IVD For *in Vitro* Diagnostic Use

CE

MERS-SARS CoV Real-TM

Handbook

Real Time PCR kit for detection and differentiation of Middle East Respiratory Sindrome (MERS-CoV) and Severe Acute Respiratory Sindrome Coronaviruses (SARS)

REF V65-50FRT

Σ **50**

NAME

MERS-SARS CoV Real-TM

INTRODUCTION

Coronaviruses are a large family of ribonucleic acid (RNA) viruses capable of infecting humans and a number of animal species. In humans, coronaviruses may cause a range of illnesses, from the common cold to severe acute respiratory syndrome (SARS).

Middle East Respiratory Syndrome (MERS) is viral respiratory illness first reported in Saudi Arabia in 2012. It is caused by a coronavirus called MERS-CoV. Early reports compared the virus to severe acute respiratory syndrome (SARS), and it has been referred to as Saudi Arabia's SARS-like virus. Most people who have been confirmed to have MERS-CoV infection developed severe acute respiratory illness. As of 31 October 2013, the World Health Organization confirmed that 149 people have contracted MERS worldwide, of which 63 have died. Affected countries in the Middle East include Jordan, Kingdom of Saudi Arabia (KSA), the United Arab Emirates (UAE), Oman, Kuwait and Qatar; in Europe countries affected include: France, Germany, the United Kingdom (UK) and Italy; and in North Africa: Tunisia. Infections presumably acquired through exposure to non-human sources have all occurred in the Middle East; limited transmission in the countries of Europe and North Africa has occurred in close contacts of recent travellers from the Middle East.

INTENDED USE

MERS-SARS CoV Real-TM is Real-Time PCR test for the qualitative detection and differentiation of **Middle East Respiratory Sindrome (MERS-CoV)** and **Severe Acute Respiratory Sindrome Coronaviruses (SARS)**

PRINCIPLE OF ASSAY

MERS-SARS CoV 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. *Coronavirus* 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. **MERS-SARS CoV 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

Part N° 1 – **“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° 2 – **“MERS-SARS CoV Real-TM”:** Real Time amplification kit

- **PCR-mix-1**, 0,6 ml;
- **PCR-mix-2-FRT**, 0,3 ml;
- **TaqF Polymerase**, 0,03 ml;
- **Pos cDNA MERS/SARS/IC C+**, 0,2 ml;
- **DNA buffer**, 0,2 ml;
- **Negative Control**, 1,2 ml*;
- **Internal Control RNA (IC)**, 5 x 0,12 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
- Biosafety cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- 60°C ± 5°C dry heat block
- Vortex mixer
- Pipettes with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks
- Refrigerator
- Freezer

Zone 2: RT and amplification:

- Real Time Thermalcycler
- Workstation
- Pipettes with aerosol barrier
- Tube racks

STORAGE INSTRUCTIONS

MERS-SARS CoV Real-TM must be stored at -20°C.The kits can be shipped at 2-8°C for 3-4 days but should be stored at -20°C immediately on receipt.

STABILITY

MERS-SARS CoV 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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Clinical specimens from MERS cases should be considered as biological substances, category B.
- 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.

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. 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

MERS-SARS CoV 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 5000g/min for 5 min. Discard the supernatant and leave about 100 µl of solution for RNA extraction.
- *aspirate, bronchial lavage, nasal wash:* centrifuge at 10000 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.
- *plasma:* whole blood collected in EDTA should be separated into plasma and cellular components by centrifugation at 800-1600 x g for 20 min within six hours. The isolated plasma has to be transferred into a sterile polypropylene tube. Plasma may be stored at 2-8°C for an additional 3 days. Alternatively, plasma may be stored at -18°C for up to one month or 1 year when stored at -70°C.
- *feces: p*repare 10-20% feces suspension, for instance adding 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces in 5 ml tube (the same can be done in 2,0 ml tube). The RNA purification must be done immediately, if it is not possible add 20% Glycerol sterile solution (cryoprotective agent that provides intracellular and extracellular protection against freezing) and store at -20°C. Vortex to get an homogeneous suspension and centrifuge for 5 min to 7000-12000g. Use the supernatant for the extraction of the viral DNA/RNA.
- *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:

- ⇨ **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.

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.
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** 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 4 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control (NCA);
   - add **10 µl** of extracted **DNA of Neg Control** to the tube labeled Negative Extraction Control (NCE).
   - add **10 µl** of **cDNA MERS/SARS/IC C+** to the tube labeled C_{posDNA};

Amplification

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

	Rotor type instruments[1]			Plate type instruments[2]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
1	95	15 min	1	95	15 min	1
2	95	10 s	10	95	10 s	10
	54	20 s		54	25 s	
	72	10 s		72	25 s	
3	95	10 s	35	95	10 s	35
	54	20 s Fluorescence detection		54	25 s Fluorescence detection	
	72	10 s		72	25 s	

[1] *For example, Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen);*
[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™/Mx300oP™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems),* SmartCycler® (Cepheid)

MERS-Cov is detected on the Rox (Orange) channel, *SARS* is detected on the JOE (Yellow) 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	10 %	off
JOE/Yellow	*from 5 Fl to 10 Fl*	0.1	10 %	off
Rox/Orange	*from 5 Fl to 10 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 *MERS* if in the channel Rox (Orange) the value of **Ct** is different from zero (must be less than 33) . 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 positive for *SARS* if in the channel Joe (Yellow) the value of **Ct** is different from zero (must be less than 33). 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.
3. The sample is considered to be negative *for MERS & SARS* if in the channel Fam (Green) 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 28.

Table. Results for controls

Control	Stage for control	Ct channel Fam (Green)	Ct channel Joe (Yellow)	Ct channel Rox (Orange)	Result
NCE	RNA isolation, PCR	Pos (< 30)	Neg	Neg	Valid result
NCA	PCR	Neg	Neg	Neg	Valid result
Pos cDNA	PCR	Pos (< 28)	Pos (< 28)	Pos (< 28)	Valid result

PERFORMANCE CHARACTERISTICS

Sensitivity

The kit **MERS-SARS CoV Real-TM** allows to detect *MERS-CoV* and *SARS* in 100% of the tests with a sensitivity of not less than 500 copies/ml.

Specificity

The analytical specificity of **MERS-SARS CoV Real-TM** PCR kit is ensured by selection of specific primers and probes as well as strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis. Specificity was confirmed on the following microorganism strains: Coronaviruses (*Cov*-E229, *Cov*-OC43, *Cov*-HKUI, *Cov*-NL63, FC0, FC1, CCV), *Enterovirus* strains, *Adenovirus*; *influenza virus* A and B; *rhinovirus*; *RS viruses*; *Parainfluenza Virus, Streptococcus* spp., *Staphylococcus aureus*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Haemophilus influenzae, Moraxella catarrhalis, Legionella pneumophila.*

Specificity of the kit was confirmed testing positive control samples of RNA MERS-CoV (hCov-EMC upE-Assay: IVT-RNA), recommended by WHO for screening tests (Institute of Virology, University of Bonn Medical Centre, Germany) and positive control samples of SARS RNA (University of Frankfurt, Germany).

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.
    - 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 Joe (Yellow)/Cy3/HEX and Rox/TexasRed channels: retesting of the sample is required.
3. 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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