Sacace
BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Swine Influenza Virus A/H1 Real-TM

Handbook

Real-Time PCR test for the qualitative detection of Swine Influenza Virus A/H1 RNA

REF V55-50FRT

REF TV55-50FRT

50

NAME

Swine Influenza Virus A/H1 Real-TM

INTRODUCTION

Swine Influenza Virus (SIV) H1N1 (referred to as "swine flu" early on) is a new influenza virus causing illness in people. This new virus was first detected in people in Mexico, United States and Canada in April 2009. Other countries, including many European countries, have reported people sick with this new virus. This virus is spreading from person-to-person and it is different from seasonal H1N1 virus, which is a human one that usually circulates widely.

Real-time RT-PCR test for influenza A virus with "Influenza A,B Real-TM" (Sacace, ref. TV36-50FRT) is recommended as a screening test for all suspected cases of swine influenza A (H1N1) virus. Confirmation of swine-origin influenza A (H1N1) virus must be performed with **Swine Influenza Virus A/H1 Real-TM** Real-Time RT-PCR test. World Health Organization (WHO) in the guidance for the surveillance of human infection with swine influenza A (H1N1) virus recommend to perform real-time RT-PCR assay with specific SIV primers as a confirmatory test for swine influenza A virus.

INTENDED USE

Swine Influenza Virus A/H1 Real-TM is Real-Time amplification test for the qualitative detection of Swine Influenza Virus A/H1 RNA in clinical specimens.

PRINCIPLE OF ASSAY

Swine Influenza Virus A/H1 Real-TM Test is based on three major processes: isolation of *virus* RNA from specimens, reverse transcription of the RNA, Real Time amplification of the cDNA. *Influenza virus* A/H1-swine detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers and detection via fluorescent dyes. These dyes are linked with probes of oligonucleotides which bind specifically to the amplified product. The real-time PCR monitoring of fluorescence intensities allows the accumulating product detection without reopening of reaction tubes after the PCR run. **Swine Influenza Virus A/H1 Real-TM** PCR kit is a qualitative test which contain the Internal Control (IC). It must be used in the isolation procedure in order to control the process of each individual sample extraction and serves also to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V55-50FRT)

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 - **“SIV A/H1”:** Real Time amplification kit

- **PCR-mix-1**, 5 x 0,12 ml;
- **PCR-mix-2-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **SIV cDNA H1 C+**, 0,1 ml;
- **Negative Control***, 1,2 ml;
- **Internal Control (IC) RNA****, 5 x 0,12 ml;
- **Internal Control (IC) DNA**, 0,1 ml;
- **DNA buffer**, 0,5 ml;

Contains reagents for 55 tests.

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

Module No.2: Complete Real Time PCR test with RNA purification kit (TV55-50FRT)

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 - **"SIV A/H1":** Real Time amplification kit

- **PCR-mix-1**, 5 x 0,12 ml;
- **PCR-mix-2-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **SIV cDNA H1 C+**, 0,1 ml;
- **Negative Control***, 1,2 ml;
- **Internal Control (IC) RNA****, 5 x 0,12 ml;
- **Internal Control (IC) DNA**, 0,1 ml;
- **DNA buffer**, 0,5 ml;

Contains reagents for 55 tests.

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

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

Swine Influenza Virus A/H1 Real-TM must be stored at 2-8°C ("Reverta-L", PCR-mix-1, PCR-mix-2-FRT, TaqF Polymerase at -20°C). Store **Ribo-Sorb** kit at 2-25°C. 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.

STABILITY

Swine Influenza Virus A/H1 Real-TM 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

***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).
- Clinical specimens from suspect influenza A (H1N1) cases should be performed in a BSL2 laboratory with BSL3 practices (enhanced BSL2 conditions). 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

Swine Influenza Virus A/H1 Real-TM can analyze RNA extracted from:

- *nasopharyngeal swabs*: swab area and place in “Eppendorf” tube with 0,5 ml of saline water or PBS sterile (Sacace Transport medium is recommended). Agitate vigorously. Repeat the swab and agitate in the same tube. Centrifuge at 1000g/min for 5 min. Discard the supernatant and leave about 100 µl of solution for RNA extraction.
- *aspirate, 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.
- *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);

⇒ **SaMag Viral Nucleic Acid Extraction kit** (Sacace, REF SM003).

Please carry out the RNA extraction according to the manufacturer’s instructions. Add 10 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the module no.2)

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** and **10 µl Internal Control (IC RNA)**. Mix by pipetting and incubate 5 min at room temperature.
3. Add **100 µl** of samples to the appropriate tube containing Lysis Solution and IC.
4. Prepare Controls as follows:
   - add **100 µl** of **C– Negative Control** to the tube labeled *Cneg.*
5. 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.
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
8. 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.
9. 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.
10. Add **500 µl** of **Etanolo al 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.
11. Repeat step 10.
12. 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.
13. Incubate all tubes with open cap for 10 min at 60°C.
14. 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).
15. 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°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:

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 µl** 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 3 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled PCR Negative Control;
   - add **10 µl** of **cDNA SIV H1 C+** to the tube labeled C_{pos};
   - add **10 µl** of **IC DNA** to the tube labeled IC DNA_{Pos}

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™/Mx3000P™ (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)*

cDNA of Swine Influenza Virus is detected on the JOE (Yellow)/Hex channel, *IC* on the FAM (Green) channel.

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

1. The sample is considered to be positive for *SIV* if in the channel Joe (Yellow)/HEX the value of **Ct** is different from zero (must be less than 33 (37 for SmartCycler). If Ct value is more than 33 the assay should be repeated and the sample is considered to be positive in case of result's repeat or in case of result is less than 33.
2. The sample is considered to be negative *for SIV* if in the channel Joe (Yellow)/HEX value is not determined (the fluorescence curve does not cross the threshold line) and in the results table on the channel Fam (Green) the Ct value is lower than 37.

Table: Results for controls

Control	Stage for control	Ct channel Fam (Green)	Ct channel Joe (Yellow)	Interpretation
NCE	RNA isolation	Pos (< 37)	Neg	Valid result
NCA	Amplification	Neg	Neg	Valid result
cDNA SIV C+	Amplification	Neg	Pos (< 37)	Valid result
IC DNA	Amplification	Pos (< 37)	Neg	Valid result

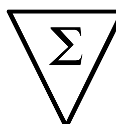
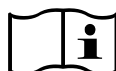
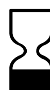
PERFORMANCE CHARACTERISTICS

The kit **Swine Influenza Virus H1 Real-TM** allows to detect *SIV A/H1* in 100% of the tests with a sensitivity of not less than 500 copies/ml.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam (Green) channel): retesting of the sample is required.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended RNA extraction method and follow the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting the extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - 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.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the RNA extraction procedure.
2. Weak (Ct > 37) sample signal on the Joe (Yellow)/Cy3/HEX channel: retesting of the sample is required.
3. Joe (Yellow)/Cy3/HEX 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

Symbol	Meaning	Symbol	Meaning
	List Number		Caution!
	Lot Number		Contains sufficient for <n> tests
	For *in Vitro* Diagnostic Use		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

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
* MX3005P®/MX3000P® are a registered trademark of Agilent Technologies
* ABI® is a registered trademark of Applied Biosystems
* LineGeneK® is a registered trademark of Bioer
* SmartCycler® is a registered trademark of Cepheid

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