ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

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

This protocol includes the workflow reported in the standard operating procedures used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 12/10/21.

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

Revision history

Revision	Date	Description
ed. 01	October 2021	• Updated references • Update of available extraction methods for organs • Specification on the number of swabs to pool by species • Minor changes to organs homogenates preparation • Minor edits for style and consistency
ed. 00	April 2021	Initial release

Contents

1. Purpose and field of application

This document describes the procedure for biological samples preparation and nucleic acids extraction using both manual and automatic extraction systems, prior to molecular diagnosis and typing of avian influenza virus (AIV) and avian orthoavulavirus 1 (AOAV-1).

The procedure can be applied to viral isolates (normally allantoic fluid), tissue homogenates, stool, tracheal and cloacal swabs properly collected and preserved.

2. References

- Intype IC-RNA Handbook (Indical Bioscience);
- NucleoSpin RNA, RNA isolation user manual (Macherey-Nagel);
- QIAamp Viral RNA Mini Handbook (Qiagen);
- QIAsymphony DSP Virus/Pathogen Kit Instructions for Use Handbook (Qiagen);
- MagMAX Pathogen RNA/DNA Kit Protocol, Applied Biosystems;
- 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, 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);
- OIE - World Organization for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.3.14. Newcastle disease (infection with Newcastle disease virus) (Version adopted in May 2021).

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. After samples preparation is completed, laboratory equipment and surfaces must be decontaminated with adequate disinfectants (e.g. Virkon, sodium chloride, ethanol etc.) in accordance with the procedures in place at individual facilities, and biological waste must be properly disposed of. 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.

- Antibiotic PBS pH 7.4 ± 0.2 (aPBS) containing 10,000 IU/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml gentamycin, and 5,000 IU/ml nystatin;
- Intype IC-RNA (Indical Bioscience);
- NucleoSpin RNA (Macherey-Nagel);

- QIAamp Viral RNA Mini Kit (Qiagen);
- QIAsymphony DSP Virus/Pathogen Kit (Qiagen);
- MagMAX Pathogen RNA/DNA Kit (Applied Biosystems);
- 100% Ethanol molecular grade;
- 70% Ethanol molecular grade;
- 100% Isopropanol molecular grade;
- Buffer ATL (Qiagen).

4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- Sterile forceps and scissors;
- TissueLyser II (Qiagen) and stainless steel beads, or mortar, pestle and sterile quartz powder;
- QIAsymphony SP system (Qiagen) and related plastic material;
- KingFisher Flex Purification System with 96 PCR head (Thermofisher Scientific) and related plastic material.

5. Procedure

5.1. Controls

To ensure the reliability of downstream molecular tests, the controls listed in Table 1 must be included in each nucleic acids isolation session.

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

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. 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

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.

Depending on the sample matrix, the most appropriate nucleic acids isolation system must be adopted. A summary of the systems validated at the IZSVe, according to the type of specimen, is provided in Table 2.

Kit	Isolate	Swabs	Organs	Stool
NucleoSpin RNA (Macherey-Nagel)		✓	✓	✓
QIAamp Viral RNA Mini Kit (Qiagen)	✓	✓		
QIAsymphony DSP Virus/Pathogen Kit (Qiagen)	✓	✓	✓	✓
MagMAX Pathogen RNA/DNA Kit (Applied Biosystems)	✓	✓	✓	

Table 2. Recommended nucleic acids isolation systems for different sample matrices

Samples can be analyzed individually or pooled. To avoid cross contamination, samples from individual epidemiological units must be processed with dedicated forceps and scissors. For the whole procedure, the use of filter tips and nuclease-free plastics is recommended.

5.2.1. Viral isolates

No preparation is required. Move forward with the nucleic acids isolation procedure processing the required sample volume, in accordance with the extraction system adopted.

5.2.2. Swabs

If samples are submitted as dry swabs, heads should be cut into tubes containing a sufficient amount of aPBS depending on the type of swab (approximately 750-1000 µl) to ensure full immersion and yield enough material for molecular tests and, if needed, virus isolation or bio-banking. Swabs from the same anatomical site and originating from the same epidemiological unit can also be pooled into groups up to 5 samples, and dipped into aPBS (approximately 1 ml for tracheal swabs and 2 ml for cloacal swabs). In particular conditions, as during an epidemic emergency, for gallinaceous species pooling up to 10 swabs into an appropriate aPBS volume may be required to increase testing capacity and shorten turnaround times. For other species (e.g., ducks and geese), it is recommended to pool maximum 5 swabs.

Vortex swabs suspension for 30 sec and centrifuge for 2 min at 15000 × *g*. While this clarification step is optional for tracheal swabs, it is strongly recommended for cloacal swabs visibly soiled with faeces.

After swabs preparation, move forward with the nucleic acids isolation procedure processing the required sample volume, in accordance with the extraction system adopted.

5.2.3. Organs

Organs can be analyzed individually or pooled. Cut the tissue into small pieces using sterile forceps and scissors and transfer approximately 150-200 mg material into a sterile tube containing a stainless steel bead. Add aPBS (450-600 µl) to obtain a 1:4 w/v dilution and homogenize at 30 Hz for 3 min. Alternatively, homogenization can be

made with mortar, pestle and sterile quartz powder. Centrifuge the suspension for 2 min at 15000 × *g*. Collect the required sample volume from the supernatant, in accordance with the extraction system adopted, and move forward with the nucleic acids isolation procedure.

5.2.4. Stool

Place the material (at least, 1 gr) into a sterile tube and add aPBS to obtain a 1:4 w/v dilution. Vortex thoroughly for 30 sec and centrifuge the fecal suspension for 2 min at 15000 × *g*. Collect the required sample volume from the supernatant, in accordance with the extraction system adopted, and move forward with the nucleic acids isolation procedure.

5.3. Isolation of nucleic acids

5.3.1. NucleoSpin RNA kit (Macherey-Nagel)

The kit can be applied for RNA isolation from tissue homogenates, stool, tracheal and cloacal swabs.

Store kit components in compliance with the manufacturer's recommendations.

Prepare the working solutions (i.e., rDNase, Wash Buffer RA3) following the instructions reported in the user manual and apply the protocol "RNA purification from cultured cells and tissue" with the following modifications (all volumes are intended per sample):

- Pipette 300 µl Buffer RA1 and 300 µl 70% ethanol into a 1.5 ml microcentrifuge tube and mix by pipetting;
- If using the internal control: add 6 µl intype IC-RNA, corresponding to 1/10 of the elution volume;
- Add 100 µl sample, and mix by pipetting;
- Place a NucleoSpin RNA Column (blue ring) in a collection tube, load the lysate to the column and centrifuge for 30 sec at 11000 × *g*;
- Discard the flow-through and place the column into a new collection tube;
- Add 350 µl Membrane Desalting Buffer and centrifuge for 1 min at 11000 × *g*;
- Prepare DNase reaction mixture into a 1.5 ml microcentrifuge tube adding for each sample 10 µl reconstituted rDNase to 90 µl Reaction Buffer for rDNase and mix by flicking;
- Apply 95 µl DNase reaction mixture directly onto the center of the silica membrane and incubate at room temperature for 15 min;
- Add 200 µl Buffer RAW2 and centrifuge for 30 sec at 11000 × *g*;
- Discard the flow-through and place the column into a new collection tube;
- Add 600 µl Buffer RA3 and centrifuge for 30 sec at 11000 × *g*;
- Discard the flow-through and place the column into a new collection tube;
- Add 250 µl Buffer RA3 and centrifuge for 2 min at 15000 × *g*;
- Place the column into a 1.5 ml nuclease-free collection tube, elute the RNA in 60 µl RNase-free H_2O and centrifuge for 1 min at 11000 × *g*.

5.3.2. QIAamp Viral RNA Mini Kit (Qiagen)

The kit can be applied for RNA isolation from viral isolates, tracheal and cloacal swabs.

Store kit components in compliance with the manufacturer's recommendations.

Prepare the working solutions (i.e., carrier RNA-Buffer AVE, Buffer AVL and carrier RNA–Buffer AVE mix, Buffer AW1, Buffer AW2) following the instructions reported in the user manual and apply the protocol "Purification of Viral RNA (Spin Protocol)" with the following modifications (all volumes are intended per sample):

- Pipette 560 µl prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube;
- If using the internal control: subtract 6 µl Buffer AVL-carrier RNA mix, and replace with the same amount of intype IC-RNA, corresponding to 1/10 of the elution volume;
- Add 140 µl sample, and mix by thoroughly by vortexing;
- Incubate at room temperature for 10 min;
- Briefly centrifuge to remove drops from the inside of the lid;
- Add 560 µl ethanol (96-100%), mix by vortexing and briefly centrifuge to remove drops from the inside of the lid;
- Apply 630 µl lysate from previous step to a QIAamp Mini column and centrifuge for 1 min at 6000 × *g*;
- Discard the flow-through and place the column into a new collection tube;
- Apply the remaining lysate (630 µl) to the column from the previous step and centrifuge for 1 min at 6000 × *g*;
- Discard the flow-through and place the column into a new collection tube;
- Add 500 µl Buffer AW1 and centrifuge for 1 min at 6000 × *g*;
- Discard the flow-through and place the column into a new collection tube;
- Add 500 µl Buffer AW2 and centrifuge for 3 min at full speed;
- Discard the flow-through and place the column into a 1.5 ml nuclease-free collection tube;
- Add 60 µl Buffer AVE and incubate at room temperature for 1 min;
- Centrifuge for 1 min at 6000 × *g*.

5.3.3. QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen) on the QIAsymphony SP instrument (Qiagen)

The kit can be applied for RNA isolation from viral isolates, tissue homogenates, stool, tracheal and cloacal swabs.

Store kit components in compliance with the manufacturer's recommendations.

Prepare the working solutions (i.e., carrier RNA (CARRIER)-Buffer AVE (AVE) mixture) following the instructions reported in the user manual.

- If using the internal control: prepare internal control-carrier RNA (CARRIER)-Buffer AVE (AVE) mixture with the intype IC-RNA, following the instructions reported in Table 3.

No. of samples	intype-IC RNA (µl)	CARRIER-AVE mixture (µl)	Buffer AVE (µl)	Total volume (µl)
1	36	12	432	480
2	45	15	540	600
3	54	18	648	720
4	63	21	756	840
5	72	24	864	960
6	81	27	972	1080
7	90	30	1080	1200
8	99	33	1188	1320
9	108	36	1296	1440
10	117	39	1404	1560
11	126	42	1512	1680
12	135	45	1620	1800
13	144	48	1728	1920
14	180	60	2160	2400
15	189	63	2268	2520
16	198	66	2376	2640
17	207	69	2484	2760
18	216	72	2592	2880
19	225	75	2700	3000
20	234	78	2808	3120
21	243	81	2916	3240
22	252	84	3024	3360
23	261	87	3132	3480
24	270	90	3240	3600

Table 3. Volumes for the preparation of the internal control-carrier RNA (CARRIER)-Buffer AVE (AVE) mixture. The intype IC-RNA volume corresponds to 1/10 of the initial elution volume, i.e. the volume of elution solution required to ensure that the actual volume of eluate is 60 µl, as selected in the touchscreen of the instrument

- Mix 300 µl sample with 250 µl Buffer ATL (Qiagen) into a 2 ml tube (Sarstedt cat. No. 72.608);
- Load reagent cartridges and plasticware into the respective drawers;
- Place the samples into the appropriate sample carrier, and load them into the “Sample” drawer;
- Place the tube(s) containing the internal control-carrier RNA (CARRIER)-Buffer AVE (AVE) mixture into the tube carrier and load into slot A of the “Sample” drawer;
- Initialize the instrument and apply the C400OBL CR22710 ID3359 script (which is a modification of the Complex400_OBL_V4_DSP script customized by Qiagen for the inclusion of the intype IC-RNA internal control).

5.3.4. MagMAX Pathogen RNA/DNA Kit (Applied Biosystems) on a KingFisher Flex Purification System with 96 PCR head (Thermofisher Scientific)

The kit can be applied for RNA isolation from viral isolates, tracheal and cloacal swabs and organs.

Store kit components in compliance with the manufacturer's recommendations.

Prepare the working solutions (i.e., Wash Solution 1, Wash Solution 2) following the instructions reported in the user manual and apply the protocol "Low-cell-content samples" for "KingFisher Flex/MagMAX Express-96 Deep Well Magnetic Particle Processor" with the following modifications (all volumes are intended per sample):

- Prepare the Lysis/Binding Solution pipetting 250 µl Lysis/Binding Solution Concentrate, 2 µl Carrier RNA, 9 µl intype IC-RNA (corresponding to 1/10 of the elution volume, if using the internal control) and 250 µl 100% isopropanol, and mix by vortexing;
- Vortex Nucleic Acid Binding Beads and prepare the Bead Mix pipetting 10 µl Nucleic Acid Binding Beads and 10 µl Lysis Enhancer, keeping the mix refrigerated;
- Prepare the tip comb plate (Standard Plate);
- Prepare two plates with 300 µl prepared Wash Solution 1 (Deep Well Plate);
- Prepare two plates with 450 µl prepared Wash Solution 2 (Deep Well Plate);
- Prepare one plate with 90 µl Elution Buffer (Standard Plate);
- In a Deep Well Plate, add 20 µl prepared Bead Mix, 500 µl Lysis/Binding Solution and 200 µl sample;
- Immediately start the processor script (4462359_DW_HV) and load the plates onto the processor as directed.

6. Analysis of nucleic acids by molecular tests

Extracted nucleic acids can be used for first intention detection of avian influenza virus (AIV) and/or avian orthoavulavirus 1 (AOAV-1) and for viral typing by molecular techniques (i.e., real-time reverse transcription polymerase chain reaction (RRT-PCR), reverse transcription polymerase chain reaction (RT-PCR), sequencing).

If required, prior to molecular tests, nucleic acids can be checked for quantity and integrity, by spectrophotometer or fluorimeter measurement and on-chip electrophoresis.

Purified nucleic acids can be refrigerated for a few hours prior to molecular testing, otherwise they must be stored at ≤ -70°C until use.

6.1. Reliability of controls

Test reliability is assured if the controls yield the expected results, as reported in the specific SOPs for RRT-PCR and RT-PCR tests. In case of invalid results, the causative reason must be investigated and proper actions have to be taken.

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. An invalid IC result (negative or weakly positive) can occur in inadequately preserved and putrescent biological samples, or in case they contain PCR inhibitors (e.g. blood).

If an invalid IC result is associated with a negative/dubious AIV/AOAV-1 sample, to rule out inhibition issues dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the molecular test. In case of recurrent invalid

IC, repeat the analysis from the nucleic acids extraction, further diluting the original biological sample to obtain a higher dilution (typically, 1:10). For stool and tissue homogenates samples, filtration with 0.45 µm filters prior to nucleic acids extraction can also be performed. Notably, an invalid IC can also be observed in positive 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.

Any modification to this SOP by third party laboratories should be supported by proper validation data assessing that the procedure is still fit-for-purpose.