ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 144

DETECTION OF EURASIAN H7 AVIAN INFLUENZA VIRUS BY REAL-TIME RT-PCR

(Slomka et al. 2009)

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.

Contents

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 Slomka et al. (2009). Modifications to the original method pertain: addition of an exogenous internal control, lack of ROX passive dye, real-time PCR apparatus and diagnostic cut-off.

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, V.J. Coward, J. Voermans, G. Koch, A. Hanna, J. Banks, I.H. Brown. Validated RealTime reverse transcriptase PCR methods for the diagnosis and pathotyping of Eurasian H7 avian influenza viruses. Influenza Other Respir Viruses 3(4):151-164, 2009. doi: 10.1111/j.1750-2659.2009.00083.x;
- 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 144.

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 LH6H7 5'-GGC CAG TAT TAG AAA CAA CAC CTA TGA-3';
- Antisense primer RH4H7 5'-GCC CCG AAG CTA AAC CAA AGT AT-3';
- Probe H7pro11 5'-FAM-CCG CTG CTT AGT TTG ACT GGG TCA ATC T-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.

4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- 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 H7 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	-	-	11.625
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 LH6H7	50 µM	0.4 µM	0.2
Antisense Primer RH4H7	50 µM	0.4 µM	0.2
Probe H7pro11	6 µM	0.15 µM	0.625
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 tubes.

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 tubes 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℃ for 30 min	1
Initial PCR activation	95℃ for 15 min	1
Denaturation	95℃ for 10 sec	40
Annealing (*)	54℃ for 30 sec	
Extension	72℃ 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). 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 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 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 H7 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 H7 subtype) 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

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.