HIV Real-TM Quant

Handbook

Real Time PCR kit for quantitative detection of Human Immunodeficiency Virus (HIV)

REF R-VM-100FRT

REF TR-VM-100FRT

REF TR-VM-100FRT C

Σ 100

NAME

HIV Real-TM Quant

INTENDED USE

kit **HIV Real-TM Quant** is a Real-Time test for the quantitative detection of Human Immunodeficiency Virus (HIV) and simultaneous detection of a HIV-specific Internal Control (IC), by dual color detection.

PRINCIPLE OF ASSAY

kit **HIV Real-TM Quant** is a Real-Time test for the quantitative detection of Human Immunodeficiency Virus in human plasma. HIV RNA is extracted from plasma, amplified using real time amplification and detected using fluorescent reporter dye probes specific for HIV or HIV IC.

Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible inhibition. IC is detected in a channel other than the HIV RNA. HIV Rec IC is a quantitative Internal Control (concentration reported in Data Card) and represents recombinant RNA-containing-structure which carried through all steps of analysis from nucleic acid extraction to PCR amplification-detection. The presence of quantitative HIV Rec IC allows not only to monitor the extraction procedure and to check possible PCR inhibition but also to verify possible losses of the RNA during extraction procedure thus enabling to calculate precisely the HIV viral load.

Monitoring the fluorescence intensities during Real Time allows the detection and quantification of the accumulating product without having to re-open the reaction tube after the real time amplification.

The kit will allow the quantification of 100 samples, including all the necessary reagents to generate the external standard curve of HIV and IC. To generate HIV and IC standard curve for quantification of the amplification products all calibrators should be used and defined as standards with specific concentrations.

MATERIALS PROVIDED

Kit R-VM-100FRT

Part N° 2 – **"Controls"**

- **HIV Rec Pos1 Control***, 4 x 0,01 ml;
- **HIV Rec Pos2 Control***, 4 x 0,01 ml;
- **Negative Control****, 4 x 0,5 ml;
- **HIV Rec IC (Internal Control)*****, 4 x 0,13 ml.

Part N° 3 – **"HIV Real-TM Quant":**

- **DTT**, 4 tubes.
- **RT-PCR-mix-1-TM HIV**, 4 x 0,3 mL.
- **RT-PCR-mix-2-TM**, 4 x 0,2 mL.
- **Hot Start TaqF Polymerase**, 4 x 0,02 mL
- **M-MLV Revertase**, 4 x 0,01 mL;
- **TE-buffer**, 4 x 0,07 mL
- **Standard HIV**[1]
  - **QS1 HIV**, 4 x 0,025 mL;
  - **QS2 HIV**, 4 x 0,025 mL;
  - **QS3 HIV**, 4 x 0,025 mL;
- **Standard IC**[1]
  - **QS1 IC**, 4 x 0,025 mL;
  - **QS2 IC**, 4 x 0,025 mL;
  - **QS3 IC**, 4 x 0,025 mL;

[1] Standards' and controls' concentrations are specific for every lot.

* *must be used in the isolation procedure as Positive Control of Extraction (when using SaMag Viral Nucleic Acids extraction kit always add 10 µl of Positive Controls of Extraction regardless of the extraction starting volume. For example add 10 µl of Pos Control and 390 µl of negative control if the extraction starting volume is 400 µl).*

** *must be used in the isolation procedure as Negative Control of Extraction.*

*** *add 5 µl of Internal Control during the RNA isolation directly to the sample/lysis mixture*

Kit TR-VM-100FRT

Part N° 1 – **"Ribo-Sorb-100":**

- **Lysis Solution**, 2 x 22,5 ml;
- **Washing Solution**, 2 x 20,0 ml;
- **Sorbent**, 2 x 1,25 ml;
- **RNA-eluent**, 10 x 0,5 ml

Part N° 2 – **"Controls"**

- **HIV Rec Pos1 Control***, 4 x 0,01 ml;
- **HIV Rec Pos2 Control***, 4 x 0,01 ml;
- **Negative Control****, 4 x 0,5 ml;
- **HIV Rec IC (Internal Control)*****, 4 x 0,13 ml.

Part N° 3 – **"HIV Real-TM Quant":**

- **DTT**, 4 tubes.
- **RT-PCR-mix-1-TM HIV**, 4 x 0,3 mL.
- **RT-PCR-mix-2-TM**, 4 x 0,2 mL.
- **Hot Start TaqF Polymerase**, 4 x 0,02 mL
- **M-MLV Revertase**, 4 x 0,01 mL;
- **TE-buffer**, 4 x 0,07 mL
- **Standard HIV**[1]
  - **QS1 HIV**, 4 x 0,025 mL;
  - **QS2 HIV**, 4 x 0,025 mL;
  - **QS3 HIV**, 4 x 0,025 mL;
- **Standard IC**[1]
  - **QS1 IC**, 4 x 0,025 mL;
  - **QS2 IC**, 4 x 0,025 mL;
  - **QS3 IC**, 4 x 0,025 mL;

[1] Standards' and controls' concentrations are specific for every lot.

* *must be used in the isolation procedure as Positive Control of Extraction.*
** *must be used in the isolation procedure as Negative Control of Extraction.*
*** *add 5 µl of Internal Control during the RNA isolation directly to the sample/lysis mixture*

Kit TR-VM-100FRT C

Part N° 1 – "**Ribo-Virus**":

- **Buffer RAV1**, 2 x 35 ml;
- **Buffer RAW**, 2 x 30 ml;
- **Buffer RAV3 (concentrate)**, 2 x 12,5 ml;
- **Buffer RE**, 2 x 5 ml;
- **Rnase-free H_2O**, 2 x 5 ml
- **Carrier RNA (lyophilized)**, 2 x 1 mg;
- **Ribo Virus columns**, 100;
- **Collecting tubes (2ml)**, 400;

Part N° 2 – **"Controls"**

- **HIV Rec Pos1 Control***, 4 x 0,01 ml;
- **HIV Rec Pos2 Control***, 4 x 0,01 ml;
- **Negative Control****, 4 x 0,5 ml;
- **HIV Rec IC (Internal Control)*****, 4 x 0,13 ml.

Part N° 3 – **"HIV Real-TM Quant":**

- **DTT**, 4 tubes.
- **RT-PCR-mix-1-TM HIV**, 4 x 0,3 mL.
- **RT-PCR-mix-2-TM**, 4 x 0,2 mL.
- **Hot Start TaqF Polymerase**, 4 x 0,02 mL
- **M-MLV Revertase**, 4 x 0,01 mL;
- **TE-buffer**, 4 x 0,07 mL
- **Standard HIV**[1]
  - **QS1 HIV**, 4 x 0,025 mL;
  - **QS2 HIV**, 4 x 0,025 mL;
  - **QS3 HIV**, 4 x 0,025 mL;
- **Standard IC**[1]
  - **QS1 IC**, 4 x 0,025 mL;
  - **QS2 IC**, 4 x 0,025 mL;
  - **QS3 IC**, 4 x 0,025 mL;

[1] Standards' and controls' concentrations are specific for every lot.

* *must be used in the isolation procedure as Positive Control of Extraction.*
** *must be used in the isolation procedure as Negative Control of Extraction.*
*** *add 5 µl of Internal Control during the RNA isolation directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (only with R-VM-100FRT kit)
- Biological 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
- Biohazard waste container
- Refrigerator, Freezer
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone

Zone 2: RT and amplification:

- Real Time Thermal cycler
- Tubes (0,2 ml)
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

STORAGE INSTRUCTIONS

Part N° 1 – **“Ribo-Sorb-100”** must be stored at 2-8°C

Part N° 1 – “**Ribo-Virus**” must be stored at 2-25°C.

Part N° 2 – **“Controls”** must be stored at 2-8°C.

Part N° 3 – **“HIV Real-TM Quant”** must be stored at -20°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

HIV Real-TM Quant Test 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

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

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.

PRODUCT USE LIMITATIONS

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

HIV Real-TM Quant can analyze RNA extracted from:

- *plasma* collected blood in ACD or EDTA tubes;

Specimens can be stored at +2-8°C for no longer than 12 hours, or freeze 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

The following kits are recommended:

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

Please carry out RNA extraction according to the manufacture's instruction. Add 5 µl of Internal Control during RNA isolation procedure directly to the sample/lysis mixture.

INTERNAL CONTROL (HIV Rec IC)

HIV Rec IC is a quantitative Internal Control (concentration reported in Data Card) and represents recombinant RNA-containing-structure which carried through all steps of analysis from nucleic acid extraction to PCR amplification-detection. The presence of quantitative HIV Rec IC allows not only to monitor the extraction procedure and to check possible PCR inhibition but also to verify possible losses of the RNA during extraction procedure thus enabling to calculate precisely the HIV viral load.

SPECIMEN AND REAGENT PREPARATION (Only for **Kit TR-VM-100FRT)**

To increase the sensitivity of test it is recommended to ultracentrifuge plasma samples (1 ml) at 24,000 x *g* at 4°C for 60 minutes. Discard the supernatant (900 µl) and use the remaining 100 µl pellet for RNA extraction.

1. **Lysis Solution** and **Washing Solution** should be warmed up to 60–65°C until disappearance of ice crystals.
2. Prepare 70% Ethanol.
3. Prepare the required quantity of 1,5 ml polypropylene tubes including one tube for **Negative Control of Extraction** and two tubes for **Positive Controls of Extraction** .
4. Add to each tube **5 µl** of **Internal Control** and **450 µl Lysis Solution**.
5. Vortex thawed patient plasma specimens for 5 sec.
6. Add **450 µl** of **Lysis Solution with IC** to each of labeled tubes.
7. Add **100 µl** of **Samples** to the appropriate tube.
8. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control**) to labeled C*neg*.
   - add **90 µl** of **C–** (**Negative Control**) and **10 µl** of **HIV Rec Pos1** to the tube labeled C*pos1*.
   - add **90 µl** of **C–** (**Negative Control**) and **10 µl** of **HIV Rec Pos2** to the tube labeled C*pos2*
9. Vortex the tubes and centrifuge for 7-10 sec.
10. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
11. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
12. Centrifuge all tubes for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
13. Add **400 µl** of **Washing Solution** to each tube. Vortex vigorously until the sorbent is completely resuspended, centrifuge for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
14. Add **500 µl** of **Ethanol al 70%** to each tube. Vortex vigorously until the sorbent is completely resuspended, centrifuge for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
15. Repeat step 14.
16. Add **500 µl** of **Acetone** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully

remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.

17. Incubate all tubes with open cap for 10 min at 56°C.
18. Resuspend the pellet in **50 µl** of **RNA-eluent.** Incubate for 10 min at 56°C and vortex periodically.

Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains RNA/DNA ready for amplification. The Real Time amplification can be performed on the same day of extraction.

SPECIMEN AND REAGENT PREPARATION (Only for **Kit TR-VM-100FRT C)**

Before starting the viral RNA isolation, prepare a 70°C incubation block and preheat an aliquot of elution buffer/water.

1. Prepare required quantity of 1,5 ml polypropylene tubes including one tube for **Negative Control of Extraction** and two tubes for **Positive Controls of Extraction** .
2. Add **600 µl buffer RAV1** containing carrier RNA into 1,5 ml tubes
3. Add to all tubes **5 µl** of **Internal Control.**
4. Add **150 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control**) to the tube labeled C*neg*.
   - add **90 µl** of **C–** (**Negative Control**) and **10 µl** of **HIV Rec Pos1** to the tube labeled C*pos1.*
   - add **90 µl** of **C–** (**Negative Control**) and **10 µl** of **HIV Rec Pos2** to the tube labeled C*pos2.*
6. Mix by pulse-vortexing for 15 sec. Incubate **for 5 min at 70°C.** Briefly centrifuge 1,5 ml polypropylene tubes to remove drops from the inside of the lid.
7. Add **600 µl ethanol (96-100%)** to the sample and mix by vortexing for 15 sec. After mixing, briefly centrifuge 1,5 ml polypropylene tubes to remove drops from the inside of the lid
8. Place **Ribo Virus** columns in 2 ml collecting tubes and load **700 µl** lysed sample. **Centrifuge** for **1 min** at **8,000 x *g***.

   Load the residual lysis solution onto the Ribo Virus column. **Centrifuge** for **1 min** at **8,000 x g**.

   The use of new 2 ml collecting tubes for every step is recommended if infectious material has to be prepared. This avoids cross-contamination and contamination of centrifuge units. Additional collecting tubes can be ordered separately. For non-infectious samples, we recommend to discard the flow-through and reuse the 2 ml tube for loading and washing steps.

   Discard the collecting tube containing the filtrate and put the Ribo Virus column into another new 2 ml collecting tube.

9. Add **500 µl buffer RAW** to the Ribo Virus column. **Centrifuge** for **1 min** at **8,000 x *g***. Discard the collecting tube containing the filtrate and put the Ribo Virus column into another new 2 ml collecting tube.
10. Add **600 µl buffer RAV3** to the Ribo Virus column. **Centrifuge** for **1 min** at **8,000 x *g***. Discard the collecting tube containing the filtrate and put the Ribo Virus column into another new 2 ml collecting tube.
11. Add **200 µl buffer RAV3**. **Centrifuge** for **5 min** at **11,000 x g** to remove ethanolic buffer RAV3 completely.
12. Incubate the columns with open cap for 1 min at 70°C to remove any remaining traces of ethanol.
13. Place the Ribo Virus column into a new, sterile 1.5 ml centrifuge tube (not provided). Add **50 µl Rnase-free H_2O preheated to 70°C** and **incubate** for **1-2 min.** Centrifuge for **1 min at 11,000 x g**

RT AND AMPLIFICATION

1. Thaw one set of reagents, vortex and centrifuge briefly the tubes.
2. Prepare 0,2 ml tubes
3. Prepare **Reaction Mix**: add into the tube with **DTT 300 µl** of **RT-PCR-mix-1 HIV**, **200 µl** of **RT-PCR-mix-2, 20 µl** of **Hot Start TaqF Polymerase** and **7,5 µl** of **M-MLV Revertase**. Vortex thoroughly and centrifuge briefly.
   *(If it is necessary to test less than 25 samples add into the tube with **DTT, 300 µl** of **RT-PCR-mix-1**, **200 µl** of **RT-PCR-mix-2** and vortex for at least 5-10 seconds. This mix is stable for 1 month at -20°C. Add for each sample (N) in the new sterile tube **12,5*N µl** of mix, **0,5 *N µl** of **TaqF Polymerase** and **0,25*N µl** of **M-MLV.**)*
4. Add **12,5 µl** of **Reaction Mix** into each tube.
5. Add **12,5 µl** of **extracted RNA** sample to the appropriate tube with Reaction Mix and 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!

6. Prepare for each run 6 standards and 1 negative control:
   7. add **12,5 µl** of **Quantitation Standards HIV** (QS1 HIV, QS2 HIV, QS3 HIV) into 3 labeled tubes;
   8. add **12,5 µl** of **Quantitation Standards IC** (QS1 IC, QS2 IC, QS3 IC) into 3 labeled tubes;
   9. add **12,5 µl** of **TE-buffer** to the tube labeled Negative Control of Amplification;

Amplification

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

	Rotor-type Instruments[1]			Plate- or modular type Instruments[2]		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**50**	30 min	1	**50**	30 min	1
2	**95**	15 min	1	**95**	15 min	
2	**95**	20 s	5	**95**	20 s	5
	52	30 s		**52**	30 s	
	72	30 s		**72**	30 s	
3	**95**	20 s	40	**95**	20 s	42
	55	30 s *fluorescent signal detection*		**55**	40 s *fluorescent signal detection*	
	72	30 s		**72**	30 s	

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

Fluorescence is detected at the 2nd step of Cycling 2 stage (55 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

HIV RNA is detected on the JOE(Yellow)/HEX/Cy3, *IC RNA* 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*	*0.03*	*10 %*	*ON*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.03*	*5 %*	*ON*

Plate-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.

Boundary value of the cycle threshold, Ct

Sample	Channel for fluorophore	Ct boundary value	
		Rotor-type instruments	Plate-type instruments
C+	FAM/Green	28	28
	JOE/Yellow/Hex/Cy3	30	30
Clinical samples, C-	JOE/Yellow/Hex/Cy3	40	40

RESULTS INTERPRETATION

For each control and patient specimen, calculate the concentration of HIV RNA using the following formula:

$$\frac{\text{HIV DNA copies/specimen}}{\text{IC DNA copies/specimen}} \text{ x coefficient* = copies HIV/mL}$$

*coefficient is specific for each lot and reported in the HIV Real-TM Quant Data Card provided in the kit.

HIV Real-TM Quant is linear from 25 to 5 x 10^6 copies/mL. Test results greater than 5.000.000 copies/mL are above the upper limit of quantitation of the test and should be reported as "greater than 5.000.000 copies/mL". If quantitation results are desired for such samples, the specimen should be diluted 1:10 with negative serum and retested. Test results less than 25 copies/mL are below the lower limit of quantitation of the test and should be reported as "less than 25 copies/mL".

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 **HIV** primers and probes. The specificity of the kit **HIV Real-TM Quant** was 100%. The potential cross-reactivity of the kit **HIV Real-TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **HIV Real-TM Quant** allows to detect **HIV** RNA in 100% of the tests with a sensitivity of not less than 25 copies/ml. The detection was carried out on the control standard and its dilutions by negative plasma using the "Magno-Sorb" extraction kit (Sacace REF K-2-16/1000).

Target region: pol gene

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

TROUBLESHOOTING

1. Weak or no signal of the IC (FAM/Green channel) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended RNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of 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
    - Improper RNA extraction.
        - ⇒ Repeat analysis starting from the RNA extraction stage
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection 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 or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. JOE(Yellow)/HEX/Cy3 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 between tubes.
        - ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special RNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ 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
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
		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® is a registered trademark of Agilent Technologies
* ABI® is a registered trademark of Applied Biosystems

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