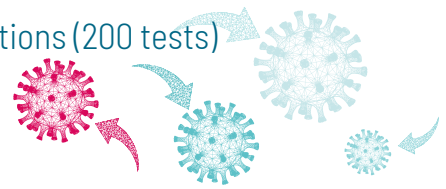


User Information

Ver. 1.1(en), 03/2021



NOTICE!

- This kit is intended for *research use only* purposes. For investigational use only.

NOTICE!

- Read all instructions in this user manual, the outer packaging, and the component labels before starting work.

CAUTION!

- SARS-CoV-2 is dangerous pathogen so follow all applicable regulations and recommendations for BSL2+ or BSL3 class laboratories.

GENERAL CAUTION

This assay targets two deletions: a 9-base deletion in NSP6 located in the ORF1a gene (NSP6 S106/G107/F108del; del9) and a 6-base deletion in the Spike gene (S H69/V70del; del6). The presence or absence of these deletions and the relative ΔCt in comparison to a reference gene allows the user to differentiate between variants, including B.1.1.7 (also 501Y.V1), B.1.351 (also 501Y.V2), P.1 (also 501Y.V3), and 1.258. Since B.1.351 and P.1 both share del9 and lack del6, this assay cannot differentiate between the two variants.

1. Explanations and abbreviations

B.1.1.7; B.1.351; B.1.258: a SARS-CoV-2 lineages characterized by increased transmissibility, virulence and evasion of immune responses

Baseline: basic background fluorescence or so-called „noise“

BHQ-1: Black Hole Quencher-1, a non-fluorescent quencher for the FAM fluorescent reporter dye

BHQ-2: Black Hole Quencher-2, a non-fluorescent quencher for the YY fluorescent reporter dye

BHQ-3: Black Hole Quencher-3, a non-fluorescent quencher for the Cy5 fluorescent reporter dye

BSL: bio-safety level

COVID-19: disease caused by the SARS-CoV-2 virus (Coronavirus Disease 2019)

Ct: threshold cycle, the cycle in which the fluorescence signal of the reaction exceeds the set fluorescence threshold

Cy5: cyanine 5, a fluorescent reporter dye

del6: internal designation for the 6 nucleotide deletion of H69/V70del

del9: internal designation for the 9 nucleotide deletion of NSP6 S106-F108del

DNA: deoxyribonucleic acid

E gene: a gene encoding the small membrane envelope protein of the SARS-CoV-2 virus

FAM: 6-carboxyfluorescein, a fluorescent reporter dye

LoD: Limit of Detection

NSP6: Non-Structural Protein 6, translated from ORF1a

NTC: No Template Control

PC: Positive Control

qPCR: quantitative Polymerase Chain Reaction

RdRP gene: a gene encoding the RNA-dependent RNA polymerase of the SARS-CoV-2 virus

RFU: relative fluorescence unit

RNA: ribonucleic acid

RNase P gene: a gene encoding human nuclear ribonuclease P

ROX: 6-carboxy-X-rhodamine, a fluorescent reference dye used to normalize the reporter dye signal

RT-qPCR: reverse transcription qPCR

rTEST: room Temperature Stable

S gene: a gene encoding the spike protein of the SARS-CoV-2 virus

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

Threshold: the point at which the fluorescence reporter signal significantly exceeds background fluorescence

YY: Yakima Yellow®, a fluorescence reporter dye developed by Epoch Biosciences

2. Intended use

The rTEST COVID-19 qPCR Multivariant kit is a qualitative *in vitro* confirmatory test designed to detect the presence of SARS-CoV-2 genetic material in biological samples obtained primarily from the human upper airways (nose and nasopharynx). The kit is intended exclusively for use in a diagnostic laboratory with the appropriate equipment, safety standards and properly trained personnel.

3. Test principle

The rTEST COVID-19 qPCR Multivariant kit is the first fully room temperature stable kit to detect genomic RNA of multiple SARS-CoV-2 lineages. It can be stored at room temperature for at least one month enabling transportation without dry ice and easier handling of the kit upon arrival. The room temperature stability is allowed by lyophilized mixes of all SARS-CoV-2 specific primers/probes sets, internal positive control (full genomic viral RNA of SARS-CoV-2 lineage B.1.1.7 spiked with human RNA), and proprietary, room temperature stable 1-step RT-qPCR reagents from Solis Biotyne.

The recent emergence of a novel variants of SARS-CoV-2, i.e. B.1.1.7 and B.1.351 has sparked global alarm due to their association with higher viral loads and increased transmissibility (i. e., more efficient and rapid transmission). These two lineages share a common 9-base pair deletion in NSP6 that is translated from Orf1 (NSP6 S106-F108del; del9), but unlike the B.1.1.7 lineage, the B.1.351 variant does not contain 6-base pair deletion in the Spike protein (S H69/V70del; del6). del6 deletion that is associated with increased virulence and evasion of immune responses has been characterised in SARS-CoV-2 B.1.258 variant. On the other hand, the absence of both deletions indicates another SARS-CoV-2 strain (i.e. B.1.1, B.1.160, B.1.177, B.1.170 or B.1.258.17).

The rTEST COVID-19 qPCR Multivariant kit is a newly designed differential test distinguishing between multiple SARS-CoV-2 lineages based on the presence or absence of del9 and del6. The kit contains two mixes of primers and hydrolysis probes (TaqMan®) targeting either SARS-CoV-2 specific E and RdRP genes or del9 and del6 whose presence determines the SARS-CoV-2 variants. Both mixes allow the detection of the human RNase P gene. The TaqMan® probes for E gene and del9 are conjugated to Yakima Yellow, the TaqMan® probes for RdRP gene and del6 are conjugated to FAM and the TaqMan® probe for RNase P is conjugated to Cy5. This enables multiplexed detection of either the SARS-CoV-2 E and RdRP gene or del9, del6, and human RNase P, which serves as an internal control to validate proper sample collection, RNA extraction, and performance of the test. **For E gene, RdRP gene, del9 and del6, we developed proprietary dual TaqMan® probes to increase the sensitivity and specificity of our 1-Step RT-qPCR method. The full**

genomic viral RNA of SARS-CoV-2 variant B.1.1.7 spiked with human RNA provided by the Biomedical Center of the Slovak Academy of Sciences is included as an internal positive control. The 5X One-step Probe CoV Mix (ROX) reagent includes ROX passive reference dye as an inert additive providing a constant fluorescent signal for sample normalization in the real-time PCR assay. Due to the unique molecular structure of the supplied ROX dye, the solution is compatible with all ROX-dependent (both high-ROX and low-ROX) and ROX-independent real-time PCR cyclers.

The RT and qPCR reactions are conducted in a one tube, one-step fashion. The rTEST COVID-19 qPCR Multivariant kit is an additional confirmatory kit used for closer characterization of SARS-CoV-2 positive samples, based on detection of del6 and del9. Patient samples that tested positive for SARS-CoV-2 in the primary test are tested for the presence of the del9 and del6 and the results are subsequently compared with the results for the E gene and RdRP gene, which run in parallel reaction. Multiplexing allows co-detection of SARS-CoV-2 genes with human RNase P, which can be used for secondary confirmation of negative samples, as well as to validate sample collection, RNA extraction, and performance of the qPCR reaction.

One package of the kit is sufficient for 400 testing reactions and is intended for the characterization of multiple variants of SARS-CoV-2 in patient samples that previously tested SARS-CoV-2 positive in the primary test. It is mandatory to run two parallel reactions to detect standard E and RdRP gene in one reaction and del9 and del6 in another one. The human RNase P internal positive control assay in multiplex mode with E and RdRP gene or del9 and del6 serves to verify the quality of the swab and RNA extraction, to determine the presence of human RNA within the extracted sample, while eliminating false negative results.

4. Kit composition

- 1x lyophilized primers/probes mix for del9, del6 and human RNase P labelled as del9/del6/RNase P Mix (must be dissolved in 400 µl of PCR water)
- 1x lyophilized primers/probes mix for SARS-CoV-2-E, SARS-CoV-2-RdRP, and human RNase P, labelled as E gene/RdRP gene/RNase P Mix (must be dissolved in 400 µl of PCR water)
- 1x lyophilized PC B.1.1.7 (must be dissolved in 100 µl of PCR water)
- 2x 800 µl 5X One-step Probe CoV Mix (ROX)
- 1x 200 µl 40X One-step SOLIScript® CoV Mix
- 2x 5 ml PCR water
- 1x Instructions for use

5. Storage and shelf life:

CAUTION!

Do not use the kit after the expiration date, which is stated on the outer box.

NOTICE!

- Wear suitable protective clothing, gloves, and eye/face protection.
- Never pipette by mouth.
- Never eat, drink, or smoke in the laboratory and do not use any cosmetics.
- Always wash your hands thoroughly when handling specimens and reagents.

All kit components can be transported and stored at room temperature (15–25 °C) up to 1 month. The kit can be routinely stored at –20 °C. The 5X One-step Probe CoV Mix (ROX) contains ROX as a reference dye that is photo-sensitive and should be protected from light whenever possible. The shelf life of the kit is a maximum of 12 months from the date of manufacture. The exact expiration date of the kit is indicated on the outer box. The exact expiration date of the individual components of the kit is indicated on the inner packaging/vials.

The kit and its individual components are designed to perform 400 reactions. For users who perform fewer reactions within one run, we recommend that you aliquot all kit components according to internal procedures and the standard number of reactions per run. Aliquoting the kit components minimizes the need to reopen individual tubes and thus minimizes the risk of contamination of the kit components. Aliquoting the components of the kit will also prevent repeated thawing and freezing of the individual components, which may lead to reduced efficiency. Before aliquoting, it is necessary to completely thaw the individual components of the kit. Thawing is optimally performed gently, by incubation in a refrigerator (at 4 °C) for approx. 2 hours. Immediately before aliquoting, it is necessary to mix the contents of the tubes thoroughly, but at the same time carefully, avoiding the formation of bubbles, until the mixture is completely homogeneous. In the case of tubes containing enzymes (5X One-step Probe CoV Mix (ROX), 40X One-step SOLIScript® CoV Mix), we recommend mixing by rotating the tube several times, pulse vortexing, or short vortexing for no longer than 5 seconds without generating any bubbles. Pipette the enzymes carefully and slowly; otherwise, the viscosity of the buffer may lead to pipetting errors.

6. Consumables and equipment not included in the kit

- Real-time PCR equipment: the rTEST COVID-19 qPCR Multivariant kit has been validated and tested on Agilent devices – Mx3005P® and AriaMx®, Thermo Fisher Scientific – QuantStudio™ 5, BioRad – CFX96™, Analytik Jena – qTOWER3
- Laminar flow box
- Vortex mixer
- Mini centrifuge
- Centrifuge with rotor for plates
- Personal protective equipment: powder-free disposable laboratory gloves, goggles, protective shield, FFP3 respirator, protective clothing
- Laboratory plasticware certified sterile and free of DNA, RNA, DNases and RNases: reagent tubes, PCR tubes, PCR strips, PCR plates, PCR foils, sterile pipette tips with filter
- Autoclavable adjustable micropipettes
- Bio-waste container
- Autoclavable test tube racks
- PCR tube/plate cooler

7. Workflow

CAUTION!

Any work with the kit must be performed by qualified personnel.

NOTICE!

- Workspaces must be arranged in such a way that there are separate, dedicated rooms (zones), laboratory equipment, and consumables for each step in the workflow: nucleic acid isolation, preparation of amplification reactions, and amplification and detection of amplification products. The amplified products must never

come into contact with space, equipment, and consumables intended for the isolation of nucleic acids or for the preparation of amplification mixtures.

- Use separate, designated lab coats, gloves, and all other personal protective equipment for each step in the workflow. Never use the same personal protective equipment in different rooms (zones).
- Always handle all biological samples as potentially infectious material and avoid direct contact with biological material. Avoid spilling samples and reagents and generating aerosols.
- After sample preparation, it is advised to avoid excessive delay before starting the reaction in a thermocycler.
- Follow the enclosed instructions for use thoroughly.

7.1. Sampling and RNA extraction

CAUTION!

During the workflow, always wear personal protective equipment and work in a laminar flow box. Viral RNA can also cause infection. Thus, handle all samples with caution and treat as potentially infectious material.

NOTICE!

- The samples analyzed are intended solely for this type of analysis.
- Strictly follow sample processing guidelines to avoid degradation of nucleic acids.
- Do not open different samples at the same time to avoid possible cross contamination.
- Vortex and centrifuge the samples in a laminar flow box to prevent aerosol contamination.
- Use pipettes designated exclusively for handling specimens and use disposable filter tips that are certified sterile and free of DNA, RNA, DNases and RNases.

Improper sample collection, transport, and storage procedures as well as RNA extraction may result in incorrect test results. Users should refer to established guidelines for collecting, transporting, and storing samples and should adhere to manufacturer instructions for specimen collection. Sterile swabs with a plastic or aluminum shaft and a synthetic swab must be used for sampling. Swabs with a wooden shaft and / or a cotton swab must not be used. Following sample collection, immerse swabs immediately into sterile tubes containing 2–3 ml of viral transport media.

RNA extraction from the samples should be performed promptly after collection according to the manufacturer's instructions. If a delay in extraction is expected, samples must be stored at 4 °C for up to 12 hours after collection or at –70 °C for long-term sample storage. Avoid repeated thawing and freezing of samples. The rTEST COVID-19 qPCR Multivariant kit was validated and tested on RNA samples obtained using the following extraction kits:

- Zymo Research, Quick-RNA Viral 96 Kit (Catalog # R1040, R1041)
- Cytiva (formerly GE Healthcare Life Sciences), RNAspin 96 Kit (Catalog # 25050075)
- RNeasy Advance Viral Genomic Reagent (magnetic beads, Catalog # C59543, C63510)

7.2. RT-qPCR

The rTEST COVID-19 qPCR Multivariant kit workflow consists in testing samples that were previously identified to be positive for SARS-CoV-2 by a primary screening test in order to confirm the presence/absence of deletions del9 and del6 using del9/del6/RNase P primers/probes mix. It is mandatory to run two parallel

multiplex confirmatory tests to determine the presence of standard SARS-CoV-2 E and RdRP gene in one reaction and del9/del6/RNase P in another one. Moreover, an internal control test for human RNase P is included in both assays with standard SARS-CoV-2 E and RdRP gene or del9/del6 to confirm swab and RNA extraction efficiency and assay performance.

NOTICE!

- RNase P is a human transcript, therefore the primers and probe mix designed for RNase P can detect both RNA and genomic DNA if present in RNA extract.

7.3. Workspace preparation

Before starting the protocol, first clean the working space of the laminar flow box and adjacent surfaces with a 10% solution of sodium hypochlorite (bleach) and then with a 70% solution of ethanol to remove residual bleach. Use the UV decontamination cycle before and after working in the laminar flow box.

7.4. Preparation of reagents

Remove the necessary kit components from the freezer and thaw completely in the refrigerator (4 °C), on ice, or in a refrigerated tube cooling rack. Once thawed, mix their contents thoroughly and gently until completely homogeneous.

We recommend mixing tubes containing enzymes (5X One-step Probe CoV Mix (ROX), 40X One-step SOLIScript® CoV Mix) by rotating the tube several times, pulse vortexing, or short vortexing for no longer than 5 seconds without generating any bubbles. Then centrifuge the tubes briefly to remove droplets from the cap and ensure all liquid is at the bottom of the tubes. Pipette enzyme mixes carefully and slowly as the viscosity of the buffer may lead to pipetting errors.

Mixtures of primers and TaqMan® probes designated SARS-CoV-2_E, SARS-CoV-2_RdRP, del9, del6, and Human RNase P (provided as E gene/RdRP gene/RNase P Mix or del9/del6/RNase P Mix) as well as the PC B.1.1.7 spiked with human RNA are supplied in lyophilized form to increase stability. It is therefore necessary to dissolve mixtures of primers and probes and the positive control in PCR water when the kit is used for the first time.

CAUTION!

Since the colored oligo pellet can become dislodged during shipping, it is crucial to briefly centrifuge every tube before opening. Failure to do so could result in yield loss, because oligo pellets that are not at the bottom of the tube could fly out of the tube when the cap is opened.

Add 400 µl of water from the PCR water tube to the tube labeled as E gene/RdRP gene/RNase P Mix (200 reactions) and 400 µl of water from the PCR water tube to the tube labeled as del9/del6/RNase P Mix (200 reactions). In addition, add 100 µl of water from the PCR water tube to the tube containing positive control PC B.1.1.7.

Thoroughly vortex the contents of the tubes. Then briefly centrifuge the tubes to remove droplets from the cap and ensure all liquid is at the bottom of the tubes.

Prepare the reaction mixture as soon as possible after mixing the contents of the individual components of the kit. If necessary, vortex and centrifuge the contents of the tubes once more just before preparing the reaction mixture.

After use, store kit components in the freezer (–20 °C). Avoid repeated thawing and freezing of kit components. If any kit components will be reused within 2 hours, store at 4 °C.

CAUTION!

Always handle reagents in a laminar flow box.

Always prepare reagents for amplification separately, preparing reagents exclusively for one analysis at a time.

Use pipettes designed exclusively for preparation of reagents and use disposable filter tips.

The tips used must be sterile and free of DNA, RNA, DNases and RNases.

NOTICE!

- Use only reagents contained in this kit and reagents recommended by the manufacturer.
- Do not combine or mix reagents from different lots.
- Do not combine reagents from kits from different manufacturers.

7.5. Preparation of the reaction mixture

The recommended total volume of one reaction is 20 µl. To prepare the reaction mixture, the individual components of the kit must be mixed in the following order and ratio:

Table 1. Reaction mixture setup

Kit component	Component volume per reaction	
	E gene/RdRP gene/RNase P	del9/del6/RNase P
PCR water	8.5 µl	8.5 µl
5X One-step Probe CoV Mix (ROX)	4 µl	4 µl
40X One-step SOLIScript® CoV Mix	0.5 µl	0.5 µl
E gene/RdRP gene/RNase P Mix or del9/del6/RNase P Mix	2 µl	2 µl
Total volume	15 µl	15 µl

Table 2. Calculated volumes for a given number of reactions

Kit component	1	2	3	4	5	6	7	8	9	10	96
PCR water	8.5 µl	17 µl	25.5 µl	34 µl	42.5 µl	51 µl	59.5 µl	68 µl	76.5 µl	85 µl	816 µl
5X One-step Probe CoV Mix (ROX)	4 µl	8 µl	12 µl	16 µl	20 µl	24 µl	28 µl	32 µl	36 µl	40 µl	384 µl
40X One-step SOLIScript® CoV Mix	0.5 µl	1 µl	1.5 µl	2 µl	2.5 µl	3 µl	3.5 µl	4 µl	4.5 µl	5 µl	48 µl
E gene/RdRP gene/RNase P Mix or del9/del6/RNase P Mix	2 µl	4 µl	6 µl	8 µl	10 µl	12 µl	14 µl	16 µl	18 µl	20 µl	192 µl
Total volume	15 µl	30 µl	45 µl	60 µl	75 µl	90 µl	105 µl	120 µl	135 µl	150 µl	1440 µl

NOTICE!

- The reaction mixture has limited stability, use it as soon as possible after preparation. If the reaction mixture cannot be used immediately, store it in a refrigerator at 4 °C.
- When preparing multiple reactions, it is recommended to make 5 - 10% extra reaction mixture to account for pipetting errors.

NOTICE!

- The reaction with E gene/RdRP gene/RNase P Mix and del9/del6/RNase P Mix must be run side by side on the same PCR plate (or PCR tubes in one PCR run) to assure the homogeneity of the results!

7.6. Plate preparation and inspection

Add 5 µl of sample to each prepared 15 µl reaction mixture, resulting in a 20 µl total reaction volume.

Prepare the required number of clean PCR tubes, PCR strips, or PCR plates and place them in a refrigerated cooling rack. Mix the prepared reaction mixture thoroughly but at the same time gently by turning the tube several times, pulse vortexing, or short vortexing for no longer than 5 seconds without generating any bubbles. Then centrifuge it briefly to remove droplets from the cap and ensure all the liquid is at the bottom of the tube. Pipette 15 µl of the prepared reaction

mixture into individual PCR tubes or wells of a PCR plate in accordance with the required number and position of reactions. Pipette the reaction mixture carefully and slowly as the viscosity of the buffer may lead to pipetting errors. Then transfer the PCR tubes or PCR plate with the pipetted reaction mixture from the laminar flow box for preparation of the reaction mixture to the laminar flow box for finalization of plate preparation. Add 5 µl of sample, or 5 µl of positive control (PC B.1.1.7), or 5 µl of PCR water (NTC) into appropriate PCR tubes or PCR plate wells. Then tightly seal the individual PCR tubes with the lids or wells of the PCR plate with optical foil. Centrifuge the PCR tubes or PCR plate briefly so that all fluid is at the bottom of the tubes/wells and insert into the real-time PCR instrument.

A minimum of one negative control must be included in each analysis to verify the presence of contamination. A no template control (NTC) containing PCR water is used as a negative control instead of an unknown sample. A separate NTC for each mix of primers/probes (E gene/RdRP gene/RNase P and del9/del6/RNase P) tested must be included.

A minimum of one positive control (PC) must be included in each analysis to validate the workflow of the analysis and the functionality of the kit components. A reaction containing the positive control (PC B.1.1.7) is used instead of an unknown sample. A separate PC for each mix of primers/probes (E gene/RdRP gene/RNase P and del9/del6/RNase P) tested must be included.

The PC B.1.1.7 consists of isolated viral genomic RNA of SARS-CoV-2 variant B.1.1.7 spiked with human RNA. The PC B.1.1.7 will yield a positive result with all primer and probe sets (SARS-CoV-2 primers/probes for E gene, RdRP gene, del9, del6, and primers/probe for human RNase P).

7.7. Real-time PCR instrument settings

Follow the instructions below to set the assay conditions for the reaction volume, temperature conditions, and optical channels.

Reaction volume:

- 20 µl

Thermocycling conditions:

- Reverse transcription: 55 °C, 10 min
- Initial denaturation: 95 °C, 10 min
- Cycling, 45 cycles:
 - » Denaturation: 95 °C, 15 s
 - » Annealing/extension: 60 °C, 30 s

Optical channels used:

- Optical channel for FAM label: blue or green channel according to the real-time PCR device - excitation maximum 495 nm, emission maximum 520 nm
- Optical channel for HEX and YY label: yellow channel (HEX, JOE or VIC) according to the real-time PCR device - excitation maximum 525 nm, emission maximum 550 nm
- Optical channel for ROX dye: orange or red channel according to the real-time PCR device - excitation maximum 575 nm, emission maximum 605 nm
- Optical channel for Cy5 label: red channel - excitation maximum 650 nm, emission maximum 670 nm

If the real-time PCR device supports normalization to the ROX passive reference dye, we recommend performing the analysis with this function enabled. The 5X One-step Probe CoV Mix (ROX) contains ROX with a unique molecular structure as a reference dye providing a constant fluorescent signal for sample normalization of the assay. The fluorescent signal

of the ROX dye does not interfere with the detection channel of the FAM, YY or Cy5 fluorescent label. As a result, the rTEST COVID-19 qPCR Multivariant kit is fully compatible with all ROX-dependent and ROX-independent real-time PCR cyclers.

Follow the real-time PCR equipment manufacturer's manual and your internal procedures for this type of assay when setting the analysis conditions for the number and type of samples, the distribution of samples on the plate, and the type of plasticware used (tubes, strips, plates).

CAUTION!

Do not modify or change the recommended protocols for PCR analyses.

CAUTION!

Handle amplification products with extreme care to avoid dispersal into the laboratory area and possible contamination of new test specimens. Use pipettes designated exclusively for handling amplification products and use disposable filter tips that are certified sterile and free of DNA, RNA, DNases, and RNases.

7.8. Analysis of the obtained data

To set the baseline and threshold for each reaction, follow the manufacturer's manual for the real-time PCR instrument in accordance with your internal procedures for this type of assay.

8. Interpretation of results

8.1. Interpretation of results and reporting (clinical samples)

• Extraction and positive control results and interpretation

• No template control (NTC)

The NTC consists of using nuclease-free water (PCR water) in the RT-qPCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence amplification curves that cross the threshold line. If any of the NTC reactions exhibit an amplification curve that crosses the cycle threshold line, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

• Positive control (PC B.1.1.7)

The PC B.1.1.7 consists of lyophilized isolated genomic RNA of SARS-CoV-2 variant B.1.1.7 spiked with human RNA and co-precipitant (such as salmon sperm DNA or Baker's yeast tRNA to increase stability). The PC B.1.1.7 will yield a positive result with all primer and probe sets (i.e., SARS-CoV-2 primers/probes for E gene, RdRP gene, del9, del6, and primers/probe for human RNase P). Standard Ct values for the PC B.1.1.7 positive control should exhibit a Ct lower than 35.00 for all tested markers. The signal level (i.e., the relative fluorescence without normalization with the ROX dye) should also result in values above 1000 RFU to be considered valid. Indications of an error or failure in the workflow or analysis of the experiment include: the complete absence of a signal, the presence of an amplified signal but with higher Ct values than usual for a given control material, or the presence of a low-level signal. In the case of a negative result in the positive control, it is not possible to unambiguously determine the correctness of other positive/negative results obtained in the

given analysis and to distinguish between negative and false negative results. Therefore, the output of such an analysis cannot be evaluated.

Table 3. Expected performance of controls included in the rTEST COVID-19 qPCR Multivariant kit

Control Type	External Control Name	E gene	RdRP gene	del9	del6	Human RNase P	Expected Ct values	Possible causes of the unexpected results
Positive	PC B.1.1.7	+	+	+	+	+	Ct < 35.00	Substantial reagent failure including primer and probe integrity
Negative	NTC	-	-	-	-	-	None detected	Reagent and/or environmental contamination

Deviation from the expected performance of the controls suggests improper assay set up and/or execution, or failure/malfunction of reagents and/or equipment could have occurred. Invalidate the run and re-test.

• RNase P (extraction control)

» All clinical samples should exhibit fluorescence amplification curves in the RNase P reaction that cross the threshold line within 35.00 cycles (Ct < 35.00), thus indicating the presence of the human RNase P gene in the RNA sample. Failure to detect RNase P in any clinical specimens may indicate:

- Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
- Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
- Improper assay set up and execution.
- Reagent or equipment malfunction.

• RNase P in E gene/RdRP gene/RNase P gene primers/probes mix

» If the RNase P assay does not produce a positive result for human clinical specimens, interpret as follows:

- If the SARS-CoV-2 E and RdRP assays are positive even in the absence of a positive RNase P, the result should be considered **valid**. It is possible that some samples may fail to exhibit RNase P amplification curves due to low cell numbers in the original clinical sample. A negative RNase P signal does not preclude the presence of SARS-CoV-2 in a clinical specimen.
- If all SARS-CoV-2 markers **and** RNase P assays are negative for the specimen, the result should be considered **invalid** for the specimen. If residual specimen is available, repeat the RNA extraction procedure and repeat the test. If all markers remain negative after re-testing, report the results as invalid and a new specimen should be collected if possible.

• RNase P in del9/del6/RNase P gene primers/probes mix

» If the RNase P assay does not produce a positive result for human clinical specimens, interpret as follows:

- If the del9 and/or del6 assay is positive even in the absence of a positive RNase P, the result should be considered **valid**. It is possible that some samples may fail to exhibit RNase P amplification curves due to low cell numbers in the original clinical sample. A negative

RNase P signal does not preclude the presence of SARS-CoV-2 in a clinical specimen.

- If all markers **and** RNase P assays are negative for the specimen, the result should be considered **invalid** for the specimen. If residual specimen is available, repeat the RNA extraction procedure and repeat the test. If all markers remain negative after re-testing, report the results as invalid and a new specimen should be collected if possible.

• SARS-CoV-2 markers – E gene and RdRP gene

- » When all controls exhibit the expected performance, a specimen is considered negative if the amplification curves for both SARS-CoV-2 genes (E, RdRP) DO NOT cross the threshold line within 40.00 cycles (Ct > 40.00) AND the RNase P amplification curve DOES cross the threshold line within 35.00 cycles (Ct < 35.00).
- » When all controls exhibit the expected performance, a specimen is considered positive if the amplification curves for both SARS-CoV-2 genes (E, RdRP) CROSS the threshold line within 40.00 cycles (Ct < 40.00). The RNase P may or may not be positive as described above, but the result is still valid.
- » When all controls exhibit the expected performance and the amplification curves for both SARS-CoV-2 genes (E, RdRP) AND the RNase P marker DO NOT cross the threshold line within 40.00 cycles (Ct < 40.00) AND 35.00 cycles (Ct < 35.00), respectively, the result is considered invalid. The extracted RNA should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is negative for all markers and RNase P, the result is invalid and collection of a new specimen from the patient should be considered.
- » When all controls exhibit the expected performance and the amplification curve for one of the SARS-CoV-2 specific genes (E, RdRP but not both) crosses the threshold line within 40.00 cycles (Ct < 40.00) the result is considered **inconclusive**. The extracted RNA should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained, report the inconclusive result. Consult with your public health authority, as appropriate, to request guidance and/or to coordinate transfer of the specimen for additional analysis.

• **SARS-CoV-2 markers – del9 and del6**

- » When all controls exhibit the expected performance, the sample is considered **B.1.351 positive** if the amplification curve for del9 (HEX channel) from del9/del6/RNase P primers/probes mix exceeds the threshold line within 40.00 cycles (Ct > 40.00) AND no later than **four** cycles compared to the averaged amplification curve for the E gene and RdRP gene. The amplification curve for del6 (FAM channel) from del9/del6/RNase P primers/probes mix does not exceed the threshold line within 40.00 cycles (Ct > 40.00) or if it exceeds the threshold line by **ten** or more cycles later when compared to the averaged amplification curve for the E gene and RdRP gene. This profile indicates the presence of SARS-CoV-2 lineage containing del9 deletion. The RNase P amplification curve may or may not be positive and cross the threshold line within 35.00 cycles (Ct < 35.00).
- » When all controls exhibit the expected performance, the sample is considered **B.1.1.7 positive** if the amplification curves for both del9 (HEX channel) AND del6 (FAM channel) from del9/del6/RNase P primers/probes mix exceed the threshold line within 40.00 cycles (Ct > 40.00) AND no later than **four** cycles compared to the averaged amplification curve for the E gene and RdRP gene. This profile indicates the presence of SARS-CoV-2 lineage containing del9 deletion as well as del6 deletion (i.e. B.1.1.7). The RNase P amplification curve may or may not be positive and cross the threshold line within 35.00 cycles (Ct < 35.00).
- » When all controls exhibit the expected performance, the sample is considered **B.1.258 positive** if the amplification curves for del9 (HEX channel) from del9/del6/RNase P primers/probes mix does not exceed the threshold line within 40.00 cycles (Ct > 40.00) or if it exceeds the threshold line by **ten** or more cycles later when compared to the averaged amplification curve for the E gene and RdRP gene and del6 (FAM channel) from del9/del6/RNase P primers/probes mix exceed the threshold line within 40.00 cycles (Ct > 40.00) AND no later than **four** cycles compared to the averaged amplification curve for the E gene and RdRP gene. This profile indicates the presence of SARS-CoV-2 lineage containing del6 deletion. The RNase P amplification curve may or may not be positive and cross the threshold line within 35.00 cycles (Ct < 35.00).
- » When all controls exhibit the expected performance, the sample is considered **other SARS-CoV-2 variant positive (B.1.1, B.1.160, B.1.177, B.1.170 or B.1.258.17)** if the amplification curves for del9 (HEX channel) AND del6 (FAM channel) from del9/del6/RNase P primers/probes mix does not exceed the threshold line within 40.00 cycles (Ct > 40.00) or if it exceeds the threshold line by **ten** or more cycles later when compared to the averaged amplification curve for the E gene and RdRP gene. **This profile indicates the presence of other SARS-CoV-2 lineages (B.1.1, B.1.160, B.1.177, B.1.170 or B.1.258.17).** The RNase P amplification curve may or may not be positive and cross the threshold line within 35.00 cycles (Ct < 35.00).
- » When all controls exhibit the expected performance and the amplification curve for only del9 (HEX channel) and/or del6 (FAM

channel) from del9/del6/RNase P primers/probes mix (but not E gene and RdRP gene) crosses the threshold line within 40.00 cycles (Ct < 40.00) the result is considered inconclusive. The extracted RNA should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained, report the **inconclusive** result. Consult with your public health authority, as appropriate, to request guidance and/or to coordinate transfer of the specimen for additional analysis.

- » When all controls exhibit the expected performance and the amplification curves for del9 (HEX channel) and del6 (FAM channel) from del9/del6/RNase P primers/probes mix, as well as the amplification curves for E gene and RdRP gene do not cross the threshold line within 40.00 cycles (Ct < 40.00) and the amplification curve for human RNase P does not cross the threshold line within 35.00 cycles (Ct < 35.00) the result is considered **invalid**. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained, the result is invalid and collection of a new specimen from the patient should be considered.

• **rTEST COVID-19 qPCR Multivariant test results interpretation guide**

The table below lists the expected results for the rTEST COVID-19 qPCR Multivariant test. If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please consult your public health authority.

NOTE!

To set the fluorescence threshold, we recommend following the manual included with the thermal cycler used or the CDC recommendations.

The appropriate baseline and threshold may differ between real time PCR instruments and may need to be established empirically to determine the optimal Δ Ct cutoff values to distinguish variants.

NOTICE!

- Before using the rTEST COVID-19 qPCR Multivariant kit, we recommend that you calibrate the real-time PCR instrument. Follow the user's instructions of your real-time PCR instrument.
- The rTEST COVID-19 qPCR Multivariant kit is designed for use by qualified and trained laboratory personnel with sufficient experience in real-time RT-qPCR testing techniques.

Table 4. Interpretation of results obtained with E gene/RdRP gene/RNase P Mix

SARS-CoV-2 E	SARS-CoV-2 RdRP	Human RNase P	Result Interpretation	Actions
+	+	+/-	SARS-CoV-2 detected	Determine the average Ct value.
+	-	+/-	Inconclusive Result	Repeat testing of nucleic acid and/or re-extract and repeat RT-qPCR. If the repeated result remains inconclusive, contact your public health authorities for further instructions or guidance.
-	+	+/-		
-	-	+	SARS-CoV-2 not detected	If the sample was tested SARS-CoV-2 positive in primary testing, repeat testing of nucleic acid and/or re-extract and repeat RT-qPCR. If the repeated result remains inconclusive, contact your public health authorities for further instructions or guidance.
-	-	-	Invalid result	Repeat extraction and RT-qPCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

Table 5. Interpretation of rTEST COVID-19 qPCR Multivariant test results and corresponding actions

E and RdRP gene (average Ct)	Difference in Ct values between del9 (HEX channel) and average Ct of E and RdRP gene	Difference in Ct values between del6 (FAM channel) and average Ct of E and RdRP gene	Human RNase P	Result Interpretation ^a	Report	Actions
+	Max 4 Ct (+)	Min 10 Ct (-)	+/-	SARS-CoV-2 del9 deletion detected	SARS-CoV-2 B.1.351 positive	Report the results to sender.
+	Max 4 Ct (+)	Max 4 Ct (+)	+/-	SARS-CoV-2 del9 and del6 deletions detected	SARS-CoV-2 B.1.1.7 positive	Report the results to sender.
+	Min 10 Ct (-)	Max 4 Ct (+)	+/-	SARS-CoV-2 del6 deletion detected	SARS-CoV-2 B.1.258 positive	Report the results to sender.
+	Min 10 Ct (-)	Min 10 Ct (-)	+/-	Other SARS-CoV-2 variant detected	SARS-CoV-2 B.1.1, B.1.160, B.1.177, B.1.1.170 or B.1.258.17 positive	Report the results to sender.
ND	+/-	+/-	+/-	Inconclusive result	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat RT-qPCR. If the repeated result remains inconclusive, contact your public health authorities for further instructions or guidance.
ND	ND	ND	ND	Invalid result	Invalid	Repeat extraction and RT-qPCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

^a Laboratories should report their diagnostic results as appropriate and in compliance with their specific reporting system.

ND – Not Detected ; (+) the result of the reaction in this channel we consider it positive; (-) the result of the reaction in this channel we consider it negative

9. Functional characteristics

9.1. Limit of detection

Evaluation of analytical sensitivity (detection limit) was performed on the combinations of primers/probes used for multiplexed detection of SARS-CoV-2 E gene/RdRP gene/RNase P and del9/del6/RNase P. The test on SARS-CoV-2 E gene/RdRP gene/RNase P primers/probes mix was performed using the positive control "SARS-CoV-2 Standard" (Exact Diagnostics) and the test on del9/del6/RNase P primers/probes mix was performed using the RNA isolated from the patient samples infected with B.1.351, B.1.1.7, and B.1.258 variants of SARS-CoV-2 as confirmed by sequencing. This RNA was diluted to 200 copies of the template per 1 µl. Serial dilutions of the stock standard were prepared resulting in samples with concentrations of 8 copies/

µl (= 40 copies/reaction), 2 copies/µl (= 10 copies/reaction), 0.8 copies/µl (= 4 copies/reaction), 0.4 copies/µl (= 2 copies/reaction) and 0.2 copies/µl (= 1 copy/reaction) that were used in the analytical sensitivity test. A synthetic matrix "SARS-CoV-2 Negative" (Exact Diagnostics) containing genomic DNA at a concentration of 75,000 copies/ml was used to dilute the control material. The assay was performed in 8 replicates for each prepared dilution. The test confirmed the high sensitivity of the rTEST COVID-19 qPCR Multivariant kit. Reliable template detection of del9 and del6 was demonstrated down to 2 copies per reaction (0.4 copies/µl) and 4 copies per reaction (0.8 copies/µl) for SARS-CoV-2 E and RdRP gene.

Table 6. Limit of detection of SARS-CoV-2 E and RdRP tests

	E gene/RdRP gene/RNase P gene		
	Total number of replicates	Number of reactions with positive results (E/RdRP/RNaseP)	Detection success (% E/RdRP/RNaseP)
40 copies/reaction	8	8/8/8	100/100/100
10 copies/reaction	8	8/8/8	100/100/100
4 copies/reaction	8	8/8/8	100/100/100
2 copies/reaction	8	8/8/8	50/75/100
1 copy/reaction	8	1/8/8	0/12.5/100

Table 7. Limit of detection of del9 and del6 tests

	Total number of replicates	Template		
		B.1.351	B.1.1.7	B.1.258
		Number of reactions with positive results (del9/del6/RNaseP)	Number of reactions with positive results (del9/del6/RNaseP)	Number of reactions with positive results (del9/del6/RNaseP)
40 copies/reaction	8	8/0/8	8/8/8	0/8/8
10 copies/reaction	8	8/0/8	8/8/8	0/8/8
4 copies/reaction	8	8/0/8	8/8/8	0/8/8
2 copies/reaction	8	8/0/8	8/8/8	0/8/8
1 copy/reaction	8	3/0/8	2/4/8	0/3/8

9.2 Clinical performance evaluation

Evaluation of the clinical performance of the rTEST COVID-19 qPCR Multivariant kit was performed for the standard SARS-CoV-2 E and RdRP gene as well as the SARS-CoV-2 B.1.351 variant. For SARS-CoV-2 E and RdRP gene, the evaluation was performed on a selected set of 38 positive and 54 negative clinical samples of patients, which were confirmed by a reference method used for routine testing by regional public health authorities

of the Slovak Republic. The analysis confirmed the results of the primary testing for all samples, except 3 samples that were exposed to one freeze-thaw cycle before RNA extraction and undetectable by reference method (Table 8.). The undetectable samples, when analysed in primary testing, expressed Ct values higher than the recommended values for retrospective validation; therefore, these samples were excluded in the final determination of sensitivity.

Table 8. Clinical performance of E gene/RdRP gene/RNase P Mix

	Reference method		E gene/RdRP gene/RNase P gene Mix		
	E gene	RdRP gene	E gene	RdRP gene	Human RNase P gene
Number of correctly identified positive samples	37	33	38	38	38
Number of false positive samples	0	0	0	0	0
Number of correctly identified negative samples	54	54	54	54	54
Number of false negative samples	1	5	0	0	0

In regards to B.1.351 variant, the evaluation was performed on a selected set of 96 SARS-CoV-2 positive clinical samples and sequencing revealed 3 of these samples belonging to the B.1.351 variant, 33 to B.1.1.7 variant and 28 to B.1.258 variant.

The remaining 33 samples showed a different genetic profile. Based on the presence or absence of del9 and del6, the rTEST COVID-19 qPCR Multivariant kit correctly identified 94 of the tested samples, including 9 samples of SARS-CoV-2 B.1.351 variant (Table 8).

Table 9. Clinical performance of del9/del6/RNase P Mix

	SARS-CoV-2 variant			
	B.1.351	B.1.1.7	B.1.258	other*
Deletion	del9	del9 + del6	del6	—
Prediction of reference method/Sequention	9	94	27	33
Number of correctly identified by rTEST COVID-19 qPCR Multivariant kit	9	94	27	33
Number of incorrectly identified by rTEST COVID-19 qPCR Multivariant kit	0	0	0	0

* the following SARS-CoV-2 variants were designated as others: B.1.1, B.1.160, B.1.177, B.1.170 or B.1.258.17

Testing of this selected set of samples was performed blinded in an external laboratory that is a member of the External Quality Assessment (EQA) scheme organized by institutions such as the European Centre of Disease Prevention and Control (ECDC), Institute of Virology, Charité, Berlin, Germany, and National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands.

The clinical evaluation of the SARS-CoV-2 samples confirmed the results of the primary testing and sequencing. The analysis demonstrated high reliability and reproducibility of the results obtained with the rTEST COVID-19 qPCR Multivariant kit.

Detection of RNase P gene showed high homogeneity in all analyzed samples, confirming the suitability of this assay as an internal control for collection and RNA extraction from a clinical sample.

10. Disposal

NOTICE!

- Decontaminate any material that has come into contact with biological samples with 3% sodium hypochlorite for a minimum of 30 minutes or autoclave at 121 °C for a minimum of 60 minutes before disposing.
- All used equipment, tips, tubes, work materials, and protective clothing should be considered potentially contaminated and disposed of in accordance with applicable infectious waste disposal regulations.
- Dispose of remaining reagents and material in accordance with applicable safety regulations.

11. Troubleshooting and safety reporting (medical device vigilance)








In case of any problems contact:

MultiplexDX, s. r. o.
Manufacturer

Address: Ilkovičova 8
841 04 Bratislava
Slovakia

Tel.: +421 2 902 68 310
Email: vigilance@multiplexdx.com

12. Symbols

	Manufacturer
	Batch number
	Recommended storage temperature
	Package size
	Research use only.
	Date of manufacturing
	Attention, follow the safety instructions and operating instructions that come with this product

 **MultiplexDX, s. r. o.**
Reg. No.: 50 111 965
Ilkovičova 8, 841 04 Bratislava
Slovakia
+421 2 902 68 308
info@multiplexdx.com



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