IVD For *in Vitro* Diagnostic Use

CE

SARS-CoV-2 Variant Typing Real-TM

Handbook

Real Time PCR kit for qualitative detection of SARS-CoV-2 Variant mutations in the Spike protein: HV 69-70 DEL, N501Y, E484K, K417N

REF V435-96FRT/V

96

NAME

SARS-CoV-2 Variant Typing Real-TM

INTRODUCTION

Viruses continuously change through mutation, and new variants of a virus are expected to emerge over time. Multiple variants of the SARS-Cov-2 virus that causes COVID-19 have been documented globally during this pandemic.

The virus that causes COVID-19 is a new type of coronavirus, a large family of viruses. Coronaviruses owe their name to the crown-like spikes on their surfaces. Scientists monitor virus changes, especially changes to the spikes on the surface of the virus. These studies, including genetic analyses, are helping scientists understand how changes to the virus might affect how it spreads and what happens to people who are infected with it.

Many variants of the virus that causes COVID-19 are circulating globally: the United Kingdom (UK) identified the first variant of concern called B.1.1.7 carrying different mutations in the fall of 2020. This variant can spread more easily and quickly than other variants. In January 2021, experts in the UK reported that this variant may be associated with an increased risk of death.

In South Africa, another variant of SARS-CoV-2 (known as 20H/501Y.V2 or B.1.351) emerged independently of B.1.1.7. Cases attributed to this variant have been detected in multiple countries outside of South Africa. This variant has multiple mutations in the spike protein, including K417N, E484K, N501Y. Unlike the B.1.1.7 lineage detected in the UK, this variant does not contain the deletion at 69/70.

In Brazil, a variant of SARS-CoV-2 (known as P.1) emerged that was first was identified in four travelers from Brazil, who were tested during routine screening at Haneda airport outside Tokyo, Japan. The P.1 lineage contains three mutations in the spike protein receptor binding domain: K417T, E484K, and N501Y. There is evidence to indicate that one of the spike protein mutations, E484K, can affect neutralization by polyclonal and monoclonal antibodies.

INTENDED USE

SARS-CoV-2 Variant Typing Real-TM is a Real-Time PCR test for the qualitative detection of SARS-CoV-2 Variant mutations in the Spike protein: HV 69-70 DEL, N501Y, E484K, K417N.

This kit allows to identify mutations of coronavirus lineage B.1.1.7 (British variant), lineage B.1.351 (South African variant) and lineage P.1 (Brazilian variant).

PRINCIPLE OF ASSAY

SARS-CoV-2 Variant Typing 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 kit is recommended for typing of SARS-CoV-2 positive samples in RNA preparations isolated from human biomaterial (nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal and nasopharyngeal aspirates, sputum). The kit detects by Real time PCR the presence of the following SARS-CoV-2 mutations in the Spike protein: HV 69-70 DEL, N501Y, E484K, K417N. The detection of only HV 69-70 DEL and N501Y mutations indicates the presence of SARS-Cov-2 lineage B.1.1.7 (VOC 202012/01, British Variant), the detection of only N501Y, E484K and K417N indicates the presence of SARS-Cov-2 lineage B.1.351 (South African Variant), the detection of only N501Y and E484K indicates the presence of SARS-Cov-2 lineage P.1 (Brazilian Variant).

SARS-CoV-2 Variant Typing Real-TM PCR kit is a multiplex Real Time PCR kit with 5 simultaneous targets: **SARS-CoV-2** wild type (FAM channel) **E gene**, Spike (S): 21765 21770 deletion (**HV 69-70 DEL**) (HEX channel), Spike (S): A23063T (**N501Y**) (ROX channel) Spike (S) G23012A (**E484K**) (Cy5/Red channel) and Spike (S): G22813T (**K417N**) (Cy5.5 channel).

Detection channel	FAM/Green	HEX/Yellow	ROX/Orange	Cy5/Red	Cy5.5
Target	**SARS-Cov-2 WT ("wild type")** E gene	Spike (S): 21765 21770 deletion (**HV 69-70 DEL**) - *mutation*	Spike (S): A23063T (**N501Y**) - *mutation*	Spike (S): G23012A (**E484K**) - *mutation*	Spike (S): G22813T (**K417N**) - *mutation*

MATERIALS PROVIDED

Format T (tube format)

- **Tubes-COVID19 Variant Typing**, 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;

Contains reagents for 96 tests.

Format S (strip format)

- **Strips-COVID19 Variant Typing**, 8x12 strip 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;

Contains reagents for 96 tests.

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 Variant Typing 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 Variant Typing 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 Variant Typing 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.
- *Sputum*

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 NA** (Sacace, REF K-2-9/2);
- ⇨ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003)

Please carry out the RNA extraction according to the manufacturer's instructions.

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 amplification (C-) and 1 tube for positive control of amplification (C+) (for example, to test 5 samples, mark 7 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 **cDNA C+** to the tube labeled C+ *(positive control of amplification)*;
   - add **10 µl** of **Negative Control buffer*** to the tube labeled C- *(negative control of amplification)*;

**not provided, use DNA/RNAse free water.*

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
	62	25 sec	
4	94	10 sec	45
	62	25 sec * **Fluorescence detection** **	

** **NOTE FOR CFX-96** and other plate type instruments: it is recommended to use at least 3 strips in each run placing them in the left, center and right columns of the thermal block to better uniform the thermolid pressure in case of not filling the complete plate.*

[1] For example, SaCycler-96™ (Sacace), CFX-96™(BioRad); Mx3005P™ (Agilent), ABI 7500 (Thermofischer)**

** On ABI® 7500 Real Time PCR instrument, please set the fluorescence acquisition time to 30 seconds.*

*** Fluorescence detection on channels FAM/Green, HEX/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson*

SARS-CoV-2 wild type (**E gene)** is detected in the FAM channel, **HV 69-70 DEL** deletion is detected in the HEX channel, **N501Y** mutation is detected in the ROX channel, **E484K** mutation is detected in the Cy5/Red channel and **K417N** mutation is detected in the Cy5.5 channel.

Detection channel	**FAM/Green**	**HEX/Yellow**	**ROX/Orange**	**Cy5/Red**	**Cy5.5/Crimson***
Target	**SARS-Cov-2 WT ("wild type")** E gene	Spike (S): 21765 21770 deletion (**HV 69-70 DEL**) - *mutation*	Spike (S): A23063T (**N501Y**) - *mutation*	Spike (S): G23012A (**E484K**) - *mutation*	Spike (S): G22813T (**K417N**) - *mutation*

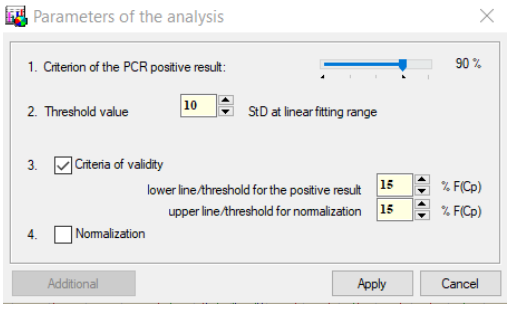
** In case ABI 7500 instrument is used, which does not have the Cy5.5/Crimson channel, it will not be possible to detect K417N mutation and so to discriminate between South African (B.1.351) and Brazilian (P.1) variants.*

INSTRUMENT SETTINGS

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.

For SaCycler-96 instrument click on the icon for changing the parameter of data analysis, a new window will show up. The settings must be precisely as in the following picture, then click **"Apply"**:



Set **90%** as "Criterion of the positive PCR result"; "Normalization data" checkbox must be **deselected.**
Criteria of validity must be selected with both thresholds set to **15%**

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.

Table 1. Results interpretation

Detection channel					**Interpretation ***
FAM *SARS-Cov-2 WT E Gene*	**Hex** *HV 69-70 DEL*	**ROX** *N501Y mutation*	**Cy5** *E484K mutation*	**Cy5.5** *K417N mutation*	
Analyzed samples					
Ct is defined	**Ct is defined**	**Ct is defined**	Ct is not defined	Ct is not defined	**SARS-CoV-2 British Variant (B.1.1.7) mutations are detected**
Ct is defined	Ct is not defined	**Ct is defined**	**Ct is defined**	**Ct is defined**	**SARS-CoV-2 South African Variant (B.1.351) mutations are detected**
Ct is defined	Ct is not defined	**Ct is defined**	**Ct is defined**	Ct is not defined	**SARS-CoV-2 Brazilian Variant (P.1) mutations are detected**
Ct is defined	Ct is not defined	Ct is not defined	Ct is not defined	Ct is not defined	**RNA of SARS-Cov-2 is detected**, RNA of SARS-CoV-2 mutations is not detected
Ct is not defined	Ct is not defined	Ct is not defined	Ct is not defined	Ct is not defined	RNA of **SARS-Cov-2 is not detected** RNA of SARS-CoV-2 mutations is **not detected**
Positive control sample					
Ct is defined	**Ct is defined**	**Ct is 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 not defined	Ct is not defined	Ct is not defined	Valid negative control result

** In case ABI 7500 instrument is used, which does not have the Cy5.5/Crimson channel, it will not be possible to discriminate between South African (B.1.351) and Brazilian (P.1) variants.*

PERFORMANCE CHARACTERISTICS

Sensitivity

The kit **SARS-CoV-2 Variant Typing Real-TM** allows to detect *SARS-Cov-2 RNA* in 100% of the tests with a sensitivity of not less than 1000 copies/ml.

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

Specificity

The analytical specificity of **SARS-CoV-2 Variant Typing 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.

TROUBLESHOOTING

1. Weak or absent signal in all channels: 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 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 Amplification (C-).
    - Contamination during 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

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* CFX™ and iQ5™ is are registered trademarks of Bio-Rad Laboratories
* MX3005P® is a registered trademark of Agilent Technologies

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