ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 014

DETECTION OF H9 AVIAN INFLUENZA VIRUS BY REAL-TIME RT-PCR (Panzarin et al., 2022)

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 17/06/2022.

The information in this document is subject to change without notice.

Revision history

Revision	Date	Description
ed. 01	June 2022	• Updated references • Updated reagents and amplification protocol • Minor edits for style and consistency
ed. 00	April 2021	Initial release

Contents

1. Purpose and field of application

This protocol describes the procedure to detect H9 avian influenza virus (AIV) in biological samples by real-time reverse transcription polymerase chain reaction (RRT-PCR). Target of the protocol is the segment 4 region encoding the HA2 subunit of the H9 subtype, based on the assay developed by Panzarin et al. (2022). This is an updated version of the assay originally developed by Monne et al., (2008) to increase sensitivity and detect any of the Y439, Y280 and G1 clades.

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

- V. Panzarin, S. Marciano, A. Fortin, I. Brian, V. D'Amico, F. Gobbo. F. Bonfante, E. Palumbo, Y. Sakoda, K.T. Le, D.-H. Chu, I. Shittu, C. Meseko, A.M. Haido, T. Odoom, M.N. Diouf, F. Djegui, M. Steensels, C. Terregino, I. Monne. Redesign and validation of a real-time RT-PCR to improve surveillance for avian influenza viruses of the H9 subtype. Viruses 14(6):1263, 2022. doi: 10.3390/v14061263;
- 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;
- WOAH - World Organization for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.3.4. Avian influenza (including infection with high pathogenicity avian influenza viruses) (Version adopted in May 2021);
- 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 014.

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

- Nuclease-free water;

- Oligonucleotides resuspension buffer (e.g. TE pH 8.0);
- Intype IC-RNA (Indical Bioscience) (required for the nucleic acids isolation phase);
- AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems);
- Sense primer Pan-H9 for 5'-ATR GGG TTT GCT GCC-3';
- Antisense primer Pan-H9 rev1 5'-TCA TAT ACA AAT GTT GCA YCT G-3';
- Antisense primer Pan-H9 rev2 5'-TTA TAT ACA GAT GTT GCA YCT G-3';
- Probe Pan-H9 (*) 5'-FAM-TTC TGG GCY A**T**G **T**CH AAY GG-BHQ1-3';
- 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.

(*) LNA-modified bases of Probe Pan-H9 are in bold and underlined.

4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- CFX96 Touch Deep Well Real-time PCR Detection System (Biorad) (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 H9 subtype and stored at ≤ -70°C, that is processed along with the samples starting from the RRT-PCR phase
Negative template control (NTC)	Sample containing all PCR reagents except for 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, considering that such 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°C) until the completion 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 Purification System (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 Table 2 and Table 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 beforehand and stored until use. The volumes reported in Table 2 are sufficient for approximately 100 RRT-PCR reactions.

Component	Initial concentration	Final concentration	Tot µl
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	1.875 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
Nuclease-free water	-	-	1.5
2X RT-PCR Buffer	2X	1X	12.5
Sense primer Pan-H9 for	10 µM	0.4 µM	1
Antisense primer Pan-H9 rev1	10 µM	0.2 µM	0.5
Antisense primer Pan-H9 rev2	10 µM	0.2 µM	0.5
Probe Pan-H9	10 µM	0.2 µM	0.5
IC-RNA assay pre-mix	-		2
RNase Inhibitor	40U/µl	20U/rxn	0.5
25X RT-PCR Enzyme Mix	25X	1X	1
Master mix minus template			20
Template			5
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 20 µl solution per sample must be pipetted in real-time PCR tube/strip/plate.

Template (5 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, PPC (if used), PTC. In case of NTC, 5 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 FAM and Cy5 (if using IC-RNA) compatible detection channels for fluorescence acquisition.

Step	Temperature and time	Repetitions
Reverse transcription	50°C for 10 min	1
RT inactivation/initial denaturation	95°C for 10 min	1

Denaturation	95°C for 15 sec	45
Annealing (*)	54°C for 30 sec	
Extension	72°C for 15 sec	

Table 4. Real-time RT-PCR thermal profile. (*) Fluorescence acquisition

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 (c.ca 50 RFU), 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 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 H9 sample, dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the analysis from the RRT-PCR to rule out inhibition issues; in case of recurrent invalid IC, repeat the analysis from the nucleic acids extraction, further diluting the original biological sample (SOP VIR 1000). Notably, an invalid IC may occur in positive H9 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 for H9 (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	Repeat the analysis from the RRT-PCR and check the PTC stock

	sigmoidal (or logarithmic) amplification curve at the expected Ct value	
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 (if used)	Positive. If required, proceed with downstream molecular and/or virological analyses for H9 characterization
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve, associated with a Ct value ≤ 30 for the IC (if used)	Negative. No action has to be taken
Weak increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct > 35, associated with a Ct value ≤ 30 for the IC (if used)	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 can be applied to the nucleic acids extracts. In case of recurrent dubious results, it is responsibility of the Head of the Laboratory to identify the actions to be taken
If the IC is used: absence of fluorescence increase from the FAM and Cy5 fluorophores, with no sigmoidal (or logarithmic) amplification curves	Invalid. Refer to Table 5 for actions. A new sampling might be required

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

7. Characteristics of the method

This method was validated 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 < 36 by M-gene real time RT-PCR, with comparable sensitivity. 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.