DIAGNOVITAL

Diagnovital SARS-CoV-2 Multiplex

Qualitative RT-PCR-based detection of SARS-CoV-2

For *in vitro* diagnostic use. For professional use only

CE IVD

REF Σ

09072025- 25 tests
09072050- 50 tests
09072100- 100 tests

TABLE OF CONTENTS

Intended Use

This document describes the use of real-time RT-PCR assays for the in vitro qualitative detection of 2019-Novel Coronavirus (SARS-CoV-2), which causes COVID-19, in respiratory specimens. The SARS-CoV-2 primer and probe sets are designed for the specific detection of SARS-CoV-2.

Diagnovital SARS-CoV-2 Multiplex Real-Time PCR Kit is an *in vitro* nucleic acid amplification assay for qualitative detection of 2019-Novel Coronavirus (SARS-CoV-2) in respiratory specimens using RTA Viral RNA Isolation Kit and BIO-RAD CFX96-IVD or Rotor-Gene 3000/6000 or Himedia Insta Q96 or Applied Biosystems 7500 or QuantStudio 5 DX Real-Time PCR Detection Systems for amplification, detection and analysis.

The kits follow CDC's and WHO's latest detection guidelines.

Product Description

Diagnovital SARS-CoV-2 Multiplex is a real-time RT-PCR-based detection system for the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). SARS-CoV-2 is considered a novel human coronavirus that is genetically distinct from the common human coronaviruses (229E, NL63, OC43, HKU1), which cause seasonal acute respiratory illness. It is also genetically distinct from the two newer human coronaviruses, MERS-CoV and SARS-CoV.

Diagnovital SARS-CoV-2 Multiplex detects the presence of 2 different and highly specific gene sequences of corona viruses (N1 and ORF1ab) at the **FAM** channel and one sequence specific for human RNA serving as a human extraction control (HEC, RNaseP) at the **HEX/VIC** channel. All 2 assays must be tested positive to confirm the sample as SARS-CoV-2-positive.

Selective amplification of RNA Internal Control is achieved by the use of non-competitive, sequence specific forward and reverse primers and a probe which have no homology with the coronavirus genome. A thermostable DNA polymerase enzyme is used for amplification.

Diagnovital SARS-CoV-2 Multiplex master mix contains detection probes for the two SARS-CoV-2 targets and one for the internal RNase P. Probes are each labelled with fluorescent dyes that act as a reporter. Each probe also has a second dye which acts as a quencher. When not bound to the target sequence, the fluorescent signals of the intact probes are suppressed by the quencher dye. During the PCR amplification step, hybridization of the probes to the specific single-stranded DNA template results in cleavage of the probe by the 5' to 3' exonuclease activity of the DNA polymerase resulting in separation of the reporter and quencher dyes and the generation of a fluorescent signal. With each PCR cycle, increasing amounts of cleaved probes are generated and the cumulative signal of the reporter dye increases concomitantly. Each reporter dye is measured at defined wavelengths, which enables simultaneous detection and discrimination of the amplified coronavirus targets.

REAL TIME PCR-BASED DETECTION OF SARS-CoV-2

The first step in the detection of SARS-CoV-2 is the conversion of viral RNA into cDNA. Afterwards, the target sequences unique for 2019-nCoV are specifically amplified with amplification monitored in real time through the use of fluorescently labelled probes: upon incorporation into the newly amplified DNA strands, the fluorophore (FAM) is released and an increase in fluorescence signal can be observed.

Due to the intrinsic mutation rate of RNA viruses, it is possible that mutations in the target sequence occur and accumulate over time. This can lead to false- negative results with a PCR- based detection approach. Diagnovital SARS-CoV-2 Multiplex addresses this issue by using 2 detection assays on 2 different target sequences to minimize the chance of false-negative results caused by an altered target sequence.

If samples are tested negative in one or more assays, additional complementary testing may be required. The original target sequences for 2019-nCoV are included as a non-infectious target positive control (TPC) to check the integrity of the detection assays.

Samples tested positive should always be confirmed through complementary methods and additional analysis in an independent laboratory.

DIAGNOVITAL® SARS-CoV-2 Multiplex is compatible with every qPCR with calibrated **FAM™** and **HEX/VIC** channel,

Materials Provided

	Reagents	Quantity and Volume (25 tests)	Quantity and Volume (50 tests)	Quantity and Volume (100 tests)
1	Diagnovital® Enzyme Mix	1 × 25 µl	1 × 50 µl	1 × 100 µl
2	Diagnovital® SARS-CoV-2 Multiplex Mix	1 × 375 µl	1 × 750 µl	1 × 1500 µl
3	Target Positive Control (TPC)	1 × 45 µl	1 × 75 µl	1 × 150 µl
4	Nuclease-free dH_2O (NTC)	1 × 1000 µl	1 × 1000 µl	1 × 1000 µl

Additional Materials Required

- Suitable means & equipment for nucleic acid extraction
- Real-time PCR detection system equipped for FAM™ and HEX/VIC detection
- Adjustable pipettes & fitting filtered pipette tips
- Appropriate personal protective equipment & workspaces for working with potentially infectious samples
- Surface decontaminants such as DNAZap™ (Life Technologies), DNA Away™ (Fisher Scientific), RNAse Away™ (Fisher Scientific), 10% bleach (1:10 dilution of commercial 5.25-6.0% sodium hypochlorite) Nuclease-free tubes / strips / plates to prepare dilutions, master mixes etc. Nuclease-free tubes / strips / plates corresponding to the PCR device
- Suitable storage options for reagents and specimen (4°C, -20°C, -70°C)

Storage

- Store all components at -15°C / -25°C and avoid repeated freeze and thaw cycles.
- Protect the Diagnovital® SARS-CoV-2 Multiplex Mix from light as prolonged exposure can diminish the performance of the fluorophores.
- If the kit components have been damaged during transport, contact RTA Laboratories. Do not use as performance may be compromised.
- Keep reagents separate from sample material to avoid contamination.
- Do not use after the designated expiry date.

Performance Characteristics

Analytical sensitivity

Analytical sensitivity was analyzed by use of a dilution series of **DIAGNOVITAL® SARS-CoV-2 Multiplex** Reference samples. A dilution series of a **DIAGNOVITAL® SARS-CoV-2 Multiplex** Reference samples was prepared to give the final concentrations of 300, 100, 30 and 10 copies/ml. Each dilution was tested in 24 replicates. Lower limit was calculated by probit analysis done by PASW Statistics 18 program. For each genotype/subtype, Limit of Detection (LoD) values and 95% confidence ranges are summarized in Table 1.

Target Gene	Limit of Detection (copies/ml)	95% confidence lower limit	95% confidence upper limit
N1+ORF1ab	38	33	50

Table 1: DIAGNOVITAL® SARS-CoV-2 Multiplex PCR Kit - Limit of Detection (LoD) values and 95% confidence ranges

Precision

In this study, precision of the kit was evaluated for intra-assay, inter-assay, inter-batch, by using RTA Viral RNA Isolation Kit (Cat No: 09010100) and different specimen types (oropharyngeal vs. nasopharyngeal swabs). For each target gene and different assay, 24 replicates of 10^3 copies/ml **DIAGNOVITAL® SARS-CoV-2 Multiplex** Reference samples were used. Descriptive statistics were analyzed by IBM SPSS Statistics program. Overall precision assays associated with Ct values were summarized in Table 2.

Descriptive Statistics					
Target Gene	**N**	**Mean**	**Std. Deviation**	**Variance**	**Coefficient of variation (%)**
N1+ORF1ab	**96**	**23,6175**	**0,139198**	**0,019269875**	**0,595032**

Table 2: Overall descriptive statistics of **DIAGNOVITAL® SARS-CoV-2 Multiplex** precision data

Diagnostic specificity

SARS-CoV-2 RNA negative clinical specimens were analyzed to determine the diagnostic specificity of **DIAGNOVITAL® SARS-CoV-2 Multiplex** Real Time PCR Kit. 30 SARS-CoV-2 RNA negative clinical oropharyngeal swab specimens and 30 SARS-CoV-2 RNA negative clinical oropharyngeal swab specimens and 30 Broncho alveolar lavage specimens were used. None of the 100 SARS-CoV-2 negative clinical specimens gave positive test result for SARS-CoV-2. Diagnostic specificity of **DIAGNOVITAL® SARS-CoV-2 Multiplex** Real Time PCR Kit is 100 %. All of the internal control gave positive result.

Cross-reactivity

To examine the specificity of an assay, cross-reactivity studies should be performed for potential cross-reactive markers. In this study, the specificity of the assay was evaluated by testing 20 reference organisms.

DIAGNOVITAL® SARS-CoV-2 Multiplex Real Time PCR Kit do not show any cross-reactivity with other potential cross-reactive markers given in the table 3 below:

Table 3: Potential cross-reactive markers tested in the study

Sample	Source	Sample ID	Replicates Detected/Total	Result
Influenza A	NIBSC	16/324	0/3	Negative
Influenza A H5	ATCC	VR-93	0/3	Negative
Influenza A H1	ATCC	VR-95	0/3	Negative
Influenza A H3	ATCC	VR-1609	0/3	Negative

Influenza A H7	ATCC	VR-1672	0/3	Negative
Influenza B	ATCC	VR-822	0/3	Negative
Parainfluenza 1	ATCC	VR-1641	0/3	Negative
Parainfluenza 2	ATCC	VR-101	0/3	Negative
Parainfluenza 3	ATCC	VR-94	0/3	Negative
Parainfluenza 4	ATCC	VR-92	0/3	Negative
RSV	ATCC	VR-93	0/3	Negative
HRV	ATCC	VR-579	0/3	Negative
HMPV	ATCC	VR-3250SD	0/3	Negative
Human coronavirus NL63	ATCC	VR-1432	0/3	Negative
Human coronavirus HKU1	ATCC	VR-154	0/3	Negative
Human coronavirus 229E	ATCC	VR-3263SD	0/3	Negative
Human coronavirus OC43	ATCC	VR-3262SD	0/3	Negative
MERS	ATCC	VR-740	0/3	Negative
Streptococcus pneumoniae	ATCC	VR-1558D	0/3	Negative
Haemophilus influenzae	ATCC	VR-3248SD	0/3	Negative
TPC			3/3	20,91
NTC			0/3	Negative

Considerations Before Starting

BIOSAFETY

- Wear appropriate personal protective equipment (e.g. gowns, powder-free gloves, eye protection) when working with clinical specimen.
- Specimen processing should be performed in a certified Class II biological safety cabinet following biosafety level 2 or higher guidelines.
- For more information, refer to:
  Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (SARS-CoV-2) https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html
- Biosafety in Microbiological and Biomedical Laboratories 5th Edition available at http://www.cdc.gov/biosafety/publications
- The use of **DIAGNOVITAL® SARS-CoV-2 Multiplex** is restricted to trained laboratory personnel only.

SPECIMENS

Only use appropriate specimens for testing, such as:

- Respiratory specimens including nasopharyngeal / oropharyngeal swabs and bronchoalveolar lavage.
- Swab specimens should be collected only on swabs with a synthetic tip (such as polyester or Dacron®) with plastic shafts. Swabs with calcium alginate or cotton tips with wooden shafts are not acceptable

SPECIMENS - HANDLING AND STORAGE

- Specimens can be stored at 4°C for up to 72 hours after collection.
- If a delay in extraction is expected, store specimens at -20°C or ideally -70°C.
- Extracted nucleic acids should be stored at -20°C or lower.

Do not use specimens if

- they were not kept at 2-4°C (≤ 4 days) or frozen at -20°C or below.
- they are insufficiently labelled or lack documentation.
- they are not suitable for this purpose (see above for suitable sample material).
- the specimen volume is insufficient.

Sample Preparation

- The performance of RT-PCR assays strongly depends on the amount and quality of sample template RNA. It is strongly recommended to qualify and validate RNA extraction procedures for recovery and purity before testing specimens.
- Suitable nucleic acid extraction systems successfully used in combination with **DIAGNOVITAL DETECTION KITS** include: RTA Viral NA Isolation Kit, bioMérieux NucliSens® systems, QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit or RNeasy® Mini Kit (QIAGEN), EZ1 DSP Virus Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit, and Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit, and Invitrogen ChargeSwitch® Total RNA Cell Kit.
- Store and keep residual specimens and extracted nucleic acids at -70°C or lower.
- Only thaw the number of specimen extracts that will be tested in a single day.
- Do not freeze/ thaw extracts more than once before testing as each freeze/thaw cycle will decrease the RNA quality.
- It may be possible to use patient samples directly, depending on the sample type. However, this may require a prior lysis step and titration of the amount on sample that can be used without inhibiting the reaction. This procedure has not been validated, use of isolated RNA is recommended.

Reaction Setup

1. Make sure that all necessary equipment and devices are suitable, calibrated and functional before starting the experiments.

2. Decontaminate equipment and workspace and prepare everything needed for the following experiment to keep the workflow short and repeatable.

3. Switch on the PCR detection system and program it to avoid delays after setting up the reactions.

4. Thaw all components of **DIAGNOVITAL® SARS-CoV-2 Multiplex** on ice and mix gently but thoroughly to ensure even distribution of components. Collect liquid at the bottom of the tube with a quick spin.

5. Prepare enough master mix for all planned reactions (n) according to your sample size, including 1 negative control (NTC) and 1 positive control (PC) for each experiment. It is recommended to prepare master mix for 2 additional reactions to compensate for pipetting inaccuracies.

   The pipetting amounts for a single reaction as given below Table 4:

Table 4: Reaction Volumes

Component Volume	Volume per Reaction
Diagnovital SARS-CoV-2 Multiplex Mix	15 µl
Diagnovital Enzyme Mix	1 µl
Isolated sample RNA / TPC/ NTC	4 µl
Total Master Mix/Reaction	**20 µl**

6. Distribute 16 µL of the master mix to each well of your PCR plate.

7. Transfer the Master mix Plate to a separate workspace to add the sample material. Preparing reagents and final reaction setup in separate workspaces helps to avoid contamination of equipment and reagents with sample material.

8. Prepare negative reactions first and seal them before handling positive samples. It is recommended to only bring potentially positive sample material and the included target positive control to the workspace once the NTC is prepared and sealed.

9. Add 4 µl Sample or Control to the respective sample and control wells and seal the plate. Keep reactions on ice until transferring them to the PCR device.

Example pipetting scheme for the distribution of master. Please see below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM
B												
C												
D												
E												
F												
G												
H												

*MM (Master Mix)

Example pipetting scheme for the addition of samples. The bottom half of the plate could be used for replicates with an identical setup. Please see below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	TPC
B												
C												
D												
E												
F												
G												
H												

Figure 1: Example pipetting scheme for the addition of samples. (S: Sample)

Setting up RT-PCR Program:

1. Switch on the PCR detection system
2. Program the following thermal protocol in Table 5:

Table 5: RT-PCR Program

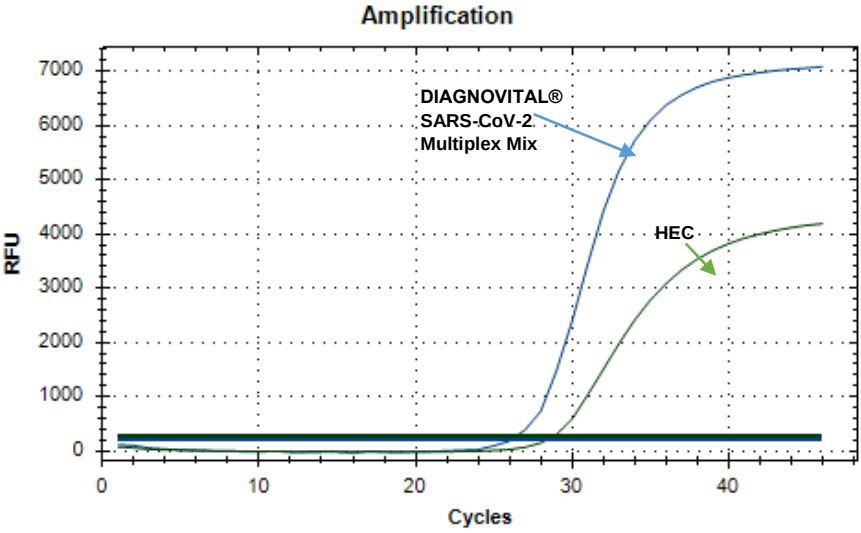
Step	Cycles	Temperature	Duration
Reverse Transcription	1	50°C	5 Min
Initial Denaturation	1	95°C	5 Min
Amplification	40	95°C	5 Sec
		60°C	30 Sec

*Enable Data Collection for **FAM™** (for virus detection) and **HEX/VIC** (for internal control).

3. Once the run is finished, do not open the reaction tubes/ plate to avoid contamination and discard according to local guidelines and regulations. Do not autoclave as this may contaminate laboratory equipment with amplicons.

Analysis & Troubleshooting

EXEMPLARY RESULT



- **dH$_2$O controls (NTC) must not give a positive Ct for any assay**. If they do, the reaction was contaminated with sample RNA / DNA. Decontaminate equipment and workspace and repeat the reactions.
- **For a sample to be considered positive for SARS-CoV-2,** both targets (N / ORF1ab) and the RNase P target must give positive Ct values of ≤35. If the RNase P target fails to amplify within ≤Ct 35, but the SARS-2 specific targets are amplified, the sample is considered valid positive.

- **For a sample to be considered negative for SARS-CoV-2,** the SARS-CoV-2 targets (N / ORF1ab) must remain undetermined (or Ct values are > 35 cycles) and the RNase P must give a positive Ct value (≤35 cycles) to ensure that sample material of suitable quality was present.
- **A sample result is invalid if the detection of RNase P in the sample fails and the sample also fails to show amplification of SARS-CoV-2 targets (N / ORF1ab) within ≤35 Ct.** Invalid results cannot be interpreted. These samples should be repeated from extraction step. If the SARS-CoV-2 targets are detected in the sample in the absence of the RNase P target, the sample is valid positive.
- **When using the TPC, a positive Ct in the FAM™ channel must be observed**. **The Ct value for the TPC should be <35 cycles**. Both, N and ORF1ab targets must be observed with Ct values of ≤35 cycles for the TPC control to be valid. If the Ct value is > Ct 35 or not all SARS-CoV-2 targets are tested positive, PCR was compromised. Check the reaction setup and PCR device settings and repeat the reactions. If any of the targets in the positive control is negative the run is invalid.
- **If no amplification signal is observed for any assay, PCR was inhibited**. Check reaction setup and device settings and repeat the RNA extraction if necessary. Results are invalid and cannot be interpreted.

Table 6: Result Interpretation of Diagnovital SARS-CoV-2 Multiplex for Samples

N / ORF1ab	RNase P	Interpretation	Report	Actions
-	+	Only the target sequence for the RNase P was amplified. The sample is considered negative for SARS-CoV-2.	Negative	Report results
+	+	Both target sequences for SARS-CoV-2, and the RNase P were amplified. The sample is considered positive for SARS-CoV-2.	Positive	Report results
-	-	PCR was inhibited, results are invalid.	Invalid	Sample is repeated once. If the result is again invalid, it is reported to the sender as invalid and collection of a new sample is recommended.

Limitations

- For reliable results, it is essential to adhere to the guidelines given in this manual. Changes in reaction setup or cycling protocol may lead to failed experiments.
- Depending on the sample matrix, inhibitors may be present in the isolated RNA and disable reverse transcription and/or PCR amplification. If this is the case, another sample type or isolation method may be beneficial.
- Spontaneous mutations within the target sequence may result in failure to detect the target sequence.
- Results must always be interpreted in consideration of all other data gathered from a sample. Interpretation must be performed by personnel trained and experienced with this kind of experiment.

Trademarks

DIAGNOVITAL®, NucliSens® (bioMérieux), QIAamp®, RNeasy® (QIAGEN), ChargeSwitch® (Invitrogen), ROX™, FAM™ (Life Technologies), DNAZap™, DNA Away™, RNAse Away™

Registered names, trademarks, etc. used in this document, even if not specifically marked as such, are not to be considered unprotected by law.

RTA LABORATUVARLARI BİYOLOJİK ÜRÜNLER İLAÇ VE MAKİNE SAN. TİC. A.Ş.
Geposb Cumhuriyet Cad. No:3
41400 Gebze/ Kocaeli / Turkey
Tel: +90 262 648 5300
E-mail: rta@rtalabs.com.tr
Web: www.rtalabs.com.tr