IVD For *in Vitro* Diagnostic Use

CE

SARS-CoV-2 Real-TM

Handbook

Real Time PCR kit for detection of SARS-CoV-2 (COVID-19) RNA in clinical samples

REF **V435-100FRT**

Σ **96**

NAME

SARS-CoV-2 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).

The novel coronavirus (SARS-CoV-2) is a new strain which has not previously been identified in humans. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), known also as 2019 novel coronavirus (2019-nCoV), is a positive-sense single-stranded RNA virus. It is contagious in humans and is the cause of the ongoing pandemic of coronavirus disease 2019 (COVID-19) that has been designated a Public Health Emergency of International Concern by the World Health Organization (WHO).

The earliest case of infection currently known is thought to have been found on November 2019. The virus subsequently spread to all provinces of China and to more than one hundred other countries in Asia, Europe, North America, South America, Africa, and Oceania. On 11th March 2020 the WHO has officially declared it a pandemic.

INTENDED USE

SARS-CoV-2 Real-TM is Real-Time PCR test for the qualitative detection of SARS-CoV-2 (COVID-19 virus, 2019-nCoV) RNA in clinical samples.

PRINCIPLE OF ASSAY

SARS-CoV-2 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. The reverse transcription and amplification are performed in a single, one step reaction. *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.

SARS-CoV-2 Real-TM is a multiplex Real-time PCR assay that detects and identifies the causative agent of the new coronavirus disease (COVID-19) using three targets: E gene and N gene specific for SARS-CoV-2 and region of E gene common for all SARS-like coronaviruses (SARS-CoV, SARS-CoV-2).

SARS-CoV-2 Real-TM PCR kit is a multiplex Real Time PCR kit with four simultaneous reactions – amplification of the SARS-like coronaviruses, including SARS-CoV, SARS-CoV-2 (FAM channel); SARS-CoV-2 E-gene (ROX channel); SARS-CoV-2, N-gene (Cy5 channel) as well as amplification of nucleic acid sequence of the Internal Control-RNA in HEX channel, which allows to exclude inhibition and nucleic acids extraction issues.

Detection channel	FAM/Green	JOE/HEX/Yellow	ROX/Orange	Cy5/Red
Result	SARS-like coronaviruses, SARS-CoV, SARS-CoV-2	IC detection	SARS-CoV-2, *E-gene*	SARS-CoV-2, *N-gene*

Detecting 2 different genes in the SARS-Cov-2 genome allows Sacace SARS-COV-2 Real-TM kit to be able to detect the virus RNA even in case of virus mutations in one of the detected genes.

MATERIALS PROVIDED

Format T (tube format)

- **Tubes-COVID19**, 96 tubes, 15 µl in each tube;
- **RT-PCR Buffer**, 2 x 0,81 ml;
- **Enzymes Taq/RT**, 0,055 ml;
- **Pos cDNA C+**, 0,13 ml;
- **Negative Control**, 1,0 ml*;
- **Internal Control RNA,** 1 ml;**

Contains reagents for 96 tests.

Format S (strip format)

- **Strips-COVID19**, 8x12 strip tubes (tot. 96 tubes, 15 µl in each tube), including optical strip caps;
- **RT-PCR Buffer**, 2 x 0,81 ml;
- **Enzymes Taq/RT**, 0,055 ml;
- **Pos cDNA C+**, 0,13 ml;
- **Negative Control**, 1,0 ml*;
- **Internal Control RNA,** 1 ml;**

Contains reagents for 96 tests.

* *must be used in the isolation procedure as Negative Control of Extraction (NCE).*
** *must be used as Internal Control during the RNA isolation: add 10 µl of Internal Control RNA directly to the sample / lysis mixture at the beginning of the extraction process*

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA extraction kit
- Real Time qPCR Thermalcycler instrument
- Workstation
- Pipettes with aerosol barrier
- Tubes and tubes racks

STORAGE INSTRUCTIONS

All reagents of **SARS-CoV-2 Real-TM kit** must be stored at 2-8°C except for **Enzymes Taq/RT vial** which has to be stored at -16°C or below. The kits can be shipped at 2-8°C for 3-4 days but should be stored at -16°C or below and 2-8°C immediately on receipt.

STABILITY

SARS-CoV-2 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.

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.

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 Sars-Cov-2 cases should be considered as biological substances and must be handled in a BSL-2 laboratory
- 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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

SARS-CoV-2 Real-TM can analyze RNA extracted from:

- *Nasopharyngeal / nasal 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. Use 100 µl of solution for RNA extraction.
- *Tracheal 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.

Specimens can be stored at +2-8°C for no longer than 48 hours, or frozen at -20°C to -80°C for longer periods. 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:

- ⇨ **M-Sorb-S** (Sacace, REF K502/100/A);
- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);
- ⇨ **DNA/RNA Prep NA** (Sacace, REF K-2-9/2);
- ⇨ **QIAmp™ DSP Viral RNA Mini Kit** (Qiagen®, , REF 61904);
- ⇨ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003)
- ⇨ **SaMag Viral/Bacterial Nucleic Acids Extraction Kit B** (Sacace, REF SM012)

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

Extracted RNA should be processed immediately or frozen at -20°C for up to 1 week (max 1 defrosting).

ONE STEP REVERSE TRANSCRIPTION AND PCR AMPLIFICATION **(40 µl reaction volume)**

1. Prepare required quantity of PCR tubes or PCR strips according to the number of samples to be analyzed, 1 tube for negative control of extraction (NCE), 1 tube for negative control of amplification (C-) and 1 tube for positive control of amplification (C+) (for example, to test 5 samples, mark 8 tubes)
2. Prepare in a new tube **Reaction Mix** with **15*N µl** of **RT-PCR Buffer** and **0,5*N µl** of **Enzymes Taq/RT**, for N tubes to be tested. Vortex the tube thoroughly. Then spin briefly for 3-5 sec.
   Mixture of RT-PCR-mix and Enzyme Taq/RT must be prepared immediately prior to use and can be stored at the temperatures from 2 °C to 8 °C for 1 hour.
3. Add **15 µl** of prepared **Reaction Mix** into each PCR tube, without touching the wax layer.
4. Add **10 µl** of extracted **RNA** sample to the appropriate tube with Reaction Mix, without touching the wax layer, close the tubes with provided caps, spin 2-3 seconds, then transfer them to the qPCR instrument.
5. Prepare for each panel 3 controls:
   - add **10 µl** of extracted **Neg Control** to the tube labeled Negative Extraction Control (NCE).
   - add **10 µl** of **cDNA C+** to the tube labeled C+ *(positive control of amplification)*;
   - add **10 µl** of **Negative Control** to the tube labeled C- *(negative control of amplification)*;

Amplification

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

	Plate-type qPCR Instruments[1]		
Step	**Temperature, °C**	**Time**	**Cycles**
1	35	20 min	1
2	94	5 min	1
3	94	10 sec	5
	64	25 sec	
4	94	10 sec	45
	64	25 sec * **Fluorescence detection ****	

[1] *For example, SaCycler-96™ (Sacace), CFX-96™*** *Deep Well / iQ5™ (BioRad); Mx3005P™/Mx3000P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems);*

	Rotor-Gene™ 6000/Q (Qiagen)		
Step	**Temperature, °C**	**Time**	**Cycles**
1	32	1200 sec	1
2	95	300 sec	1
3	94	10 sec	50
	60	15 sec **Fluorescence detection**	

**** **NOTE FOR CFX-96** and other plate type instruments: it is recommended to use at least two additional empty strips placing them in the last left and right columns of the thermal block to better uniform the thermolid pressure in case of not filling the complete plate.*

** On ABI® 7500 Real Time PCR instrument, please set the fluorescence acquisition time to 30 seconds.*
*** Fluorescence detection on channels FAM/Green, Joe/HEX/Yellow, ROX/Orange, Cy5/Red*

SARS-CoV-2 E-gene is detected on the **Rox (Orange) channel, SARS-CoV-2 N-gene** is detected on the **Cy5 (Red) channel, SARS-like coronaviruses (SARS-CoV, SARS-CoV-2) on the FAM (Green) channel** and **Internal Control-*IC*** on the **JOE/HEX (Yellow) channel.**

Detection channel	FAM	JOE/HEX	ROX	Cy5
Result	SARS-like coronaviruses, SARS-CoV, SARS-CoV-2	IC detection	SARS-CoV-2, *E-gene*	SARS-CoV-2, *N-gene*

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation	Eliminate threshold cycles before / Ignore first	Threshold	Slope Correct
All channels	*From 5 FI to 10 FI**	*5 cycles*	*0.04*	*On*

**** Perform Optimisation Before 1st Acquisition***

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 in the log-linear phase of amplification, approximately 10-20% of the fluorescence level of the positive control in the last amplification cycle.

RESULTS ANALYSIS

When using SaCycler-96 software, results are analysed automatically in the software RealTime PCR. For manual analysis of results refer to Table 1, Table 2 and text below.

1. The sample is considered to be ***positive for SARS-Cov-2 RNA*** if there is amplification signal with defined Ct value in the channel FAM (SARS-like coronaviruses), AND there is amplification signal with the defined Ct value in channel Rox (E-gene) and Cy5 (N-gene); If there is amplification signal in FAM channel and amplification signal only in Rox or only in Cy5 the sample is considered positive for SARS-Cov-2, but additional epidemiological research is recommended (possible mutation in one of the SARS-CoV-2 genes detected).
2. The sample is considered to be ***negative for SARS-Cov-2 RNA*** *and positive for* SARS-like coronaviruses RNA (SARS-Cov coronavirus, Bat SARS-like coronavirus, Rhinolophus affinis coronavirus; Coronavirus BtRs-BetaCoV) if in the channels Rox and Cy5 the Ct value is not determined (the fluorescence curve does not cross the threshold line) and there is amplification signal in the channel FAM with defined Ct value.
3. The sample is considered to be ***negative for SARS-Cov-2 RNA*** *and negative for* SARS-like coronaviruses RNA if in the channels FAM, Rox and Cy5 value is not determined (the fluorescence curve does not cross the threshold line) and in the results table on the channel Hex the Ct value is defined and lower than 38.
4. The sample is considered **doubtful** if there is no signal in the FAM channel (SARS-like) and there is amplification signal in both or only one SARS-Cov-2 gene (positive signal in ROX and/or Cy5). Test should be repeated and if same results appear, additional investigation will be needed.
5. If in the channels FAM, Rox, Cy5 the Ct value is not determined (the fluorescence curve does not cross the threshold line) and in the results table on the channel Hex the Ct value is higher than 38 or absent, the sample is considered **invalid** and the test should be repeated starting from the RNA extraction stage.

Table 1. Results interpretation

Detection channel				**Interpretation**
FAM *SARS-LIKE Cov*	**Hex** *IC*	**Rox** *SARS-Cov-2 E gene*	**Cy5** *SARS-Cov-2 N gene*	
Analyzed samples				
Ct is defined	Not considered	**Ct is defined**	**Ct is defined**	**RNA of SARS-CoV-2 is detected**
Ct is defined	Not considered	Ct is not defined	Ct is not defined	**RNA of SARS-like coronaviruses is detected**, RNA of SARS-CoV-2 is not detected
Ct is not defined	**Ct is defined**	Ct is not defined	Ct is not defined	RNA of SARS-like coronaviruses is **not detected** RNA of SARS-CoV-2 virus is **not detected**
Positive control sample				
Ct is defined	Ct is not defined	**Ct is defined**	**Ct is defined**	Valid positive control result
Negative control sample (NCE and C-)				
Ct is not defined	Ct is not defined (C-) Ct is defined (NCE)	Ct is not defined	Ct is not defined	Valid negative control result

Table 2. Other possible results

Detection				Interpretation
FAM *SARS-LIKE Cov*	**Hex** *IC*	**Rox** *SARS-Cov-2 E gene*	**Cy5** *SARS-Cov-2 N gene*	
Analyzed samples				
Ct is defined	Not considered	**Ct is defined**	Ct is not defined	**Sars-Cov-2 RNA detected**, Additional epidemiological research is recommended, possible mutation in one of the SARS-CoV-2 genes detected
		Ct is not defined	**Ct is defined**	
Ct is not defined	Not considered	**Ct is defined**	Not considered	Unreliable result*
		Not considered	**Ct is defined**	Unreliable result*
Ct is not defined	Ct is not defined	Ct is not defined	Ct is not defined	Unreliable result*

**repeat PCR amplification or RNA extraction for the given sample, performed sequentially*

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity

The kit **SARS-CoV-2 Real-TM** allows to detect *SARS-Cov-2 RNA* in 100% of the tests with an analytical sensitivity of not less than 500 copies/ml (10 copies/PCR, elution volume 50 µl).

The sensitivity of the kit **SARS-CoV-2 Real-TM** was tested using the serial dilutions of "Laboratory Positive Sample".

Table 3. Results of dilutions of Laboratory Positive Sample

No	Copies/reaction	SARS-CoV-2 Real-TM					
		LOT 1	LOT 2	LOT 3	LOT 4	LOT 5	LOT 6
		Quantity of positive results testing 24 samples					
1	20	24	24	24	24	24	24
2	10	24	24	24	24	24	23
3	5	17	19	19	19	16	19
4	0	-	-	-	-	-	-

Analytical Specificity

The analytical specificity of **SARS-CoV-2 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: *Influenza A virus, Influenza B virus, Human coronavirus HKU-1, Human coronavirus NL-63, Human rhinovirus, Mycoplasma pneumonia, Streptococcus pneumonia, Chlamydophila pneumoniae, Haemophilus influenza, Klebsiella pneumoniae, Moraxella catarrhalis, Bordetella pertussis, Bordetella parapertussis.* There was no significant homology between the tested microorganisms genome and our target primers / probes.

Diagnostic characteristics evaluation

As a result of clinical tests, the diagnostic characteristics of the kit were established: diagnostic sensitivity and diagnostic specificity total for all types of biomaterial.

Number of studies conducted to establish diagnostic characteristics:

Result	Data of Main Clinical Evaluation	Data of Additional Clinical Evaluation	Total
True positive	72	10	82
False positive	0	0	0
True negative	100	10	110
False negative	0	0	0
Total	172	20	192

Diagnostic sensitivity

Diagnostic sensitivity= TP/(TP+FN)x100% with 95% confidence interval, where TP - true positive results obtained by tested kit and reference methods, FN - false negative results in case of negative result obtained by tested kit and positive result obtained by reference methods.

Diagnostic specificity

Diagnostic specificity= TN/(TN+FP)x100% with 95% confidence interval, where TN - true negative results obtained by tested kit and reference methods, FP - false positive results in case of positive result obtained by tested kit and negative result obtained by reference methods.

Diagnostic characteristics:

Number of samples (n) - 192;

Diagnostic sensitivity - 100,0 % (95 % CI: 95,6-100 %);

Diagnostic specificity - 100,0 % (95 CI: 96,7-100 %).

Thus, according to the data analysis (n=192 samples) it was found that the diagnostic sensitivity of the kit is 100,0 % (95 % CI: 95,6-100 %), diagnostic specificity is 100,0 % (95 % CI: 96,7-100 %).

Interfering substances

The presence of PCR inhibitors in a sample of biological material may cause dubious (uncertain) results. A sign of PCR inhibition may be a simultaneous lack of amplification of the internal control and of a specific amplification product.

According to the results of risk analysis and R&D, the following substances are classified as PCR inhibitors, which may be present in extracted RNA: hemoglobin, which is present in the RNA sample as a result of incomplete removal of the blood-containing biomaterial, as well as isopropyl alcohol and methyl acetate present in the RNA sample as a result of incomplete removal of washing solutions during RNA extraction process.

The maximum concentrations of interfering substances at which there was no effect on the amplification of the laboratory control sample and of the internal control sample are:

- hemoglobin - 0.35 mg / ml cDNA sample
- isopropyl alcohol - 100 µl / ml cDNA sample
- methyl acetate - 100 µl / ml cDNA sample

Impurities contained in the biomaterial sample, such as mucus, blood, elements of tissue breakdown and inflammation, local drugs, including those contained in nasal sprays, etc. are removed during the isolation of RNA using kits for sample preparation. To reduce the number of PCR inhibitors, it is necessary to follow the rules for sampling the biological material. If there is a doubt of the presence of a large number of PCR inhibitors in the sample, it is recommended to choose appropriate methods for isolating nucleic acids that allow maximum removal of PCR inhibitors; express methods for isolating nucleic acids are not recommended.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Joe/HEX 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 incorrect treatment of clinical material, incorrect RNA extraction, which resulted in the loss of RNA, or by the presence of PCR inhibitors.
        ⇒ Repeat RNA extraction process, take attention during the sample preparation.
2. Any signal with signal with Negative Control of extraction (NCE) or with the Negative Control of Amplification (C-).
    - Contamination during RNA extraction or PCR 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.
        ⇒ 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

* 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
* QIAmp™ is a registered trademark of Qiagen

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