ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 1005

MOLECULAR PATHOTYPING OF EURASIAN H5 AVIAN INFLUENZA VIRUS BY REAL-TIME RT-PCR

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 28/07/26.

The content of this document is subject to modification without prior notice.

The commercial products referenced in the SOP are the ones currently in use at the EURL for AI/ND. Their inclusion is intended exclusively to support diagnostic laboratories in the implementation of the protocol and does not constitute any form of commercial promotion. The use of alternative reagents or instruments that prove fit for the intended purpose is not restricted under any circumstances.

Revision history

Revision	Date	Description
ed. 01	July 2026	• Updated references • Updated field of application and extension to mammalian samples and dairy cattle milk samples • Updated probe sequence • Updated criteria for results interpretation • Minor edits to improve clarity and format harmonization
ed. 00	March 2022	Initial release

Contents

1. Purpose and field of application

This procedure applies to Eurasian avian influenza viruses (AIV) of the H5 subtype, and describes the protocols to discriminate highly pathogenic viruses (HPAIV) of the goose/Guangdong (gs/GD) lineage, clade 2.3.4.4b and low pathogenic viruses (LPAIV) by real-time reverse transcription polymerase chain reaction (RRT-PCR). The procedure can be applied to RNA purified from AIV isolates, organ/tissue homogenates, stool and swabs either from birds and mammals and dairy cattle milk properly collected and preserved.

The protocol targets different regions of segment 4 encoding the hemagglutinin (HA) gene of H5 Eurasian viruses, corresponding to the HA1 subunit and to the endoproteolytic cleavage site (CS), and consists of two independent RRT-PCR reactions to be performed in parallel:

- a duplex reaction consisting of the 'H5-HPAI CS assay' and the 'clade 2.3.4.4b assay'
- a simplex reaction consisting of the 'H5-LPAI CS assay'

The assays are intended as downstream methods for H5 confirmed cases. However, under epidemic circumstances, and where the epidemiological context has been established, the procedure may also be applied downstream of M-gene screening to support the early identification of clade 2.3.4.4b viruses in the following situations: i) to quickly confirm a strong clinical suspicion (e.g. abnormal mortality during an HPAI epidemic), ii) to confirm secondary cases epidemiologically liked to H5 primary cases for which the CS sequence was obtained, iii) in case of widespread circulation of H5 in wild birds within a defined geographical area. Importantly, for the first outbreak in any disease-free area and for those cases in which epidemiological information is limited or unavailable, or clinical evidences is inconclusive (e.g. absence of mortality in suspected HPAI outbreaks) it is highly recommended to confirm viral pathotype by sequencing.

2. References

- 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 September 2025);
- 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 1005.

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 assessment, 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);
- AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems);
- Sense primer H5HP_DE_22_F3 5'-CCTTGCGACTGGGCTYAG-3'
- Antisense primer H5HP_R2 5'-ATCAACCATTCCCTGCCA-3'
- Probe H5HP_ 25_probe 5'-FAM-AGRAGAAAAAGRGGCCTGTTTGGGGC-BHQ1-3'
- Sense primer H5_2.3.4.4_F_2022 5'-ACCAGGGAGCRCCYTCCT-3';
- Antisense primer H5_2.3.4.4_R_2022 5'-GTATTATTGTAGCTTATCTTTATTG-3';
- Probe H5_2.3.4.4_probe_2022 5'-Cy5-TTGGGTATGCATCGTTCTTTTTGATAAGCCA-BHQ2-3';
- Sense primer H5LP-EA_F_2022 5'-CCCAAATAYGTGAAATCAGAT-3';
- Antisense primer H5LP-EA_R_2022 5'-GCCAYCCTCCTTCTATAAAG-3';
- Probe H5LP1_EA_probe_2022 5'-FAM-CCAAAYAGYCCTCTYGTTTCT-MGB-3'.

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 and for all the designated RRT-PCR assays/targets.

Control Type	Definition
Positive template control (amplification control, PTC)	Sample containing a known amount of the target RNA 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 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 and for the H5 subtype by a screening and a subtyping molecular diagnostic assay. In case this procedure is applied as a frontline method from a new sample aliquot, a negative process control (NPC) (i.e. sample containing no target organism that is processed along with the samples starting from the nucleic acids isolation phase), and 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 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 list of nucleic acids isolation systems validated at the IZSVe on different types of matrices and related procedures are available in 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. The reagents should be kept refrigerated throughout the entire preparation phase. Probes should be protected from light. For the whole procedure, the use of filter tips and nuclease-free plastics is recommended.

Oligonucleotides pre-mixes preparation

Oligonucleotides pre-mixes can be prepared in advance and stored until use. The volumes reported in Tables 2-4 are sufficient for approximately 100 RRT-PCR reactions.

Component	Initial concentration	µl
Nuclease-free water	-	165
Sense primer H5HP_DE_22_F3	100 µM	15
Antisense primer H5HP_R2	100 µM	15
Probe H5HP_25_probe	100 µM	5
Total volume		200

Table 2. Volume of the ‘H5-HPAI CS assay’ pre-mix components

Component	Initial concentration	µl
Nuclease-free water	-	165
Sense primer H5_2.3.4.4_F_2022	100 µM	15
Antisense primer H5_2.3.4.4_R_2022	100 µM	15
Probe H5_2.3.4.4_probe_2022	100 µM	5
Total volume		200

Table 3. Volume of the ‘clade 2.3.4.4b assay’ pre-mix components

Component	Initial concentration	µl
Nuclease-free water	-	165
Sense primer H5LP-EA_F_2022	100 µM	15
Antisense primer H5LP-EA_R_2022	100 µM	15
Probe H5LP1_EA_probe_2022	100 µM	5
Total volume		200

Table 4. Volume of the 'H5-LPAI CS assay' 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 Tables 5 and 6 are per single reaction.

Component	Initial concentration	Final concentration	µl per reaction
Nuclease-free water	-	-	2.5
2X RT-PCR Buffer	2X	1X	12.5
'H5-HPAI CS assay' pre-mix	-	0.6 µM primers, 0.2 µM probes	2
'clade 2.3.4.4b assay' pre-mix	-		2
25X RT-PCR Enzyme Mix	25X	1X	1
Master mix minus template			20
Template			5
Total reaction volume			25

Table 5. Volume and concentration of the reaction mix components for the duplex RRT-PCR reaction

Component	Initial concentration	Final concentration	µl per reaction
Nuclease-free water	-	-	4.5
2X RT-PCR Buffer	2X	1X	12.5
'H5-LPAI CS assay' pre-mix	-	0.6 µM primers, 0.2 µM probe	2
25X RT-PCR Enzyme Mix	25X	1X	1
Master mix minus template			20
Template			5
Total reaction volume			25

Table 6. Volume and concentration of the reaction mix components for the simplex RRT-PCR reaction

The master mix should be mixed thoroughly and 20 µl solution per sample must be pipetted in real-time PCR tube/strip/plate.

Template 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 (if used), nucleic acids isolated from diagnostic samples, PTC and PPC (if used). 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 7, and select FAM and Cy5 compatible detection channels for fluorescence acquisition.

Step	Temperature and time	Repetitions
Reverse transcription	45°C for 10 min	1
RT inactivation/initial denaturation	95°C for 10 min	1
Denaturation	95°C for 30 sec	40
Annealing/extension (*)	58°C for 15 sec	
Final extension	72°C for 15 sec	

Table 7. 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 100 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 8. 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
NPC (if used)	Absence of fluorescence increase from the FAM/Cy5 fluorophores, with no sigmoidal (or logarithmic) amplification curve	Repeat the analysis from the nucleic acids extraction and check nucleic acids isolation reagents
PPC (if used)	Increase in fluorescence from the FAM/Cy5 fluorophores yielding a sigmoidal (or logarithmic) amplification curve at the expected Cq value	Repeat the analysis from the nucleic acids extraction
NTC	Absence of fluorescence increase from the FAM/Cy5 fluorophores, with no	Repeat the analysis from the RRT-PCR and check RRT-PCR reagents

	sigmoidal (or logarithmic) amplification curve	
PTC	Increase in fluorescence from the FAM/Cy5 fluorophores yielding a sigmoidal (or logarithmic) amplification curve at the expected Cq value	Repeat the analysis from the RRT-PCR and check the PTC stock

Table 8. Assessment of test reliability. Cq = quantification cycle

6.2. Diagnostic samples

Criteria for data interpretation of diagnostic samples and subsequent actions are reported in Tables 9 and 10.

Result	Interpretation and action
Increase in fluorescence from both the FAM and the Cy5 fluorophores of the 'H5-HPAI CS assay' and the 'clade 2.3.4.4b assay', respectively, yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 36	Positive for H5 HPAI clade 2.3.4.4b. In samples characterized by a low viral load, positive results for the 'H5-HPAI CS assay' may be associated with negative results for the 'clade 2.3.4.4b assay' due to the lower sensitivity of this amplification reaction
Increase in fluorescence from both the FAM and the Cy5 fluorophores of the 'H5-HPAI CS assay' and the 'clade 2.3.4.4b assay', respectively, yielding a sigmoidal (or logarithmic) amplification curve with Cq > 36	Doubtful for H5 HPAI clade 2.3.4.4b. In samples characterized by a low viral load, positive results for the 'H5-HPAI CS assay' may be associated with negative results for the 'clade 2.3.4.4b assay' due to the lower sensitivity of this amplification reaction. If inhibition of the amplification reaction is suspected, dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the analysis from the RRT-PCR; alternatively, repeat the analysis from the nucleic acids extraction, further diluting the original biological sample (SOP VIR 1000). It is responsibility of the Head of the Laboratory to identify the actions to be taken if no conclusive results are obtained from the same animal/epidemiological unit
Absence of fluorescence from both the FAM and the Cy5 fluorophores of the 'H5-HPAI CS assay' and the 'clade 2.3.4.4b assay', respectively, with no sigmoidal (or logarithmic) amplification curve	Negative for H5 HPAI clade 2.3.4.4b

Table 9. Interpretation of real-time RT-PCR diagnostic results for the 'H5-HPAI CS assay' and the 'clade 2.3.4.4b assay'

Result	Interpretation and action
Increase in fluorescence from the FAM fluorophore of the simplex 'H5-LPAI assay' yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 36	Positive for H5 LPAI
Increase in fluorescence from the FAM fluorophore of the simplex 'H5-LPAI assay' yielding a sigmoidal (or logarithmic) amplification curve with Cq > 36	Doubtful for H5 LPAI. If inhibition of the amplification reaction is suspected, dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the analysis from the RRT-PCR; alternatively, repeat the analysis from the nucleic acids extraction, further diluting the original biological sample (SOP VIR 1000). It is responsibility of the Head of the Laboratory to identify the actions to be taken.
Absence of fluorescence increase from the FAM fluorophore of the simplex 'H5-LPAI assay', with no sigmoidal (or logarithmic) amplification curve	Negative for H5 LPAI
All other cases	Not typeable. If needed, proceed with alternative molecular and/or virological analyses. It is responsibility of the Head of the Laboratory to identify the actions to be taken

Table 10. Interpretation of real-time RT-PCR diagnostic results for the 'H5-LPAI assay'

Positive or doubtful results for the 'H5-HPAI CS assay' and the 'clade 2.3.4.4b assay' may be associated with positive or doubtful results for the 'H5-LPAI assay'. Although this occurrence is rare, it might be indicative of co-infection with both high and low pathogenic H5 viruses in an individual or in a population, or might reflect the shift from LPAI to HPAI in poultry species. Such circumstances require confirmation of the viral pathotype by *in vivo* tests or high-throughput sequencing. However, the latter I prefereable as this approach can provide sufficient depth of coverage across the HA cleavage site region and supports the detection of mixed infections and within-sample viral population diversity.

The 'H5-HPAI assay' detects predominantly viruses of clade 2.3.4.4b. However, cross-reaction with other clades of the gs/GD lineage may be expected due to conserved motifs among clades in the target region. Samples characterized by a high viral load testing positive for the 'H5-HPAI CS assay' and negative for the 'clade 2.3.4.4b assay', and originating from regions where clades other than 2.3.4.4b are reported, require careful assessment and in depth characterization by sequencing.

Samples testing negative with the 'H5-HPAI CS assay', the 'clade 2.3.4.4b assay' and the 'H5-LPAI assay' have to be considered as untypeable, and testing with additional methods (e.g. SOP VIR 125) might be required.

In the presence of a high viral load associated with untypeable samples, or with inconsistent results between the 'H5-HPAI CS assay' and the 'clade 2.3.4.4b assay' should be critically assessed.

7. Characteristics of the method

This method was validated at the IZSVe according to the ISO/IEC 17025, employing AIV samples and clinical specimens 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 pathotyping protocol is based on the update of the oligonucleotide sets originally developed by Naguib et al. (2017), after the observation of false negative results during the H5-HPAI epidemic in 2021-2022, and surveillance activities in wild birds.

The method usually yields positive results for samples with Cq 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.