**ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE**

**EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE**


# SOP VIR 1004

# HA AND NA SUBTYPING OF AVIAN INFLUENZA VIRUS BY REAL-TIME RT-PCR

# (Hoffmann et al., 2016; James et al., 2018)

Standard operating procedure used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 08/04/21.

# Contents

## 1. Purpose and field of application

This protocol describes the procedure to subtype the HA and NA of avian influenza virus (AIV) in biological samples by real-time reverse transcription polymerase chain reaction (RRT-PCR).

The protocol targets segment 4 (HA) and segment 6 (NA) regions conserved within different AIV subtypes using multiple oligonucleotides sets, based on the assays developed by Hoffmann et al. (2016) and James et al. (2018) and their subsequent updates.

The H5, H7 and H9 subtype identification is not included in this document. Please refer to specific RRT-PCR assays available at SOPs VIR 014, 143, 144, 1001.

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


- B. Hoffmann, D. Hoffmann, D. Henritzi, M. Beer, T.C. Harder. Riems influenza A typing array (RITA): an RT-qPCR-based low density array for subtyping avian and mammalian influenza A viruses. Sci Rep 6:27211, 2016. doi: 10.1038/srep27211;
- J. James, M.J. Slomka, S.M. Reid, S.S. Thomas, S. Mahmood, A.M.P. Byrne, J. Cooper, C. Russel, B.C. Mollett, E. Agyeman-Dua, S. Essen, I.H. Brown, S.M. Brookes. Development and application of real-time PCR assays for specific detection of contemporary avian influenza virus subtypes N5, N6, N7, N8 and N9. Avian Dis 63(sp1):209-218, 2018. doi: 10.1637/11900-051518-Reg.1;
- 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.


## 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);

- AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems);
- Subtype-specific oligonucleotides sets (see Annex I).

### 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 |
|---|---|
| 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 searched AIV 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

This procedure is applied as a downstream method to samples which have tested positive for AI by a different molecular diagnostic assay. In case this procedure is applied as a frontline method from a new sample aliquot, 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 (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).

### 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. For the whole procedure, the use of filter tips and nuclease-free plastics is recommended.

**Subtype-specific oligonucleotides pre-mix preparation**

Subtype-specific oligonucleotides sets can be assembled in advance as pre-mixes using TE pH 8.0, and stored until use. Final concentrations of each oligonucleotide in the reaction mix are detailed in Annex I.

**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 | - | - | 4.5 |
| 2X RT-PCR Buffer | 2X | 1X | 12.5 |
| Subtype-specific oligonucleotides pre-mix | - | - | 2 |
| 25X RT-PCR Enzyme Mix | 25X | 1X | 1 |
| 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, 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.

| Step | Temperature and time | Repetitions |
|---|---|---|
| Reverse transcription | 45℃ for 10 min | 1 |
| RT inactivation/initial denaturation | 95℃ for 10 mi n | 1 |
| Denaturation | 95℃ for 15 sec | 45 |
| Annealing (*) | 56℃ for 20 sec | |
| Extension | 72℃ for 30 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 (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 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, if required |
| 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 confirmatory method (i.e. with alternative oligonucleotides sets) can be applied to the nucleic acids extracts.<br>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).<br>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

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.

For any technical query, please contact eurl.ai.nd.secretariat@izsvenezie.it

**Annex I.** Oligonucleotides sets targeting all the HA and NA subtypes; [1] Hoffmann et al., (2016); [2] James et al. (2018)

| Target | Oligonucleotide | Sequence 5'-3' | Reference | Concentration in the reaction mix |
|---|---|---|---|---|
| H1 | IAV-H1-115F | ACA CAA TAT GTA TAG GYT AHC ATG C | [1] | 1.6 µM |
| | IAV-H1-199R | GAG TGT GTY ACT GTY ACA TTC TT | | 1.6 µM |
| | IAV-H1-147FAM | FAM-TCD ACM GAC ACT GTW GAC ACA GTA CTN GA-BHQ1 | | 0.4 µM |
| H2 | IAV-H2-470-F | GAC ACA GCA YAC RAC AAC TGG | [1] | 1.2 µM |
| | IAV-H2-601-R | GTG TTG TTG TAT GAT CYT TTR GCA A | | 1.2 µM |
| | IAV-H2-522-FAM | FAM-CCN TCA TTC TTC AGG AAC ATG GTY TGG-BHQ1 | | 0.4 µM |
| H3 | IAV-H3-1667-F | TGG ATT TCC TTT GCC ATA TCA TG | [1] | 1.2 µM |
| | IAV-H3-1784-R | ATR CAC TCA AAT GCA AAT GTT GCA | | 1.2 µM |
| | IAV-H3-1753-FAM | FAM-CTA ATG TTG CCT YTY TGG CAG GCC CAC AT-BHQ1 | | 0.4 µM |
| H4 | IAV-H4-1586F | GAC TCA RGG ATA CAA RGA CAT | [1] | 1.2 µM |
| | IAV-H4-1599F | AAG GAC ATC ATY CTY TGG ATT TC | | 1.2 µM |
| | IAV-H4-1686R | ACA AGC CCA CAA AAT RAA GGC | | 1.2 µM |
| | IAV-H4-1696R | TTC CRT TYT GAC AAG CCC ACA A | | 1.2 µM |
| | IAV-H4-1628FAM | FAM-TCC ATA TCA TGC TTY TTR CTC GTT GC-BHQ1 | | 0.4 µM |
| H6 | IAV-H6-1666F | CTT GGT GTG TAT CAA ATY CTT GC | [1] | 1.6 µM |
| | IAV-H6-1776R | CAT TGA RCC ATT TGA RCA CAT CCA | | 1.6 µM |
| | IAV-H6-1693FAM-MGB | FAM-TAT AGT ACG GTA TCG AGC AGY CT-MGB | | 0.4 µM |
| H8 | IAV-H8-1604F | TAC AAA ATT CTY AGC ATY TAC AGT AC | [1] | 1.6 µM |
| | IAV-H8-1677R | ATT ARA CCT CCA GCA AYC AGG A | | 1.6 µM |
| | IAV-H8-1654FAM | FAM-TGC CAA GCA RAG ACT GGC CGC CA-BHQ1 | | 0.4 µM |
| H10 | IAV-H10-991F | GTT GCT TGC WAC MGG AAT GAG | [1] | 1.2 µM |
| | IAV-H10-1041F | GCC TGT TTG GDG CRA TAG C | | 1.2 µM |
| | IAV-H10-1122R | TTT TGR TGT CKG AAR CCA TAC CA | | 1.2 µM |
| | IAV-H10-1173R | ATA GCT GCY TGA GTA CTY TTG TA | | 1.2 µM |
| | IAV-H10-1092FAM | FAM-ACC ATY CCT TCC CAT CCR TTY TCT A-BHQ1 | | 0.5 µM |
| H11 | IAV-H11-1510F | ARG TYA GGA ATG GAA CAT ATG AYC A | [1] | 1.6 µM |
| | IAV-H11-1723R | CAA ATG GTA CAT CTA CAT GAY CCA | | 1.6 µM |

| Target | Oligonucleotide | Sequence 5'-3' | Reference | Concentration in the reaction mix |
|---|---|---|---|---|
| H11 | IAV-H11-1626FAM | FAM-ATT TAC AGC TGC ATY GCA AGY AGT CT-BHQ1 | [1] | 1 µM |
| H12 | IAV-H12-1607F | AGC ATC TAC AGC AGT GTY GC | [1] | 1.6 µM |
| | IAV-H12-1707R | CAG AAA GTA CAA CGA AYA TTT CCA | | 1.6 µM |
| | IAV-H12-1674FAM | FAM-CCG AAA ATG AAA CCC CCA ATA ATC ATG A-BHQ1 | | 0.4 µM |
| H13 | IAV-H13-1241F | ATT GAC AAA ATG AAT GGR AAY TAT GAY TC | [1] | 1.6 µM |
| | IAV-H13-1372R | AAG AAG YTT DGC ATT RTA TGA CCA | | 1.6 µM |
| | IAV-H13-1304FAM | FAM-ATA AAY ATG CTY GCA GAY AGR ATA GAT GAY GC-BHQ1 | | 0.4 µM |
| H16 | IAV-H16-1589F | GGG ATA AAR TTG AAR ACT GAR GA | [1] | 1.6 µM |
| | IAV-H16-1708R | ACT GCT RCA TGC CCA CAK TAT | | 1.6 µM |
| | IAV-H16-1635FAM-MGB | FAM-TTT AYA GYT GCA TTG CAA GCA G-MGB | | 0.4 µM |
| N1 | IAV-N1-3-F | AGR CCT TGY TTC TGG GTT GA | [1] | 2 µM |
| | IAV-N1-3-R | ACC GTC TGG CCA AGA CCA | | 2 µM |
| | IAV-N1-3-FAM | FAM-ATY TGG ACY AGT GGG AGC AGC AT-BHQ1 | | 0.4 µM |
| N2 | IAV-N2-1367F | AGT CTG GTG GAC YTC AAA YAG | [1] | 2.4 µM |
| | IAV-N2-1488R | AAT TGC GAA AGC TTA TAT AGV CAT | | 2.4 µM |
| | IAV-N2-1444.1FAM-MGB | FAM-CCA TCA GGC CAT GAG CCT-MGB | | 0.4 µM |
| N3 | IAV-N3-1348F | AAY AGT ATA GTT ACT TTC TGY GG | [1] | 1.6 µM |
| | IAV-N3-1422R | CCA ATG TTR GAA CCA TCH GG | | 1.6 µM |
| | IAV-N3-1373FAM | FAM-TAR ACA ATG AAC CTG GAT CGG GVA A-BHQ1 | | 0.4 µM |
| N4 | IAV-N4-1335F | GAC YAG TGG TAG TAG YAT YGC | [1] | 1.6 µM |
| | IAV-N4-1345F | AGT AGY ATT GCR TTY TGT GGT GTT | | 1.6 µM |
| | IAV-N4-1437R | AAA TYA CTT GTC TAT GTC AAA DGG | | 1.6 µM |
| | IAV-N4-1387FAM | FAM-TGG TCR TGG CCY GAT GGC GCT CT-BHQ1 | | 0.4 µM |
| N5 | IAV-N5-1322F | AAG AGA GRA CWA GCA TTT GGA C | [1] | 1.2 µM |
| | IAV-N5-1353F | CTC CAC KGT RTT TTG TGG TGT | | 1.2 µM |
| | IAV-N5-1421R | GGA AGA ATT GCK CCA TCA YC | | 1.2 µM |
| | IAV-N5-1426R | CAA AKG GAA GAA TTG CKC CAT CA | | 1.2 µM |
| | IAV-N5-1375FAM | FAM-TCM AGT GAG GTC CCA GGR TGG TC-BHQ1 | | 0.4 µM |
| | Mod-N5-1353F | TTC CAC TGT GTT TTG TGG KGT | [2] | 0.6 µM |
| | Mod-N5-1426R | CGA ATG GTA GAA TTG CTC CAT CA | | 0.6 µM |

| Target | Oligonucleotide | Sequence 5'-3' | Reference | Concentration in the reaction mix |
|---|---|---|---|---|
| N5 | Mod-N5-1375FAM | FAM-TCY AGT GAG GTC CCA GGA TGG TC-BHQ1 | [2] | 0.4 µM |
| N6 | IAV-N6-14F-New | TGA ARA TGA ATC CAA ATC ARA AGA TAA | FLI update of [1] | 0.8 µM |
| | IAV-N6-100R | GCG ATT CCT ATT AGC AGG CTT AC | | 0.8 µM |
| | IAV-N6-43FAM-New | FAM-TGC ATT TCA GCA ACA GGA GTA ACA CTA TC-BHQ1 | | 0.4 µM |
| | Mod-N6-10F | AGG GTG AAA ATG AAT CCA AAT CA | [2] | 1.6 µM |
| | Mod-N6-14F | TGA AAA TGA ATC CAA ATC ARA AGR TAA | | 1.6 µM |
| | Mod-N6-97R | AAT TCC TAT YAG CAG RCT TAC YAC | | 1.6 µM |
| | Mod-N6-43FAM | FAM-TGC RTT TCA GCM ACA GGA RTR ACA CTA TC-BHQ1 | | 0.4 µM |
| N7 | IAV-N7-1305F | GTT GAA TTA ATW AGA GGA AGR CC | [1] | 1.6 µM |
| | IAV-N7-1430R | GAT YTG TGC CCC ATC RGG GA | | 1.6 µM |
| | IAV-N7-1383FAM | FAM-AGC CCA DTC YCA GTT GGG TCY GGT TC-BHQ1 | | 0.4 µM |
| | Mod-N7-1305F | GTT GAA TTR ATT AGA GGR AGR CC | [2] | 1.6 µM |
| | IAV-N7-1430R | GAT YTG TGC CCC ATC RGG GA | | 1.6 µM |
| | IAV-N7-1383FAM | FAM-AGC CCA DTC YCA GTT GGG TCY GGT TC-BHQ1 | | 0.4 µM |
| N8 | IAV-N8-1296F | TCC ATG YTT TTG GGT TGA RAT GAT | [1] | 1.6 µM |
| | IAV-N8-1423R | GCT CCA TCR TGC CAY GAC CA | | 1.6 µM |
| | IAV-N8-1354FAM | FAM-TCH AGY AGC TCC ATT GTR ATG TGT GGA GT-BHQ1 | | 0.8 µM |
| | Mod-N8-1296F | YCC CTG YTT TTG GGT CGA AAT GAT | [2] | 0.8 µM |
| | Mod-N8-1423R | GCT CCA TCG TGC CAT GAC CA | | 0.8 µM |
| | Mod-N8-1354FAM | FAM-TCT AGT AGC TCC ATT GTA ATG TGT GGA GT-BHQ1 | | 0.4 µM |
| N9 | IAV-N9-1363F | AGY ATA GTA TCR ATG TGT TCC AG | [1] | 1.6 µM |
| | IAV-N9-1439R | AAG TAC TCT ATT TTA GCC CCA TC | | 1.6 µM |
| | IAV-N9-1393FAM | FAM-TTC CTB GGA CAA TGG AAC TGG CC-BHQ1 | | 0.4 µM |
| | IAV-N9-1363F | AGY ATA GTA TCR ATG TGT TCC AG | [2] | 1.6 µM |
| | Mod-N9-1439R | AAG TAC TCT ATT YTA GCC CCR TC | | 1.6 µM |
| | IAV-N9-1393FAM | FAM-TTC CTB GGA CAA TGG AAC TGG CC-BHQ1 | | 0.4 µM |