replace the IC-RNA assay premix volume with 2 µl of nuclease-free water

The master mix should be mixed thoroughly and 23 µl solution per sample must be pipetted in real-time PCR tube/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 tube/strip/plate in the real-time PCR apparatus. Set up the loading scheme as well as the thermal profile as per Table 4, and select Cy5 and FAM compatible detection channels for fluorescence acquisition.

| Step | Temperature and time | Repetitions |
|---|---|---|
| Reverse transcription | 50°C for 30 min | 1 |
| Initial PCR activation | 95°C for 15 min | 1 |
| Denaturation | 95°C for 10 sec | 40 |
| Annealing (*) | 54°C for 30 sec | |
| Extension | 72°C for 10 sec | |

**Table 4.** 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 5. 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 |
|---|---|---|
| IC (if used) | Positive, i.e. increase in fluorescence from the Cy5 fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct ≤ 30 in all samples, including NPC | If an invalid IC result (negative or with Ct > 30) is associated with a negative/dubious AIV sample, to rule out inhibition issues dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the analysis from the RRT-PCR; in case of recurrent invalid IC, repeat the analysis from the nucleic acids extraction, further diluting the original biological sample (SOP VIR 1000).<br>Notably, an invalid IC can occur in positive AIV samples, typically characterized by a high viral load, as a result of competition for reagents during the duplex amplification reaction. In this case, the sample can be considered conforming and no action has to be taken |
| 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 |
| 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 Cy5 and FAM fluorophores, with no sigmoidal (or logarithmic) amplification curve | Repeat the analysis from the RRT-PCR and check RRT-PCR reagents |
|---|---|---|

**Table 5.** 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 6.

| Result | Interpretation and action |
|---|---|
| Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct ≤ 35, associated with any Ct value for the IC | Positive. Proceed with downstream molecular and/or virological analyses for H5 AIV pathotyping |
| Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve, associated with a Ct value ≤ 30 for the IC | 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 35 and 40, associated with a Ct value ≤ 30 for the IC | 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 H5 subtype) can be applied to the nucleic acids extracts.<br>In case of recurrent dubious results, it is responsibility of the Head of the Laboratory to identify the actions to be taken |

**Table 6.** Interpretation of real-time RT-PCR diagnostic results

## 7. Characteristics of the method

This method was validated and accredited at the IZSVe according to the ISO/IEC 17025, employing AIV samples, selected avian viruses and bacteria available at the IZSVe repository, as well as materials, equipment and procedures as described above. The validation dossier is accessible upon request by contacting eurl.ai.nd.secretariat@izsvenezie.it

The method usually yields positive results for samples with Ct values < 35 by M-gene real time RT-PCR. However, sensitivity can be strain-dependent and might be affected by poor RNA quality, low viral load and the presence of PCR inhibitors.

Any modification to this SOP by third party laboratories should be supported by proper validation data assessing that the method is still fit-for-purpose.