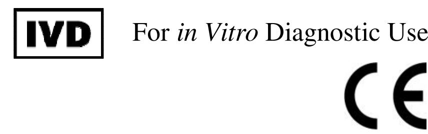

Influenza A H1N1 & H3N2 Real-TM

Handbook

Real Time Amplification test for the detection of Influenza A H1N1 and H3N2 viruses

REF **V54-50 FRT**

REF **TV54-50 FRT**

Σ **50**

NAME

Influenza A H1N1 & H3N2 Real-TM

INTENDED USE

Influenza A H1N1 & H3N2 Real-TM is Real-Time amplification test for typing of Influenza virus A (identification to subtypes H1N1 and H3N2) RNA in Influenza virus cultures and in clinical material (nasal and oropharyngeal swabs; sputum, bronchial lavage, autopsy material).

PRINCIPLE OF ASSAY

Influenza A H1N1 & H3N2 Real-TM Test is based on four major processes: isolation of *Influenza A virus* RNA from specimens, reverse transcription of the RNA and Real Time amplification of the cDNA of *Influenza A virus*. **Influenza A H1N1 & H3N2 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 (V54-50 FRT)

Part N° 2 – **"Reverta-R":**

- **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 – **"Influenza A H1N1 & H3N2 Real-TM":**

- **PCR-mix-1 H1N1**, 5 x 0,12 ml;
- **PCR-mix-1 H3N2**, 5 x 0,12 ml;
- **PCR-mix-2-FRT**, 0,6 ml;
- **TaqF Polymerase**, 0,06 ml;
- **Positive Control cDNA H1N1 C+**, 0,1 ml;
- **Positive Control cDNA H3N2 C+**, 0,1 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control***, 1,2 ml;
- **Internal Control (IC) RNA****, 5 x 0,12 ml;
- **Internal Control (IC) DNA**, 0,1 ml;

Contains reagents for 55 tests.

* *must be used in the extraction procedure as Negative control of extraction.*
** *add 10 µl of Internal Control (IC) RNA during the RNA extraction procedure directly to the sample/lysis mixture (see extraction kit protocol)*

Module No.2: Complete Real Time PCR test with RNA purification kit (TV54-50 FRT)

Part N° 1 – **“Ribo-Sorb”:**

- **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-R”:**

- **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 – **“Influenza A H1N1 & H3N2 Real-TM”:**

- **PCR-mix-1 H1N1**, 5 x 0,12 ml;
- **PCR-mix-1 H3N2**, 5 x 0,12 ml;
- **PCR-mix-2-FRT**, 0,6 ml;
- **TaqF Polymerase**, 0,06 ml;
- **Positive Control cDNA H1N1 C+**, 0,1 ml;
- **Positive Control cDNA H3N2 C+**, 0,1 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control***, 1,2 ml;
- **Internal Control (IC) RNA****, 5 x 0,12 ml;
- **Internal Control (IC) DNA**, 0,1 ml;

Contains reagents for 55 tests.

* *must be used in the extraction procedure as Negative control of extraction.*

** *add 10 µl of Internal Control (IC) RNA during the RNA extraction procedure directly to the sample/lysis mixture (see extraction kit protocol)*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- RNA extraction kit (Module No. 1)
- Biosafety cabinet
- Desktop microcentrifuge for "eppendorf" type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with aerosol barriers
- 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

Reverta-L and **Influenza A H1N1 & H3N2 Real-TM** must be stored at ≤ -16°C. Store **Ribo-Sorb** at 2-8°C. The kits can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and ≤ -16°C immediately on receipt.

STABILITY

Influenza A H1N1 & H3N2 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 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

Influenza A H1N1 & H3N2 Real-TM 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.
- *autopsy material*: Collect autopsy material into sterile containers and freeze immediately or analyze within 1 h. The samples can be stored at ≤ –68 °C for 1 year. Homogenize the samples using sterile porcelain mortars and pestles and then prepare 10 % suspension in sterile saline or PBS. Transfer the suspension into a 1.5-ml tube and centrifuge at 10000 rpm for 5 min. Use 100 µl of the supernatant for RNA extraction. Freeze the rest of aspirate if it is necessary to repeat analysis later.

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.
- *animal 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

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 Acids 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 DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8ºC) should be warmed up to 60–65ºC 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 **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.
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 37ºC 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 -20ºC for a week or at -70ºC during a year.

PCR Reagents preparation

Reaction Mix 25 µl

1. Prepare required quantity of tubes or PCR plate (2 tubes for each sample if H1N1 and H3N2 have to be tested simultaneously).
2. Prepare for each sample in the new sterile tube:

    10*N µl of **PCR-mix-1 (**PCR-mix-1 H1N1 or/and PCR-mix-1 H3N2),

    5*N µl of **PCR-mix-2**-**FRT**

    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.
4. Prepare for each panel 3 controls:
    - add **10 µl** of **DNA-buffer** to the tube labeled PCR Negative Control;
    - add **10 µl** of **Positive Control cDNA H1N1 (or H3N2) C+** to the tube labeled C_{pos};
    - add **10 µl** of **Internal Control (IC) DNA** to the tube labeled IC DNA_{Pos}

Create a temperature profile on your Real-time instrument as follows:

<table>
<tr><th colspan="4">Plate type or modular type instruments[1]</th><th colspan="4">Rotor type instruments[2]</th></tr>
<tr><td>Temp, ℃</td><td>Time</td><td>Fluorescence detection</td><td>Cycle repeats</td><td>Temp, ℃</td><td>Time</td><td>Fluorescence detection</td><td>Cycle repeats</td></tr>
<tr><td>95</td><td>15 min</td><td>–</td><td>1</td><td>95</td><td>15 min</td><td>–</td><td>1</td></tr>
<tr><td rowspan="3">95</td><td rowspan="3">15 s</td><td rowspan="3">–
–
–</td><td rowspan="6">42</td><td>95</td><td>10 s</td><td>–</td><td rowspan="3">10</td></tr>
<tr><td>54</td><td>20 s</td><td>–</td></tr>
<tr><td>72</td><td>10 s</td><td>–</td></tr>
<tr><td rowspan="2">54</td><td rowspan="2">25 s</td><td rowspan="2">FAM, JOE/HEX/Cy3, ROX/Texas Red</td><td>95</td><td>10 s</td><td>–</td><td rowspan="3">35</td></tr>
<tr><td>54</td><td>20 s</td><td>FAM/Green, JOE/Yellow, ROX/Orange</td></tr>
<tr><td>72</td><td>25 s</td><td>–</td><td>72</td><td>10 s</td><td>–</td></tr>
</table>

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

INSTRUMENT SETTINGS

Rotor-type instruments

<table>
<tr><th>PCR-mix-1</th><th>Channel</th><th>Calibrate/Gain Optimisation…</th><th>Threshold</th><th>More Settings/ Outlier Removal</th><th>Slope Correct</th></tr>
<tr><td rowspan="3">PCR-mix-1-H1N1</td><td>FAM/Green</td><td>from 5 Fl to 10 Fl</td><td>0.1</td><td>0 %</td><td>off</td></tr>
<tr><td>JOE/Yellow</td><td>from 5 Fl to 10 Fl</td><td>0.1</td><td>5 %</td><td>off</td></tr>
<tr><td>Rox/Orange</td><td>from 5 Fl to 10 Fl</td><td>0.1</td><td>5 %</td><td>on</td></tr>
<tr><td rowspan="3">PCR-mix-1-H3N2</td><td>FAM/Green</td><td>from 5 Fl to 10 Fl</td><td>0.1</td><td>0 %</td><td>off</td></tr>
<tr><td>JOE/Yellow</td><td>from 5 Fl to 10 Fl</td><td>0.05</td><td>5 %</td><td>off</td></tr>
<tr><td>Rox/Orange</td><td>from 5 Fl to 10 Fl</td><td>0.1</td><td>5 %</td><td>on</td></tr>
</table>

Plate-type instruments

<table>
<tr><th>PCR-mix-1</th><th>Channel</th><th>Threshold</th></tr>
<tr><td rowspan="3">PCR-mix-1- H1N1,
PCR-mix-1- H3N2</td><td>FAM/Green</td><td rowspan="3">The threshold is set manually at a level corresponding to 10-20% of maximum fluorescence detected for the positive controls during the last amplification cycle. Moreover, the fluorescence curve of the positive controls should cross the threshold line in the area of exponential growth passing through linear phase.</td></tr>
<tr><td>JOE/Yellow</td></tr>
<tr><td>Rox/Orange</td></tr>
</table>

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RESULTS ANALYSIS

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

Channels for detection of gene targets

PCR-mix-1	Detection in channel		
	FAM/Green	JOE/HEX/Yellow	ROX/Orange/Texas Red
PCR-mix-1- H1N1	IC	Influenza virus A H1	Influenza virus A N1
PCR-mix-1- H3N2	IC	Influenza virus A H3	Influenza virus A N2

Results are accepted as relevant if both positive and negative controls of amplification are passed.

Analysis of results for clinical samples:

- AH1 (or A/H3) Influenza virus is detected in a clinical sample if the Ct value is detected in the JOE/Yellow channel and if it is less than the Ct value specified in the table below;
- AN1 (or A/N2) Influenza virus is detected in a clinical sample if the Ct value is detected in the ROX/Orange channel and if it is less than the Ct value specified in the table below;
- If Ct value is not detected in one or both channels (JOE/Yellow or ROX/Orange) and the Ct value for the Internal Control is less than the Ct value specified for the IC in the table below in the FAM/Green channel, the analysed A/H1N1 (or A/H3N2) subtype of the epidemic Influenza virus is not found.

Results and boundary values for controls and clinical samples

Sample	Instruments								
	Rotor type			Plate types			SmartCycler		
	FAM Green	JOE Yellow	ROX Orange	FAM	JOE HEX	ROX	FAM	Cy3	Texas Red
NCE	<28	-	-	<28	-	-	<38	-	-
NCA	-	-	-	-	-	-	-	-	-
IC DNA	<26	-	-	<26	-	-	<36	-	-
C+A H1N1 C+A H3N2	-	<25	<25	-	<25	<25	-	<35	<35
Clinical samples	<28	≤35	≤35	<28	≤35	≤35	<38	≤42	≤42

PERFORMANCE CHARACTERISTICS

The kit **Influenza A H1N1 & H3N2 Real-TM** allows to detect *Influenza A* in 100% of the tests with a sensitivity of not less than 1000 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 signal on the Fam(Green) (Ct > 28 RG, > 38 others), Joe (Yellow)/Cy3/HEX (Ct > 35 RG, > 42 others) or Rox (Orange)/TexasRed channel (Ct > 35 RG, > 42 others): retesting of the sample is required.
3. 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
REF	List Number	!	Caution!
LOT	Lot Number	Σ	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

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* iCycler™ and iQ5™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Corbett Research
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* Applied Biosystems® is trademarks of Applera Corporation
* SmartCycler® is a registered trademark of Cepheid

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