IVD For *in Vitro* Diagnostic Use

CE

Influenza A H5 H7 H9 Typing FRT

Handbook

Real Time Amplification test for the detection of Influenza A virus H5, H7 and H9 subtypes.

REF V47-50FRT

REF TV47-50FRT

Σ 50

NAME

Influenza A H5 H7 H9 Typing FRT

INTRODUCTION

Avian influenza is an infectious disease of birds caused by type A strains of the influenza virus. The disease, which was first identified in Italy more than 100 years ago, occurs worldwide. All birds are thought to be susceptible to infection with avian influenza, though some species are more resistant to infection than others. Infection causes a wide spectrum of symptoms in birds, ranging from mild illness to a highly contagious and rapidly fatal disease resulting in severe epidemics. The latter is known as “highly pathogenic avian influenza”. This form is characterized by sudden onset, severe illness, and rapid death, with a mortality that can approach 100%.

Fifteen subtypes of influenza virus are known to infect birds, thus providing an extensive reservoir of influenza viruses potentially circulating in bird populations. To date, all outbreaks of the highly pathogenic form have been caused by influenza A viruses of subtypes H5, H7 and H9.

INTENDED USE

Influenza A H5 H7 H9 Typing FRT is Real-Time amplification test for the qualitative detection of Influenza A virus H5, H7 and H9 subtypes.

PRINCIPLE OF ASSAY

Influenza A H5 H7 H9 Typing FRT Test is based on four major processes: isolation of *Influenza A virus* RNA from specimens, reverse transcription of the RNA, Real Time amplification of the cDNA Of *Influenza A virus* with subsequent identification of subtypes H5, H7 and H9. Real-time RT-PCR test for influenza A virus with “Influenza A Real-TM” (Sacace, ref. TVET46-50FRT) is recommended as a screening test for all suspected cases of influenza A virus. **Influenza A H5 H7 H9 Typing FRT** kit must be used for H5, H7 and H9 genotyping.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V47-50FRT)

Part N° 2 – **"Reverta-L":** Reverse transcription of the RNA

- **RT-G-mix-1**, 5 x 0,01 ml;
- **RT-mix**, 5 x 0,125 ml;
- **Reverse transcriptase** (M-MLV), 0,03 ml;
- **TE-buffer**, 1,2 ml.

Contains reagents for 60 tests.

Part N° 3 – **"A H5 H7 H9 Typing FRT":** Real Time amplification kit

- **PCR-mix-1 H5/H7/H9**, 0,6 ml;
- **PCR-mix-2-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Positive Control cDNA H5/H7/H9 C+**, 0,1 ml;
- **TE-buffer**, 0,2 ml;

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with RNA purification kit (TV47-50FRT)

Part N° 1 – **"Ribo-Sorb":** Sample preparation

- **Lysis Solution**, 22,5 ml;
- **Washing Solution**, 20 ml;
- **Sorbent**, 1,25 ml.
- **RNA-eluent**, 5 x 0,5ml;

Contains reagents for 50 tests.

Part N° 2 – **"Reverta-L":** Reverse transcription of the RNA

- **RT-G-mix-1**, 5 x 0,01 ml;
- **RT-mix**, 5 x 0,125 ml;
- **Reverse transcriptase** (M-MLV), 0,03 ml;
- **TE-buffer**, 1,2 ml.

Contains reagents for 60 tests.

Part N° 3 – **"A H5 H7 H9 Typing FRT":** Real Time amplification kit

- **PCR-mix-1 H5/H7/H9**, 0,6 ml;
- **PCR-mix-2-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Positive Control cDNA H5/H7/H9 C+**, 0,1 ml;
- **TE-buffer**, 0,2 ml;

Contains reagents for 55 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- RNA extraction kit (Module No. 1)
- Biosafety cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone
- Refrigerator
- Freezer

Zone 2: RT and amplification:

- Real Time Thermalcycler
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

STORAGE INSTRUCTIONS

The kits can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and -20°C immediately on receipt. **Influenza A H5 H7 H9 Typing FRT** must be stored at -20°C. Store **Ribo-Sorb** kit at 2-25°C.

STABILITY

Influenza A H5 H7 H9 Typing FRT is is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Influenza A H5 H7 H9 Typing FRT can analyze RNA extracted from:

Human diagnostics:

- *mucosal swabs (nasal, oral)*: swab area and place in “Eppendorf” tube with 0,5 ml of saline water or PBS sterile. Agitate vigorously. Repeat the swab and agitate in the same tube. Centrifuge at 1000g/min for 5 min. Remove and discard the supernatant. Resuspend the pellet in 100 µl of Saline water.
- *bronchial lavage, nasal wash:* centrifuge at 2000 g/min for 10-15 min. If the pellet is not visible add 10 ml of liquid and repeat centrifugation. Remove and discard the supernatant. Resuspend the pellet in 100 µl of Saline water.

Animal diagnostics:

- *feces*:
  - Prepare the 10% suspension of feces (4-5 g) with Saline solution. Vortex to get a homogeneous suspension and incubate for 10 min at room temperature. Transfer the supernatant in the sterile 1,5 ml polypropylene tube and centrifuge for 5 min to 7000-12000g. Use the supernatant for RNA extraction.
- *animals feeds and feeds for poultry:* homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles
- *cloaca swabs*: resuspend in 1,0 ml of saline water or PBS sterile and centrifuge at 10000 g/min for 10 min. Remove and discard the supernatant. Resuspend the pellet in 100 µl of Saline water.
- *tissue:* 1,0 gr (parenchimatous organs, trachea, lung, brain) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇒ **Ribo-Sorb-** (Sacace, REF K-2-1);
- ⇒ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇒ **SaMag Viral Nucleic Acid Extraction kit** (Sacace, REF SM003).

Please carry out the RNA extraction according to the manufacturer’s instructions.

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction**.
2. Add to each tube **450 µl Lysis Solution** Mix by pipetting and incubate 5 min at room temperature.
3. Add **100 µl** of samples to the appropriate tube containing Lysis Solution.
4. Vortex the tubes and centrifuge for 5 sec at 5000g. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 1 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for RNA extraction
5. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
6. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
7. Centrifuge all tubes for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
8. Add **400 µl** of **Washing Solution** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
9. Add **500 µl** of **Ethanol 70%** to each tube. Vortex vigorously, centrifuge for 1 min at 10000g. and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Repeat step 10.
11. Add **400 µl** of **Acetone** to each tube. Vortex vigorously , centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
12. Incubate all tubes with open cap for 10 min at 60°C.
13. Resuspend the pellet in **40 µl** of **RNA-eluent.** Incubate for 10 min at 60°C and vortex periodically. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g).
14. The supernatant contains RNA ready for use. The RT-PCR can be performed the same day. If this is not possible, the RNA preparations can be stored at -80°C for up to one month.

RT AND AMPLIFICATION

Reverse Transcription:

1) Prepare Reaction Mix: for 12 reactions, **add 5,0 µl RT-G-mix-1** into the tube containing **RT-mix** and vortex for at least 5-10 seconds, centrifuge briefly. This mix is stable for 1 month at -20°C. Add **6 µl M-MLV** into the tube with Reagent Mix, mix by pipetting, vortex for 3 sec, centrifuge for 5-7 sec (must be used immediately after the preparation).

   *(If it is necessary to test less than 12 samples add for each sample (N) in the new sterile tube **10*N µl** of **RT-G-mix-1 with RT-mix** and **0,5*N µl** of **M-MLV**).*

2) Add **10 µl** of **Reaction Mix** into each sample tube.
3) Pipette **10 µl RNA** samples to the appropriate tube. (*If the Ribo-Sorb isolation kit is used as a RNA extraction kit, re-centrifuge all the tubes with extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction)*.Carefully mix by pipetting.
4) Place tubes into thermalcycler and incubate at 37^{o}C for 30 minutes.
5) Dilute 1: 2 each obtained cDNA sample with TE-buffer (add **20 µl TE-buffer** to each tube).

cDNA specimens could be stored at -20^{o}C for a week or at -70^{o}C during a year.

PCR Reagents preparation

Reaction Mix 25 µl

1. Prepare required quantity of tubes or PCR plate.
2. Prepare for each sample in the new sterile tube **10*N µl** of **PCR-mix-1**, **5*N µl** of **PCR-mix-2-FRT** and **0,5*N** of **TaqF Polymerase.**
3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **cDNA** sample to appropriate tube with Reaction Mix.
5. Prepare for each panel the following controls:
   - add **10 µl** of **TE-buffer** to the tube labeled PCR Negative Control;
   - add **10 µl** of **Positive Control cDNA H5/H7/H9 C+**, to the tube labeled C_{pos};

Amplification

1. Create a temperature profile on your instrument as follows:

	Rotor type instruments[1]				*Plate type or modular instruments*[2]			
Stage	***Temp, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***	***Temp,°C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	95	15 min	–	1	95	15 min	–	1
Cycling	95	10 s	–	10	95	10 s	–	10
	54	20 s	–		54	25 s	–	
	72	10 s	–		72	25 s	–	
Cycling 2	95	10 s	–	35	95	10 s	–	35
	54	20 s	FAM(Green), JOE(Yellow), Rox(Orange)		54	30 s	FAM, JOE/HEX/Cy3, Rox/TexasRed	
	72	10 s	–		72	25 s	–	

[1] For example Rotor-Gene™ 3000/6000 (Corbett Research, Australia)
[2] For example,SaCycler-96™ *(Sacace),* CFX96/iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid).

RESULTS ANALYSIS

The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line.

Influenza A H5 is detected on the FAM (Green) channel, *Influenza A H7* on the JOE (Yellow)/HEX/Cy3 channel and *Influenza A H9* on the Rox/TexasRed channel.

Results are accepted as relevant if both positive and negative controls of amplification are passed (see table 1).

Table 1. Results for controls

Control	Stage for control	Ct channel Fam (Green)	Ct channel Joe (Yellow)/ HEX/Cy3	Ct channel Rox/TexasRed	Interpretation
NCA	Amplification	Neg	Neg	Neg	Valid result
cDNA C+	Amplification	Pos (< 33)	Pos (< 33)	Pos (< 33)	Valid result

PERFORMANCE CHARACTERISTICS

The kit **Influenza A H5 H7 H9 Typing FRT** allows to detect *Influenza A H5, H7 and H9 types* in 100% of the tests with a sensitivity of not less than 1000 copies/ml.

TROUBLESHOOTING

1. Weak or absent signal on the FAM (Green), Joe (Yellow)/Cy3/HEX and Rox/TexasRed) with the positive control: retesting of the sample is required.
    - The PCR was inhibited.
    - The reagents storage conditions didn't comply with the instructions.
        ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
2. Weak signal on the FAM (Green), Joe (Yellow)/Cy3/HEX and Rox/TexasRed channels: retesting of the sample is required.
3. FAM (Green), Joe (Yellow)/Cy3/HEX or Rox/TexasRed signal with Negative Control of extraction.
    - Contamination during RNA extraction procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
        ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR preparation procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        ⇒ Pipette the Positive controls at the end.
        ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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