ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 1003

DETECTION OF TYPE A INFLUENZA VIRUS BY REAL-TIME RT-PCR (Nagy et al., 2021)

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

This protocol describes the procedure to detect type A influenza virus (AIV) of all subtypes and species of origin, in biological samples by real-time reverse transcription polymerase chain reaction (RRT-PCR).

The protocol targets segment 7 encoding the matrix protein (MP) of any AIV subtype, based on the assay developed by Nagy et al. (2021). Reagents and equipment reported in this procedure have been selected among those recommended by the authors in the original paper.

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

- A. Nagy, L. Černíková, K. Kunteová, Z. Dirbáková, S.S. Thomas, M.J. Slomka, A. Dán, T. Varga, M. Máté, H. Jiřincová, I.H. Brown. A universal RT-qPCR assay for “One Health” detection of influenza A viruses. PloS One 16(1):e0244669, 2021. doi: 10.1371/journal.pone.0244669;
- OIE - World Organization for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.3.4. Avian influenza (Infection with avian influenza viruses) (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 SVIP-MP-F 5’-GGC CCC CTC AAA GCC GA-3’;
- Antisense primer SVIP-MP-R 5’-CGT CTA CGY TGC AGT CC-3’;
- Probe SVIP-MP_P2-MGB 5’-FAM-TCA CTK GGC ACG GTG AGC GT-MGB-3’.

4.2. Equipment

- General molecular biology laboratory equipment;

- 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
Positive process control (PPC)	Sample containing the target organism that is processed along with the samples 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 AIV and stored at ≤ -70℃, that is processed along with the samples starting 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

5.2. Samples preparation 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 this method in combination with the QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen) on the QIAsymphony SP instrument testing swabs, tissue homogenates and stool.

For additional details on the nucleic acids isolation procedure, 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. 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	-	-	3.72
2x QuantiTect Probe RT-PCR Master Mix	2X	1X	12.5
Sense primer SVIP-MP-F	10 µM	0.6 µM	1.5
Antisense primer SVIP-MP-R	10 µM	0.6 µM	1.5
Probe SVIP-MP_P2-MGB	10 µM	0.21 µM	0.53
QuantiTect RT Mix	-	-	0.25
Master mix minus template			20
Template			5
Total reaction volume			25

Table 2. Volume and concentration of the reaction mix components

The master mix should be mixed thoroughly and 20 µl solution per sample must be pipetted in real-time PCR 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, PTC. In case of NTC, 5 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. When using a CFX96 Touch Deep Well Real-time PCR Detection System (Biorad), a ramp/rate of 1℃/sec between the annealing and extension steps should be set.

Step	Temperature and time	Repetitions
Reverse transcription	50℃ for 30 min	1
PCR initial activation	95℃ for 15 min	1
Denaturation	95℃ for 10 sec	45
Annealing (*)	64℃ for 30 sec	
Extension	72℃ for 10 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	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 ≤ 36	Positive. Proceed with downstream molecular and/or virological analyses for AIV subtype identification
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 36 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 screening method can be applied to the nucleic acids extracts for confirmatory diagnosis. 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). 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

This method was extensively validated by NRLs and veterinary/human diagnostic laboratories in the Czech Republic, the Slovak Republic, the United Kingdom and Hungary. 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. The report is accessible upon request by contacting eurl.ai.nd.secretariat@izsvenezie.it

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.