Instructions for Use

RealLine SARS-CoV-2

QUALITATIVE ASSAY KIT FOR THE DETECTION OF THE TARGETS E-GENE AND N-GENE OF SARS-COV-2 RNA BY REAL TIME PCR

In vitro Diagnostics

CE

The kit consists of two packs, please store **immediately** after delivery:
PART1 at (2 - 8) °C
PART2 at (-18 …-22) °C

RealLine SARS-CoV-2 (A-Format)	**BI1019-96**	**96 Tests**
RealLine SARS-CoV-2 (B-Format)	**BI1020-96**	**96 Tests**
valid from:	**July 2021**	

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Explanation of symbols used in labelling

IVD	*In-vitro* Diagnostics
LOT	Batch code
REF	Catalogue number
	Contains sufficient for <n> tests
	Use-by-date
	Temperature limit
	Consult instructions for use
	Manufacturer
	Keep away from sunlight

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TABLE OF CONTENT:

Validated Cyclers	
BI1019-96 – A-format	**BI1020-96 – B-format**
RealLine Cycler and equivalent CFX96 (Bio-Rad)	RealLine Cycler and equivalent Rotorgene Cyler (Qiagen) CFX96 (Bio-Rad

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QUALITATIVE ASSAY KIT FOR THE DETECTION OF SARS-COV-2 RNA BY REAL TIME PCR

1. INTENDED USE

The **RealLine SARS-CoV-2 Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – pathogen-detection-based product. The **RealLine SARS-CoV-2 Detection Kit** is designed to detect SARS-CoV-2 (COVID-19 virus, 2019-nCoV) and SARS-like coronaviruses in human biological samples with the Polymerase Chain Reaction (PCR) method.

Samples are human biological materials: nasopharyngeal swabs, oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, sputum.

Indications for the use:

- persons with acute respiratory viral infection (ARVI) symptoms and who have been in contact with COVID-19 infected, regardless of their age;
- persons of all ages without ARVI symptoms (in the centers of infection/ in the conditions of infection spread) for the purpose of early detection of coronavirus to prevent further spread of infection.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use the **RealLine SARS-CoV-2 Detection Kit.**

The **RealLine SARS-CoV-2 Detection Kit** can be used in clinical and diagnostic laboratories of medical institutions and research practice. The safety of laboratories should be ensured in accordance with the requirements of legislation in the field of sanitary and epidemiological welfare.

Potential users: personnel qualified in molecular diagnostics methods and working in the clinical and diagnostic laboratory.

It is necessary to apply the kit only as directed in this user manual.

Table1: Overview to the detected genes and channels:

FAM	HEX	ROX	Cy5
SARS-like Corona viruses, including: SARS-CoV and SARS-CoV-2	Internal Control (RNA-IC)	SARS-CoV-2, E-gene	SARS-CoV-2, N-gene

The extraction of RNA from samples is performed using the RealLine Prep NA-S (BI1007, BIORON Diagnostics), RealLine Prep NA (BI1010, BIORON Diagnostics GmbH) or other extraction Kits intended for the extraction of virus RNA. See respective IFUs.

The kit is intended for use with RealLine Cycler (BIORON Diagnostics GmbH) with equivalent cyclers and CFX96 (Bio-Rad). For the use of Rotorgene Cyclers (Qiagen) RealLine SARS-CoV-2 (B-format (REF BI1020-96)) kit , with tubes is approved.

The limit of detection is 10 DNA copies per amplification tube, dependent on the quality of sampling and extraction of RNA.

State of the art July 2021: these RealLine SARS-CoV-2 kits detect all known variants of the SARS-CoV-2 Virus (Alpha, Beta, Gamma, Delta).

The use of:

- **Extraction Kits for nucleic acids from clinical specimen from other suppliers**
- **Other real-time PCR devices than described**
- **Appropriate reaction volumes, other than described**

has to be validated in the lab by the user. Instructions for the use of the Internal Control (IC) have to be followed.

2. KIT CONTENTS

The **RealLine SARS-CoV-2 Detection Kit** is intended for single use and designed for 96 tests (94 defined samples, one positive control and one negative control).

It is not recommended to perform less than 8 samples (6 defined samples, one positive control and one negative control) in one run. It can lead to situation when the volume of enzyme will be insufficient.

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Reagent	Quantity	Appearance	Long-time Storage
BI1019-96: RealLine SARS-CoV-2 (A-format)			
Paraffin-sealed PCR-Mix	12 x 8 strip-tubes, each with 15 µl PCR-Mix	Colourless transparent liquid under white wax layer	Box of supplier (2 - 8) C
RT-PCR-buffer	2 tubes, 810 µl each	Colourless transparent liquid	Box of supplier (2 - 8) C
Mineral Oil	2 tubes, 1 ml each	Colourless transparent viscous oily liquid	Box of supplier (2 - 8) °C
Positive Control	1 tube, 130 µl	Colourless transparent liquid	Box of supplier (2 - 8) °C
Internal Control (RNA-IC)	1 tube, 1 ml	Colourless transparent liquid	Box of supplier (2 - 8) °C
Strips caps	12 x 8 strip-caps	---	Box of supplier (2 - 8) °C
Enzyme Taq/RT	1 tube, 55 µl	Colourless transparent viscous liquid	Pouch in freezer (-18 .- -22) °C

Reagent	Quantity	Appearance	Long-time Storage
BI1020-96: RealLine SARS-CoV-2 (B-format)			
Paraffin-sealed PCR-Mix	96 tubes, each with 15 µl PCR-Mix	Colourless transparent liquid under white wax layer	Box of supplier (2 - 8) °C
RT-PCR-buffer	2 tubes, 810 µl each	Colourless transparent liquid	Box of supplier (2 - 8) °C
Mineral Oil	2 tubes, 1 ml each	Colourless transparent viscous oily liquid	Box of supplier (2 - 8) °C
Positive Control	1 tube, 130 µl	Colourless transparent liquid	Box of supplier (2 - 8) °C
Internal Control (RNA-IC)	1 tube, 1 ml	Colourless transparent liquid	Box of supplier (2 - 8) °C
Enzyme Taq/RT	1 tube, 55 µl	Colourless transparent viscous liquid	Poach in freezer (-18 - -22) °C

3. METHOD

The implemented method of reverse transcription followed by polymerase chain reaction is based on RNA reverse transcription process and subsequent amplification of cDNA.
The RNA reverse transcription stage and PCR amplification of cDNA stage are performed in one test tube.

To increase the sensitivity and specificity of the amplification reaction, the use of a hot-start is provided. Hot-start is provided by reaction mixture preparation consisting of two layers separated by a layer of paraffin. The polymerase chain reaction starts only when paraffin is melted. It excludes non-specific annealing of primers to targets DNA during the initial heating of the tube.
The **RealLine SARS-CoV-2 Detection Kit** is based on fluorescent modification of the PCR method. The PCR-Mix contains four target-specific probes bearing reporter fluorescent dyes (FAM, HEX, ROX and Cy5) and quencher molecules. Once hybridized to a target sequence, the probes become activated. As a result of activation fluorescence increases proportionally to target sequence amplification. The intensity of fluorescence is measured at every cycle of reaction with a Real-time PCR thermal cycler data collection unit and analyzed with the software provided.

The **RealLine SARS-CoV-2 Detection Kit** includes the Internal Control (RNA-IC), which is intended to assess the quality of the RNA extraction and polymerase chain reaction. DNA probe used for the detection of the SARS-CoV-like coronaviruses product amplification includes fluorescent dye FAM. DNA probe used for the detection of the SARS-CoV-2 (E-gene) product amplification includes fluorescent dye ROX. DNA probe used for the detection of the SARS-CoV-2 (N-gene) product amplification includes fluorescent dye Cy5. DNA probe used for the detection of the Internal Control amplification product includes the fluorescent dye HEX. The application of four fluorescent dyes makes it possible to register the results of different amplification reactions taking place simultaneously in one tube. Table 1 shows the detection channels of amplification products.

The automatic analysis is available on BIORON Diagnostics GmbH made instruments: RealLine Cyclers 48 and 96.
The **RealLine SARS-CoV-2 Detection Kit** is also approved for use with Rotor-Gene Q (Qiagen) and CFX96 (Bio-Rad) real-time thermal cyclers

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4. PRODUCT USE LIMITATIONS

- Diagnostic sensitivity of the kit may vary depending on the pathogen prevalence, insufficiency of patient sample and characteristics of the enrolled cohort.
- Reliable results depend on adequate specimen sampling.
- Positive results indicate active or/and asymptomatic infection; results should be interpreted with consideration of clinical and laboratory findings.
- Negative results indicate lack of detectable RNA but do not exclude the infection or disease.
- Potential mutations within the target regions of the SARS-CoV-2 genome covered by the primers and/or probes used in the kit may result in failure to detect the presence of the pathogens.
- Using of results in combination with COVID-19 lies in the responsibility of the user and clinicians.
- The kit is intended to be used for the detection of SARS-CoV-2 RNA and should be interpreted with consideration of clinical and laboratory findings.

The detection result of this product is only for clinical reference, and it should not be used as the only evidence for clinical diagnosis and treatment. The clinical management of patients should be considered in combination with their symptoms/signs, history, other laboratory tests and treatment responses. The detection results should not be directly used as the evidence for clinical diagnosis, and are only for the reference of clinicians.

5. SPECIFICATIONS

5.1. Analytical specificity:

The analytical **specificity** of the **RealLine SARS-CoV-2 Detection Kit** was assessed by bioinformatics analysis using available on-line databases with up-to-date comprehensive genetic information. The specific oligonucleotides used in the test were checked against GenBank database sequences. None of the sequences showed sufficient similarity for unspecific detection.

Since it is impossible to exclude the occurrence of new mutations in the genome of the SARS-CoV-2 coronavirus, three genome sites were selected as targets to improve the reliability of diagnostics: the N- and E-genes sites specific to the SARS-CoV-2 coronavirus, as well as the conservative E-gene site common to the group of SARS-CoV-like coronaviruses (including SARS-CoV and SARS-CoV-2).

In the samples of human biological material with SARS-CoV-2 coronavirus RNA, the detecting amplifier should register an increase in fluorescence on the FAM/*Green*, ROX/*Orange* and Cy5/*Red* detection channels.

In the samples of human biological material free of SARS-CoV-2 coronavirus RNA and SARS-CoV-like coronaviruses RNA, the detecting amplifier should register an increase in fluorescence on the HEX/*Yellow* detection channel, the increase in fluorescence on the FAM/*Green*, ROX/*Orange*, and Cy5/*Red* channels should be absent.

In samples of biological material free of SARS-CoV-2 coronavirus RNA, but which contain SARS-CoV-like coronaviruses RNA:

- SARS coronavirus (various isolates),
- Bat SARS-like coronavirus (various isolates),
- Bat SARS coronavirus (various isolates),
- SARS-like coronavirus (various isolates),,
- SARS-related coronavirus (various isolates);
- Rhinolophus affinis coronavirus;
- Coronavirus BtRs-BetaCoV,

the detecting amplifier should register an increase in fluorescence in the FAM/*Green* detection channel. Any increase in fluorescence in ROX/*Orange* and Cy5/*Red* detection channels should be absent.

For cross-reactivity tests, there was no evidence of unspecific positive results of amplification of RNA samples in the presence of *Influenza A virus, Influenza B virus, Human coronavirus HKU-1, Human coronavirus NL-63, Human rhinovirus,* for DNA of *Mycoplasma pneumonia, Streptococcus pneumonia, Chlamydophila pneumoniae, Haemophilus influenza, Klebsiella pneumoniae, Moraxella catarrhalis, Bordetella pertussis, Bordetella parapertussis*, as well as human DNA in concentrations up to 1.0×10^8 copies/ml of the sample.

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5.2. Analytical sensitivity

In a determination of analytical **sensitivity,** the **RealLine SARS-CoV-2 Detection Kit** demonstrated the ability to reproducibly detect 10 copies of RNA per amplification tube. Sensitivity is determined by the analysis of serial dilutions of the laboratory control sample (LCS).

No.	Concentration of LCS, copies per amplification tube	Strips				Tubes	
		Kit No.1		Kit No2		Kit No1	
		LCS, series 1	LCS, series 2	LCS, series 1	LCS, series 2	LCS, series 1	LCS, series 2
		Number of positive samples from 24 repetitions					
1	20	24	24	24	24	24	24
2	10	24	23	24	24	24	23
3	5	19	18	19	20	20	17
4	0	-	-	-	-	-	-

Sensitivity depends on the sampling and the final volume of the extracted NA (elution volume).

Sensitivity of 10 RNA copies per amplification tube corresponds to the following values of the RNA concentration in the sample in case of using **RealLine Prep NA-S (BI1007, BIORON Diagnostics)** or **RealLine Prep NA (REF BI1010, BIORON Diagnostics GmbH).**

No.	Detected Virus	RealLine Prep NA (BI1010) Elution volume 50 µl,	RealLine Prep NA-S (BI1007) Elution volume 50 µl,
1	SARS-like Coronaviruses	500 copies / ml sample	500 copies / ml sample
2	SARS-CoV-2 coronavirus E-Gene	500 copies / ml sample	500 copies / ml sample
3	SARS-CoV-2 coronavirus N-Gene	500 copies / ml sample	500 copies / ml sample

5.3. Diagnostic characteristics

Number of samples (n) - 192;

Diagnostic sensitivity (95 % CI) – 100 % (95.6-100 %);
Diagnostic specificity (95 % CI) – 100 % (96.7-100 %).

The claimed specifications are guaranteed when RNA extraction is performed with the extraction kits **RealLine Prep NA-S (REF BI1007, BIORON Diagnostics)** or **RealLine Prep NA (REF BI1010, BIORON Diagnostics GmbH).**

6. WARNING AND PRECAUTIONS

- Handle and dispose all biological samples, reagents and materials used to carry out the assay as if they were able to transmit infective agents.
- The samples must be exclusively employed for certain type of analysis. Samples must be handled under a laminar flow hood.
- Tubes containing different samples must never be opened at the same time.
- Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA.
- The reagents must be handled under a laminar flow hood.
- The reagents required for amplification must be prepared in such a way that they can be used in a single session.
- Pipettes used to handle reagents must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA**.** Avoid direct contact with the biological samples reagents and materials used to carry out the assay.
- Use powder-free surgical gloves.
- Avoid producing spills or aerosol.
- Any material coming in contact with the biological samples must be treated for at least 30 minutes with disinfecting solution or autoclaved for 1 hour at 121ºC before disposal.
- Molecular biology procedures, such as nucleic acids extraction, reverse transcription, amplification and detection require qualified staff to avoid the risk of erroneous results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.
- All oligonucleotide components are produced by artificial synthesis technology according to internal quality control protocol and do not contain blood or products of blood processing.
- Positive control is produced by artificial synthesis technology. Positive control does not include parts of infectious agents.
- All the liquid solutions are designed for single use and cannot be used more than once in amplification reactions.
- Plastic tubes do not contain phthalates.
- Do not breathe gas/fumes/vapour/spray produced by the components of the kit.
- Do not eat/drink components of the kit. Avoid contact with the eyes. Only use the reagents provided in the kit and those recommended by manufacturer.
- Do not mix reagents from different batches.
- Do not use reagents from third party manufacturers' kits.
- All laboratory equipment, including dispensers, test tube racks, laboratory glassware, lab coats, bouffant caps, etc., as well as reagents should be strictly stationary. It is not allowed to move them from one room to another. Equip separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products.

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- Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- Wear lab coats, gloves and tools, which are exclusively employed for the extraction/preparation of the amplification reaction and for the amplification/detection of the amplification products.
- Never transfer lab coats, gloves and tools from the area designed for amplification/detection of the amplification products to the area designed for extraction/preparation of amplification reactions.
- Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination.
- Pipettes used to handle amplification products must be exclusively employed for this specific purpose.
- Remove PCR waste only in a closed form. Do not open the tubes after amplification.
- Waste materials are disposed of in accordance with local and national standards. All surfaces in the laboratory (work tables, test tube racks, equipment, etc.) must be treated daily with disinfecting solution.

Emergency actions

Inhalation: Inhalation of the Master Mix contained within this kit is unlikely, however care should be taken.

Eye Contact: If any component of this kit enters the eyes, wash eyes gently under potable running water for 15 minutes or longer, making sure that the eyelids are held open. If pain or irritation occurs, obtain medical attention.

Skin Contact: If any component of this kit contacts the skin and causes discomfort, remove any contaminated clothing. Wash affected area with plenty of soap and water. If pain or irritation occurs, obtain medical attention.

Ingestion: If any component of this kit is ingested, wash mouth out with water. If irritation or discomfort occurs, obtain medical attention.

- **Do not use the kit:**

If the transportation and storage conditions are breached;

If the reagents' appearance does not respond to the kit passport;

If the kit components packaging is breach;

After the expiry date provided.

- **Significant health effects are NOT anticipated from routine use of this kit when adhering to the instructions listed in the current manual.**

7. EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

Specimen collection

Specimen collection swabs: use only dacron, rayon, or calcium alginate tipped collection swabs with plastic or non-aluminium wire shafts;

RNA extraction and PCR

Specimen and control preparation

- Biological safety cabinet class II;
- Vortex mixer;
- Refrigerator;
- Nucleic acid extraction kit (RealLine Prep NA (REF BI1010) or RealLine Prep NA-S (REF BI1007) Kits are recommended);
- High speed centrifuge (RCF 16000 x g);
- Solid-state thermostat (temperature range 40-95°C);
- PCR tube rack for 1.5 ml tubes;
- 1.5 ml microcentrifuge tubes with caps;
- Physiological saline solution 0.9 % NaCl (Sterile);
- Container for used pipette tips;
- Electric laboratory aspirator with trap flask for the removal of supernatant;
- RNase and DNase free filtered pipette tips for aspirator with trap flask;
- Powder-free surgical gloves;
- Disinfectant solution.

Pre-amplification-reagent preparation area

- UV PCR cabinet;
- Vortex mixer;
- Refrigerator;
- PCR tube rack for 0.2 ml tubes or strips;
- Rotor for strips (if package in strips is used);
- Single channel pipettes (volume range 2-20 µl, 20-200 µl, 200-1000 µl);
- RNase and DNase free filtered pipette tips (volume range 20 µl, 200 µl, 1000 µl);
- Powder-free surgical gloves;
- Disinfectant solution;

Post-Amplification – Amplification detection area

- Real-Time PCR Cycler

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8. SAMPLES

The **RealLine SARS-CoV-2 Detection Kit** is designed to detect RNA extracted from the nasopharynx and oropharynx swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, sputum, depending on professional prescription.

Interfering substances

The presence of PCR inhibitors in a sample may cause controversial (uncertain) results. The sign of PCR inhibition is the simultaneous absence of signal in Internal Control and specific product of amplification.

PCR inhibitors are the presence of haemoglobin in the RNA sample as a result of incomplete removal during the extraction of RNA from a biomaterial sample containing an impurity of blood, as well as the presence of isopropyl alcohol and methyl acetate in the extracted RNA sample as a result of incomplete removal of washing solutions during sample preparation.

The maximum concentration of interfering substances, which do not affect the amplification of the laboratory control sample and Internal Control, are: haemoglobin – 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 medicines, including those that are contained in nasal sprays, etc. should be removed during the NA extraction using sample preparation kits. To reduce the count of PCR inhibitors, it is necessary to follow the principles of taking biological material. Suspecting a large count of PCR inhibitors in the sample, it is recommended to choose NA extraction methods that allow to remove PCR inhibitors from the sample as much as possible. It is not recommended to use express methods of NA extraction.

The features of biomaterial sampling

Work with biomaterials should be performed in accordance with Laboratory testing for coronavirus disease (COVID-19) in suspected human cases, Interim guidance, 19 March 2020 (WHO) and national legislation.

The collection of clinical material and its packaging is carried out by an employee of a medical organization who is trained in the requirements and rules of biological safety when working and collecting material suspected of being infected with pathogenic microorganisms.

The timing of biomaterial sampling is very important. Presumably, the highest content of the virus in the respiratory organs of person can be within the first 4 days after the appearance of symptoms of the disease. Samples should be collected within 3 days after the appearance of clinical symptoms of the disease.

At least three types of clinical material should be collected from one patient.

It is necessary to take swabs from the nasal cavity, naso-and oropharynx.
Each sample of biomaterial should be placed in a separate transport container.

Transportation and storage of the samples

Type of the sample	Collecting material requirements	Transpor-tation	Storage conditions before transportation	Comments
Nasopharynx and oropharynx swabs	Plastic test tubes and tampons for swabs **	4 °C	≤5 days: 4 °C >5 days *: -70 °C	Nasopharyngeal and oropharyngeal tampons should be placed in the same tube to increase the viral load
Broncho-alveolar lavage	Sterile container	4 °C	≤48 hours: 4 °C >48 hours *: -70 °C	A small sample dilution is possible
Endotracheal aspirate, naso-pharyngeal aspirate or nasal lavage	Sterile container	4 °C	≤48 hours: 4 °C >48 hours *: -70 °C	
Sputum	Sterile container	4 °C	≤48 hours: 4 °C >48 hours *: -70 °C	Make sure that the material is from the lower respiratory tract

* if it is not possible to store samples at minus 70 °C, store samples at minus 20 °C.

** Use a transport medium for storage and transportation of the respiratory swabs or saline solution (if transportation to the laboratory no more than 24 hours after taking the sample) or a dry probe-tampon (if transportation to the laboratory no more than 4 hours after taking the sample).

Avoid repeated freezing and thawing of samples.

Samples must be transported in accordance with the requirements of the sanitary legislation in relation to pathogenic microorganisms

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9. PROCEDURE

The range of SARS-CoV-2 viral load can vary widely from very low values (10^4 or less copies/ml) in the biomaterial of asymptomatic carriers and patients in the recovery stage to extremely high values (more than 10^9 copies/ml) in the biomaterial of patients with a clinical picture of acute viral pneumonia. In this regard, when performing research in a clinical laboratory, the risk of cross-contamination between samples at all stages of work is a serious danger, especially during aliquoting and RNA extracting. Cross-contamination with high-copy biomaterial can lead to sporadic false-positive results.

To prevent cross-contamination of the biological material in the laboratory, the following rules are recommended:

- it is necessary to conduct a visual assessment of the incoming biomaterial and cull test tubes with broken integrity;
- if possible, it is recommended to analyze samples of patients from a hospital with symptoms of acute infection separately from the rest of the samples (the biological material for screening exposed individuals and patients with mild disease). It is desirable to work with the supposed high-copy samples in a separate box or after working with the supposed low-copy samples;
- it is necessary to use negative control samples, starting from the stage of extracting RNA in each protocol;
- use tips with aerosol filters at all stages of the assay;
- strictly follow the assay procedure, open the Eppendorf test tubes with tweezers (do not touch inside the tube cap by the gloved hand); when applying reagents, do not touch inside the test tube by the tip (if this happened, immediately replace the tip).

9.1. RNA-Extraction

For RNA extraction from the nasopharynx and oropharynx swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, sputum, RNA extraction kits are used (see Table 3).

Table 3. The reagent kits validated for RNA extraction and further study with the **RealLine SARS-CoV-2 Detection Kit**

Reagent Kit	Biomaterial
RealLine Prep NA (REF BI1010)	nasopharynx and oropharynx swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, sputum
RealLine Prep NA-S (REF BI1007)	nasopharynx and oropharynx swabs

RNA extraction is carried out according to the extraction kit instructions.

The volume of the resulting RNA preparation should not exceed 50 µl.

The resulting RNA preparation must be used immediately for RT-PCR. If it is needed, the resulting RNA preparation can be stored at temperatures from minus 18 °C to minus 22 °C for no longer than a week with a single defrost before reverse transcription.

9.2. The features of biomaterial preparation for SARS-CoV-2 coronavirus RNA testing

Do not perform centrifugation as a pre-treatment of nasopharyngeal and oropharyngeal swabs (smears) taken into transport medium.

For RNA extraction, 100 µl of the sample is used.

9.3. The use of control samples at the stage of nucleic acid extraction

9.3.1 Internal control sample

To exclude false negative results of the study and to control the quality of the study, it is necessary to use an internal control sample to the clinical samples at the stage of nucleic acid extraction.
The Internal Control (RNA-IC) from the **RealLine SARS-CoV-2 Detection Kit** should be used as an internal control sample. The RNA-IC is an artificial RNA packed in phage particle. It is irrelevant to SARS-CoV-2 and amplified with separate pair of primers and probe.
The RNA-IC should be used in the amount of 10 µl per sample.

9.3.2 Negative control sample

To exclude false positive results of the study and to control the quality of the study, it is necessary to use a negative control sample from the nucleic acid extraction stage.

Independently of DNA/RNA extraction kit used, a negative control sample should go through all stages of DNA/RNA extraction simultaneously with the RNA extraction from clinical samples.

Physiological saline solution can be used as a negative control sample in volumes as indicated in the instructions for use of extraction kits or negative control sample that is include in the corresponding extraction kit.

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9.4. PCR Amplification with Reverse Transcription (RT-PCR)

The reagents and tubes should be kept away from direct sun light.

When using package A-format (REF BI1019-96), strips, strictly observe the completeness of the strips and caps to them. Do not use the caps to the strips of the other kits!

9.4.1. Mark the required number of tubes with paraffin sealed RT-PCR-mix according to the number of samples to be analyzed, 1 tube for Positive Control **PC** and 1 tube for Negative Control **NC**

Example: To test 6 samples, mark 6 tubes (one for each sample), 1 tube for **PC** and 1 tube for **NC**. Total number of tubes – 8.

9.4.2. Vortex the **RT-PCR-buffer** and **Enzyme Taq/RT** thoroughly for 3-5 sec, then spin briefly for 1-3 sec.

Enzyme Taq/RT should be take out from the freezer immediately prior to use.

9.4.3. Prepare the mixture of **RT-PCR-buffer** and **Enzyme Taq/RT**. Add to one mixture tube:

15 x (N+1) **µl** of **RT-PCR-buffer**,

0.5 x (N+1) **µl** of **Enzyme Taq/RT**,

Where: N is the amount of the samples, including PC and NC.

Example: To test 6 samples, mark 8 tubes. Prepare the mixture of RT-PCR-buffer and Enzyme Taq/RT for 9 (8+1) tubes. Mix 135 µL of RT-PCR-buffer and 4.5 µL of Enzyme Taq/RT.

Taking the Enzyme Taq/RT, it is necessary to dip the tip no more than 1.0 mm and observe the rules for dosing viscous liquids. Thoroughly flush the remaining Enzyme Taq/RT from the tip by pipetting at least 5 times

9.4.4. Vortex the tube with the mixture of RT-PCR-buffer and Enzyme Taq/RT thoroughly. Then spin briefly for 1-3 sec.

Mixture of RT-PCR-buffer and Enzyme Taq/RT should be prepared immediately before use, it must be used within 2 hours after preparation, storage at temperatures from (2 – 8) °C.

9.4.5. Add **15 µl** of Enzyme Taq/RT and -RT-PCR-buffer **mix** into the each PCR tube. Avoid paraffin layer break.

9.4.6. Add one drop (~20 µl) of mineral oil into the each tube. Close tubes.

9.4.7. Vortex the tubes with samples and PC and NC for 3-5 sec and spin down the drops by centrifuging for 1-3 sec.

⚠ Open the cap of the tube/strip, add RNA sample (or control sample), then close the tube/strip before proceeding to the next tube/strip to prevent contamination. Use filter tips.

9.4.8. Add **10 µl RNA** sample into corresponding RT-PCR tube. Close the tubes tightly. Add **10 µl NC** which passed the whole NA extraction procedures into corresponding tube. Add **10 µl PC** into PC tube. Avoid paraffin layer break. Close the tubes tightly.

Reagent	Patient Sample(s)	PC	NC
Extracted RNA	10 µl	-	-
NC	-	-	10 µl
PC	-	10 µl	-
Mineral Oil	one Drop	one Drop	one Drop

9.4.9. Vortex tubes for 3-5 sec and spin the tubes briefly for 3-5 sec.

9.4.10. Place the tubes into the Thermal Cycler.

For the RealLine Cycler (BIORON Diagnostics GmbH), we can provide the PCR program for easy use on request: techsupport@bioron.de

Launch the RealTime_PCR application in “Device operation” mode. Upload the **“.ini”** file before the first run. Add test in subsequent runs. Specify the number and identificator of samples. Define position of tubes in software interface according to position they were set in thermal unit. Run PCR.

If you use another Real-Time PCR cycler, please contact us for support: techsupport@bioron.de

9.4.11. Choose channels: **FAM** / ***Green***
HEX / ***Yellow***
ROX / ***Orange***
Cy5 / ***Red***

9.4.12. PCR Volume: **40 µl**

9.4.13. Program the positions of test tubes with samples, positive and negative controls according to the instruction manual for the Real-Time PCR system in use.

⚠ Amplification products can be stored at temperatures from 2 °C to 8 °C for one month or at temperatures from minus 20 °C for 12 months.

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9.4.14. Program the RealLine Cycler as follows

For RealLine Cyclers

Step	Temperature	Time	Number of the Cycles	Type of step
1	35 °C	20 min	1	Cycle
2	95 °C	5 min	1	Cycle
3	94 °C 64 °C *	10 s 10 s *	5 *	Cycle
4	94 °C 64 °C *	5 s 10 s *	45 *	Cycle
5	80 °C	1 s	1	Cycle
6	10 °C	-	Holding	Holding
* measurement of fluorescence FAM, HEX, ROX, Cy5				

For Rotor-Gene Cyclers

Cycling	Temperature	Hold time, s	Cycle repeats
Cycling	32 °C	1200	1 time
Cycling 2	95 °C	300	1 time
Cycling 3	94 °C 60 °C *	10 15 *	50 times *
* measurement of fluorescence in channels *Green* (FAM), *Yellow* (HEX), *Orange* (ROX), *Red* (Cy5)			

For CFX96 (Bio-Rad)

Step	Temperature	Time, min:s	Cycle repeats
1	35 °C	20:00	1
2	95 °C	5:00	1
3	94 °C	0:15	50 *
4	64 °C *	0:20 *	
* measurement of fluorescence in channels FAM, HEX, ROX, Cy5			

Amplification products can be stored at temperatures from (2 – 8) °C for one month or at temperatures from minus 20 °C for 12 months.

10. DATA ANALYSIS

In case of using RealLine Cyclers, the analysis is performed automatically. In all other cases, the analysis is based on the presence or absence of specific signal.

The Real-time PCR Thermal Cyclers detects and interprets results automatically. Analysis will be performed by Real-Time PCR application.

PCR results interpretation should be carried out in accordance with following Tables:

Detection Channel				**Interpretation**
FAM/*Green*	**HEX/*Yellow***	**ROX/*Orange***	**Cy5/*Red***	
SARS-CoV	IC	SARS-CoV-2 E-gene	SARS-CoV-2 N-gene	
Analyzed samples				
Cp/Ct/Cq is specified	Not considered	**Cp/Ct/Cq is specified**	**Cp/Ct/Cq is specified**	**RNA of SARS-CoV-2 is detected ***
Cp/Ct/Cq is specified	Not considered	Cp/Ct/Cq is **not** specified	Cp/Ct/Cq is **not** specified	**RNA of SARS-like Coronaviruses is detected,** RNA of SARS-CoV-2 is **not** detected
Cp/Ct/Cq is **not** specified	**Cp/Ct/Cq is specified**	Cp/Ct/Cq is **not** specified	Cp/Ct/Cq is **not** specified	RNA of SARS-like Coronaviruses is **not** detected and RNA of SARS-CoV-2 is **not** detected
Positive Control sample				
Cp/Ct/Cq is specified	Not considered	**Cp/Ct/Cq is specified**	**Cp/Ct/Cq is specified**	Positive result. The results are valid!
Negative Control sample				
Cp/Ct/Cq is **not** specified	**Cp/Ct/Cq is specified**	Cp/Ct/Cq is **not** specified	Cp/Ct/Cq is **not** specified	Negative result. The results are valid!

* The simultaneous presence of SARS-CoV-2 coronavirus and other coronaviruses like SARS-CoV in the RNA sample is possible

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Other possible results:

Detection Channel				Interpretation
FAM/*Green*	**HEX/*Yellow***	**ROX/*Orange***	**Cy5/*Red***	
Analyzed samples				
Cp/Cq ≤ 37 **Ct ≤ 35**	Not considered	**Cp/Ct/Cq is specified**	Cp/Ct/Cq is **not** specified	Additional research is required, there is a possible mutation in one of the SARS-CoV-2 genes
		Cp/Ct/Cq is **not** specified	**Cp/Ct/Cq is specified**	
Cp/Ct/Cq is **not** specified or **Cp/Cq ≥ 37** **Ct ≥ 35**	Not considered	**Cp/Ct/Cq is specified**	Not considered	Probably, there is low RNA load of the SARS-CoV-2. Repeat NA extraction or re-collect of a clinical sample, performed sequentially
		Not considered	**Cp/Ct/Cq is specified**	
Cp/Ct/Cq is **not** specified	Cp/Ct/Cq is **not** specified	Cp/Ct/Cq is **not** specified	Cp/Ct/Cq is **not** specified	Unreliable result. Repeat PCR amplification or NA extraction or re-collect of a clinical sample, performed sequentially

Unreliable results may be caused by the presence of inhibitors in the nucleic acid preparation obtained from the clinical material, errors in the pre-analytical stage, incorrect implementation of the analysis protocol, non-compliance with the temperature mode of amplification, etc. In this case, either re-staging of reverse transcription and polymerase chain reaction, or re-extracting of the nucleic acid preparation, or re-collect of clinical material (performed sequentially) is required.

If the expressed growing fluorescence (Cp/Ct/Cq is specified) through the FAM/*Green*, ROX/*Orange*, or Cy5/*Red* channels is expressed for Negative Control (NC), the results of whole series are considered false. It is required to eliminate contamination.

A single negative test result, especially if it is a sample from the upper respiratory tract, does not exclude infection. Lower respiratory tract sampling should be checked for SARS-CoV-2 coronavirus, especially in cases of severe and progressive disease.

Negative results do not eliminate the possibility of SARS-CoV-2 infection and should not be used as the only reason for taking a decision about patient treatment. Negative results should go together with clinical observations and epidemical information.

The controls should be also considered to exclude false positive and false negative results (see Paragraph 9 of the current manual). Please use the **cut-off Ct ≤ 40** (specified product) and **33 (PC)** for Rotor-Gene thermal cycler. The result characterized by Ct above this value should be considered doubtful and the whole assay should be repeated.

11. STORAGE AND TRANSPORTATION

- Expiry date – 12 months from the date of production.
- Both parts of the kits must not delivered and keep with dry ice
- All components of **RealLine SARS-CoV-2 Detection Kit PART 1** must be stored out of light at temperatures from (2 – 8) °C during the storage period. Excessive temperatures and light can be detrimental to product performance.
- The Enzyme Taq/RT, component in **RealLine SARS-CoV-2 Detection Kit PART 2** must be stored at temperatures from (- 18°C to – 22) °C during the storage period.
- The kit can be transported by all types of roofed transport at temperatures corresponding to the storage conditions of the kit components over the transportation. Transportation is allowed in thermal containers with icepacks by all types of covered transport at a temperatures up to 25 °C inside the container, but for no longer than 5 days.
- An expired **RealLine SARS-CoV-2 Detection Kit** should not be used
- It is strongly recommend to follow the given instructions in order to obtain accurate and reliable results.
- Do not use kits with damaged inner and outer packaging and get in contact with BIORON Diagnostics GmbH.

Shelf-life of the kit following the first opening of the primary container:

- Components of the kit should be stored at temperatures from (2 – 8) °C during the storage period;
- PCR-mix for amplification should be stored at temperatures from (2 – 8) °C and out of light during the storage period;
- Enzyme Taq/RT should be stored at temperatures from (- 18°C to – 22) °C during the storage period.

Please avoid freeze/thaw cycles of the Enzyme Taq/RT component.

The conformity of the **RealLine SARS-CoV-2 Detection Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

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12. TROUBLESHOOTING

	Possible cause	Solution	Result
PC	-	Operation error PCR inhibition Violation of storage and handling requirements	Repeat whole test Dispose current batch
NC	+	Contamination	Dispose current batch Perform decontamination procedures
IC	-	PCR inhibition RNA extraction violation	Repeat RNA extraction Repeat whole test Resample

If you face any undescribed issue, remarks, requests and comment, please contact: techsupport@bioron.de

Document Revision Information

Rev09	Changes were not released
Rev10	**New Revision is based on Rev08**
Chapter 1	Addition of a further validated Extraction Kit RealLine Prep-NA-S
Chapter 9.	Controls from former chapter 10 were included
Chapter 9.4 15.	Changed PCR times with the RealLine Cycler, highlighted in yellow

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SPACE FOR YOUR NOTES:

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