IVD For *in Vitro* Diagnostic Use

CE

Avian A Screening & H5N1 Typing FRT

Handbook

Real Time PCR kit for the qualitative detection of Avian influenza A virus RNA in clinical specimens and genotyping of positive samples for H5N1 subtype

REF R-V33-FRT

50

NAME

Avian A Screening & H5N1Typing 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%.

Of the 15 avian influenza virus subtypes, H5N1 is of particular concern for several reasons. H5N1 mutates rapidly and has a documented propensity to acquire genes from viruses infecting other animal species. Its ability to cause severe disease in humans has now been documented in many cases. In addition, laboratory studies have demonstrated that isolates from this virus have a high pathogenicity and can cause severe disease in humans.

INTENDED USE

Avian A Screening & H5N1Typing FRT is Real-Time amplification test for the qualitative detection of Avian influenza A virus RNA in clinical specimens and genotyping of positive samples for H5N1 subtype.

PRINCIPLE OF ASSAY

Avian A Screening & H5N1Typing FRT RG Test is based on four major processes: isolation of *Avian influenza A virus* RNA from specimens, reverse transcription of the RNA, Real Time amplification of the cDNA of *Avian influenza A virus* with subsequent identification of subtype H5N1.

MATERIALS PROVIDED

Real Time PCR kit (R-V33-FRT)

Part N° 2 – **"Reverta-R ":** 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 – **"Avian A":** Real Time screening amplification kit

- **PCR-mix-1 Screening**, 5 x 0,11 ml;
- **PCR-buffer-Flu**, 2 x 0,28 ml;
- **TaqF Polymerase**, 0,06 ml;
- **Positive Control cDNA Avian A C+**, 0,1 ml;
- **Negative Control**, 3 x 1,2 ml;*
- **Internal Control**, 5 x 0,12 ml;**
- **DNA buffer**, 0,5 ml;

Contains reagents for 55 tests.

Part N° 4 – **"Avian H5N1":** Real Time genotyping amplification kit

- **PCR-mix-1 H5N1**, 5 x 0,11;
- **Positive Control cDNA H5 C+**, 0,1 ml;
- **Positive Control cDNA N1 C+**, 0,1 ml

Contains reagents for 55 tests.

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- RNA extraction kit
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- 60°C ± 5°C dry heat block
- Vortex mixer
- Pipettes
- Tips with filter
- 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
- Pipettes
- Tips with filter
- Tube racks

STORAGE INSTRUCTIONS

Avian A Screening & H5N1Typing FRT must be stored at -20°C. Store **Ribo-Sorb** kit at +2-25°C). The kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

Avian A Screening & H5N1Typing FRT 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:

- 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.

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

Avian A Screening & H5N1Typing FRT can analyze RNA extracted with **Ribo-Sorb** (REF K-2-1) 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/DNA 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 kit:

- ⇨ **Ribo-Sorb-** (Sacace, REF K-2-1);
- ⇨ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003);

Please carry out RNA extraction according to the manufacture's instruction.

Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

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. Carefully mix by pipetting.

   (*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)*.
4. Place tubes into thermalcycler and incubate at 37°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°C for a week or at -70°C during a year.

Real Time amplification screening:

1. Prepare required quantity of reaction tubes for samples and controls.
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1 Screening**, **5*(N+1) µl** of **PCR-buffer-Flu** and **0,5*(N+1) µl** of **TaqF Polymerase.** Vortex the tube and centrifuge for 3-5 sec.
3. Add to each tube **15 µl** of **Reaction Mix.**
4. Add **10 µl** of **cDNA** to appropriate tube.
5. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Negative Control Amplification;
   - add **10 µl** of **cDNA Avian A C+** to the tube labeled Avian Positive Control;
6. Insert the tubes in the thermalcycler and program the instruments as indicated below

Real Time amplification (H5N1 Genotyping) for positive Avian Influenza A Viruses:

1. Prepare required quantity of reaction tubes for positive Avian cDNA samples and controls.
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1 H5N1**, **5*(N+1)** of **PCR-buffer-Flu** and **0,5*(N+1)** of **TaqF Polymerase.** Vortex and centrifuge the tube.
3. Add to each tube **15 µl** of **Reaction Mix.**
4. Add **10 µl** of **cDNA** to appropriate tube.
5. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **Positive Control cDNA H5 C+** to the tube labeled Avian H5 Positive Control;
   - add **10 µl** of **Positive Control cDNA N1 C+** to the tube labeled Avian N1 Positive Control;
6. Insert the tubes in the thermalcycler and program the instruments as indicated below

Amplification

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

	Rotor and plate type instruments[1]			Modular type instruments[2]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
1	95	15 min	1	95	900 s	1
2	95	10 s	10	95	15 s	42
	54	25 s		54	25 s Fluorescence detection*	
	72	25 s		72	25 s	
3	95	10 s	35			
	54	30 s Fluorescence detection*				
	72	25 s				

[1] For example SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen), LineGeneK® (Bioer)
[2] For example, SmartCycler® (Cepheid)

**Fluorescence is detected is in FAM/Green and JOE/Yellow/HEX/Cy3 fluorescent channels.*

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	0.1	5 %	off
JOE/Yellow	*from 4 Fl to 8 Fl*	0.1	10 %	off

Plate- or modular type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

RESULTS ANALYSIS (Screening kit)

Influenza Virus A is detected on the FAM (Green) channel, *Internal Control* is detected on the *JOE/Yellow/HEX/Cy3* channel

1. Specimens with Ct < 33 (37 for SmartCycler) in the *Fam (Green)* channel are interpreted as positive for Avian A Virus.
2. Specimens absent Ct in the *Fam (Green)* channel are interpreted as negative for Avian A Virus.
3. If the Ct value of the specimen in the *JOE/Yellow/HEX/Cy3* channel (Internal Control) is higher than 33 (37 for SmartCycler) a retesting of the sample is required.

RESULTS ANALYSIS (Genotyping kit)

Influenza Virus A H5 is detected on the FAM (Green) channel, *Influenza Virus A N1* is detected on the *JOE/Yellow/HEX/Cy3* channel

cDNA N1 amplification analysis

1. Specimens with Ct < 33 (37 for SmartCycler) in the *JOE/Yellow/HEX/Cy3* channel are interpreted as positive for Avian A N1 subtype.
2. Specimens with absent Ct in the *JOE/Yellow/HEX/Cy3* channel are interpreted as negative for Avian A N1 subtype

cDNA H5 amplification analysis

1. Specimens with Ct < 33 (37 for SmartCycler) in the *Fam (Green)* channel are interpreted as positive for Avian A H5 subtype.
2. Specimens with absent Ct in the *Fam (Green)* channel are interpreted as negative for Avian A H5 subtype.

The contemporaneous detection of signal in the Joe and Fam channels in the specimen indicates presence of Avian influenza H5N1 subtype or contemporaneous presence of different types of Avian Influenza viruses that have in their structure haemoagglutinin 5 and neuraminidase 1 types.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific **Avian H5N1** primers and probes. The specificity of the kit **Avian A Screening & H5N1Typing FRT** was 100%. The potential cross-reactivity of the kit **Avian A Screening & H5N1Typing FRT** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **Avian A Screening & H5N1Typing FRT** allows to detect **Avian H5N1** RNA in 100% of the tests with a sensitivity of not less than 2500 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Target region: A-matrix, Ha5, N1

TROUBLESHOOTING

Results of analysis are not being registered in the following cases:

1. If the signal is registered in Negative Control of extraction on FAM/Green channel (in case of *Influenza virus* A RNA detection) or on any of the channels (in case of *Influenza virus* A H5N1 subtype identifying) and in Negative Control of amplification (NCA) in any of the channels, it indicates the contamination of reagents or samples. In this case results of the analysis for all samples are considered invalid. It is necessary to repeat the analysis of all tests, and also to take measures to detect and eliminate the source of contamination.
2. If no signal is detected for Positive Controls of amplification, it can suggest incorrect programming of the temperature profile, incorrect configuration of the PCR reaction or storage conditions for kit components has not complied with manufacturer instruction, or the reagents kit has expired. Programming of the instruments, storage conditions, and the expiration date of the reagents should be checked, and then the PCR should be repeated.
3. If Ct value in results grid for IC (the JOE/Yellow/Cy3 channel for PCR-mix-1 Screening) exceed 37, analysis should be repeated, beginning with first step.

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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