RealLine SARS-CoV-2 / Influenza A+B

Instructions for Use

RealLine SARS-CoV-2 / Influenza A+B

QUALITATIVE ASSAY KIT FOR THE DIFFERENTIAL DETECTION OF THE SARS-COV-2 RNA WITH TARGETS E-GENE AND N-GENE AND INFLUENZA A AND INFLUENZA B VIRUS 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 / Influenza A+B (A-Format)	**BI1029-96**	**96 Tests**
RealLine SARS-CoV-2 / Influenza A+B (B-Format)	**BI1030-96**	**96 Tests**
valid from:	**January 2021**	

BIORON Diagnostics GmbH
info@bioron.de - www.bioron.de

RealLine SARS-CoV-2 / Influenza A+B

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

BIORON Diagnostics GmbH

In den Rauhweiden 20
67354 Römerberg
Germany

Phone +49 6232 298 44 0
Fax +49 6232 298 44 29
info@bioron.de

RealLine SARS-CoV-2 / Influenza A+B

TABLE OF CONTENT:

Validated Cyclers	
BI1029-96 – A-format	**BI1030-96 – B-format**
RealLine Cycler and equivalent	RealLine Cycler and equivalent Rotorgene Cyler (Qiagen)

RealLine SARS-CoV-2 / Influenza A+B

QUALITATIVE ASSAY KIT FOR THE DIFFERENTIAL DETECTION OF THE SARS-COV-2 RNA AND INFLUENZA A AND INFLUENZA B VIRUS RNA BY REAL TIME PCR

1. INTENDED USE

The **RealLine SARS-CoV-2** / **Influenza A+B** Detection Kit is intended for research and diagnostic applications. The **RealLine SARS-CoV-2** / **Influenza A+B** Detection Kit is an in vitro Nucleic Acid Test (NAT) – pathogen-detection-based product.

The **RealLine SARS-CoV-2** / **Influenza A+B** Detection Kit is designed to detect SARS-CoV-2 coronavirus, Influenza A virus and Influenza B virus in human biological samples with by 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:

- Acute respiratory viral infection (ARVI) symptoms;
- Contact with COVID-19 or Influenza infected patients, regardless of their age;
- Stay in centers of infection (for the purpose of early detection of possible infection and prevention of further spread of infection).

The application of the kit does not depend on population and demographic aspects. There are no contradictions for the use of **RealLine SARS-CoV-2** / **Influenza A+B** Detection Kits.

The **RealLine SARS-CoV-2** / **Influenza A+B** Detection Kit can be used in clinical and diagnostic laboratories of medical institutions and research practice.

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.

The kit is intended for use with RealLine Cycler (BIORON Diagnostics GmbH), and equivalent cyclers.

For the use of Rotorgene Cyclers (Qiagen) the RealLine SARS-CoV-2 / Influenza A+B (B-format), REF BI1030-96 with tubes is approved and should be used.

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 40 µl**

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 CONTENT

The **RealLine SARS-CoV-2** / **Influenza A+B** Kit content is represented in Table 2.

Table 2: Content of the kits RealLine SARS-CoV-2 / Influenza A+B (A-format) REF BI1029-96 and RealLine SARS-CoV-2 / Influenza A+B (B-format) REF BI1030-96

Reagent	Description	Total volume	Amount
Paraffin-sealed PCR-Mix	Colourless transparent liquid under white wax layer	1440 µl (15 µl per tube)	**BI1030-96**: 96 tubes **or** **BI1029-96:** 12 x 8 strip-tubes with strip-caps
RT-PCR-buffer	Colourless transparent liquid	1620 µl (810 µl each)	2 tubes,
Enzyme Taq/RT	Colourless transparent viscous liquid	55 µl	1 tube
Internal Control RNA-IC "A"	Colourless transparent liquid	1 ml	1 tube
Positive Control	Colourless transparent liquid	130 µl	1 tube

The SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit is intended for single use and designed for 96 tests (94 defined samples, one positive control and one negative control). It is recommended to perform no more than 12 samples per run otherwise it could lead to the situation of lacking enzyme volume.

3. PRINCIPLE OF THE PROCEDURE

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, that increases the sensitivity of the method, reduces the likelihood of contamination and reduces the time of the study.

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 nonspecific annealing of primers to targets DNA in the initial heating of the tube.

The **RealLine SARS-CoV-2** / **Influenza A+B** 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.

RealLine SARS-CoV-2 / Influenza A+B

The **RealLine SARS-CoV-2** / **Influenza A+B** Detection Kit includes the Internal control **RNA-IC "A",** which is intended to assess the quality of the RNA extraction and polymerase chain reaction. DNA probe used for the detection of the Influenza A virus product amplification includes fluorescent dye FAM. DNA probe used for the detection of the SARS-CoV-2 (E, N - genes) product amplification includes fluorescent dye ROX. DNA probe used for the detection of the Influenza B virus 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.

Table 1: Detection channels of amplification products:

FAM	HEX	ROX	Cy5
Influenza A-Virus	Internal Control RNA-IC "A"	SARS-CoV-2, E-gene and N-gene	Influenza B-Virus

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 virus and the Influenza A and B virus 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 virus RNA, Influenza A virus RNA and Influenza B virus 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. Sensitivity:

Analytical sensitivity is 10 copies of RNA per amplification tube. Sensitivity is determined by the analysis of serial dilutions of the laboratory control sample (LCS).

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

Sensitivity of 10 copies per amplification tube corresponds to the following values of the RNA concentration in the sample in case of using NA extraction kits produced by BIORON Diagnostics GmbH:

Biomaterial	RealLine PREP NA Extraction kit REF BI1010 (elution volume 50 µl)
Nasopharynx and oropharynx swabs in 500 µl of transport medium	1000 copies / ml sample
Bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate	1000 copies / ml sample
Sputum	2000…5000 copies / ml sample, depending on the preprocessing method

5.2. Specificity:

The analytical **specificity** of the **RealLine SARS-CoV-2** / **Influenza A+B** 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, two genome sites were selected as targets to improve the reliability of diagnostics: the N and E genes sites.
In the samples of human biological material with target viruses RNA, the detecting amplifier should register an increase in fluorescence on the corresponding detection channels.

In the samples of human biological material free of target viruses 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.

RealLine SARS-CoV-2 / Influenza A+B

There are no non-specific positive results of amplification of RNA sample in the presence of:

RNA	DNA,
Human coronaviruses HKU-1	Mycoplasma pneumoniae
Human coronaviruses NL-63	Streptococcus pneumoniae
Human coronaviruses 229E	Chlamydophila pneumoniae
Human coronaviruses OC-43	Haemophilus influenzae
Human rhinovirus	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.

There are specific results of amplification of RNA sample in the presence of four strains of SARS-CoV- 2 (studied under the international system QCMD (Quality Control for Molecular Diagnostics), Great Britain), 11 strains of influenza B, 15 strains of different types of Influenza A

5.3. Diagnostic characteristics

The diagnostic sensitivity and specificity were established for each virus of the ARVI pathogen which RNA is detected using the RealLine SARS-CoV-2 / Influenza A+B Kit.
Number of samples: (n) – 263.

Diagnostic characteristics	Viruses caused ARVI which RNA is detected by tested kit		
	SARS-CoV-2 coronavirus	Influenza A virus	Influenza B virus
Diagnostic sensitivity (95 % CI)	100 % (96.55–100 %)	100 % (96.15–100 %)	100 % (95.94–100 %)
Diagnostic specificity (95 % CI)	100 % (97.69–100 %)	100 % (97.84–100 %)	100 % (97.90–100 %)

The claimed specifications are guaranteed when RNA extraction is performed with RealLine PREP-NA Extraction Kit REF BI1010

6. WARNING AND PRECAUTIONS

The SARS-CoV-2 coronavirus is classified as particularly pathogenic. Laboratories performing research on the detection of SARS-CoV-2 RNA are required to ensure the safety of work in accordance with the requirements of national legislation in the field of sanitary and epidemiological welfare.

Only trained personnel with medical or biological (veterinary) education who have been trained at licensed courses of primary specialization in working with pathogenic microorganisms and who have received additional special training at advanced training courses on molecular and biological methods of diagnostics are allowed to work with the kit of reagents.

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 and protective clothing (work clothes and personal protective equipment). 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/vapor/spray produced by the components of the kit. Do not eat/drink components of the kit. Avoid contact with 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

RealLine SARS-CoV-2 / Influenza A+B

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. 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 special closed container containing a disinfectant solution.

Do not open the tubes after amplification. Work surfaces, as well as rooms where PCR is performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work. 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 breached;
- After the expiry date provided at the box;

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

7. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

7.1 Specimen collection

- Specimen collection swabs: use only dacron, rayon, or calcium alginate tipped collection swabs with plastic or non-aluminium wire shafts;
- For bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, sputum: use sterile containers with a volume of up to 60 ml;
- Use transport media for clinical samples or sterile saline for the transportation of the sample.

7.2 RNA extraction and PCR

Specimen and control preparation

- Biological safety cabinet class II;
- Vortex mixer;
- Refrigerator;
- Nucleic acid extraction kit (RealLine Prep NA Kit is recommended);
- High speed centrifuge (RCF 16,000 x g) for 1.5 ml tubes;
- Solid-state thermostat (temperature range 40 - 95°C);
- 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);
- PCR tube rack for 1.5 ml tubes;
- 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 racks for 1.5 ml tubes;
- 1.5 ml microcentrifuge tubes with caps;
- PCR tube racks for 0.2 ml tubes or strips;
- Rotor for strips (if detection kit is used: REF BI1029-96);
- 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;
- Container for used pipette tips;

Post-Amplification – Amplification detection area

- Real-Time PCR Cycler: RealLine Cycler or Qiagen Rotor-Gene Q

RealLine SARS-CoV-2 / Influenza A+B

8. SAMPLES

The **RealLine SARS-CoV-2** / **Influenza A+B Kit** is designed to detect extracted RNA from 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: haemoglobin – 0.35 mg/ml RNA sample, isopropyl alcohol – 100 µl/ml RNA sample, methyl acetate – 100 µl/ml RNA 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.

Each sample of biomaterial should be placed in a separate transport container providing requirements in accordance with the table of guidance. (s. Table 3)

Table 3: Transportation and storage of the samples

Type of the sample	Collecting material requirements	Transportation	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
Bronchoalveolar lavage	Sterile container	4 °C	≤48 hours: 4 °C >48 hours *: -70 °C	A small sample dilution is possible
Endotracheal aspirate, nasopharyngeal 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**.
** To transport samples, use a transport medium for storing and transporting respiratory swabs or saline solution (if transportation to the laboratory is no more than 24 hours after taking the sample) or a dry swab probe (if transportation to the laboratory is no more than 4 hours after taking the sample).

It is recommended to use transport media containing preservatives, intended for further study of samples by PCR.

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.

RealLine SARS-CoV-2 / Influenza A+B

9. PROCEDURE

The range of Influenza and SARS-CoV-2 viral load can vary widely. In this regard, when performing research in a clinical laboratory, the risk of cross-contamination between samples at quality management system 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 lowcopy 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, RealLine PREP NA Extraction kits are used

RNA extraction is carried out according to the IFU of 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 - minus 22) °C** for no longer than a week with a single defrost before reverse transcription.

The features of biomaterial preparation

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

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

The use of control samples at the stage of nucleic acid extraction:

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 "A" from the **RealLine SARS-CoV-2** / **Influenza A+B** detection kit must be used as an internal control sample.

The RNA-IC "A' should be used in the amount of 10 µl per sample.

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 must 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 included in the corresponding extraction kit.

9.2 PCR Amplification with Reverse Transcription (RT-PCR)

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

When using kits with A-format (REF BI1029-96) with strips, strictly observe the completeness of the strips and caps. Do not use the caps with the strips of another kit!

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

Example: if you need 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**.

2. Vortex the **RT-PCR-buffer** and **Enzyme Taq/RT** thoroughly for 3-5 s, then centrifuge briefly for 1-3 s.

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

RealLine SARS-CoV-2 / Influenza A+B

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

15.0 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.

4. Vortex the tube with the mixture Enzyme Taq/RT and RT/-PCR-buffer thoroughly, then centrifuge briefly for 1-3 s.

Mixture of RT-PCR-buffer and Enzyme Taq/RT must be prepared immediately prior to use, and should be used within one hour after preparation. If it is needed, the prepared mixture can be stored at the temperatures from (2 – 8) °C, but not longer than one hour.

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

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

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.

7. Add **10 µl** of **RNA sample** into corresponding RT-PCR tube. Close the tubes tightly.

Add **10 µl** of **NC** which passed the whole NA extraction procedures into corresponding tube. Add **10 µl** of **PC** into **PC** tube. Close the tubes tightly. Avoid paraffin layer break.

Reagent	Patient Sample(s)	PC	NC
Extracted RNA	10 µl	-	-
NC	-	-	10 µl
PC	-	10 µl	-

8. Spin the tubes briefly for 3-5 s to collect the drops.

9. Place the tubes into the Thermal Cycler

RealLine SARS-CoV-2 / Influenza A+B

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

10. Table 4: Program the **RealLine Cycler**:

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	5 s	5 *	Cycle
	64 °C	10 s		
4:	94 °C	5 s	45 *	Cycle
	64 °C	10 s		
5	80 °C	1 s	1	Cycle
6	10 °C	---		Hold
* measurement of fluorescence				

Table 5: PCR Program 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	10	50 times*
	60 °C*	15 *	
* measurement of fluorescence			

11. Choose channels:

FAM / *Green*	HEX / *Yellow*	ROX / *Orange*	Cy5 / *Red*
Influenza A-Virus	Internal Control RNA-IC "A"	SARS-CoV-2, E-gene and N-gene	Influenza B-Virus

12. PCR Volume: **40 µl**

13. Match the positions of test tubes with samples, positive and negative controls according to the instruction manual for the real time PCR system in use.

14. Run the program.

RealLine SARS-CoV-2 / Influenza A+B

10. CONTROLS

The **RealLine SARS-CoV-2** / **Influenza A+B Kit** contains **Positive Control** sample **PC**. Positive control is a cloned part of the virus genome. It is produced with genetic engineering techniques and characterized by automatic sequencing.

The kit includes the **Internal control RNA-IC "A".** This control is intended to assess the quality of the RNA extraction and polymerase chain reaction. To reveal possible contamination a negative control is required.

A **Negative Control NC** sample should go through all stages of RNA extraction. Physiological saline solution can be used as a negative control sample in volumes indicated in supplied instructions.

The test result is considered valid if:

- the exponential growth of the fluorescence level for the specific product is present, in this case the internal control is not taken into account;
- the exponential growth of the fluorescence level for the specific product is absence and for internal control is present.

The test result is considered **invalid** when the exponential growth of the fluorescence level for the specific product and for internal control are not observed.

If **Positive Control (PC)** does **not** express growing fluorescence of the specific product or positive result, it is required to repeat the whole test. It may be caused by inhibitors, operation error or violation of storage and handling.

If **Negative control (NC)** expresses growing fluorescence of the specific product or positive result, all tests of the current batch are considered false. Decontamination is required.

11. DATA ANALYSIS

In case of using Real-Line 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.

Table 6: The interpretation of assay results for control samples

Detection Channel				Interpretation
FAM/*Green*	**HEX/*Yellow***	**ROX/*Orange***	**Cy5/*Red***	
Influenza A	RNA IC	SARS-CoV-2	Influenza B	
Positive Control sample				
+	**-**	**+**	**+**	**Positive result** **The results are valid**
Negative Control sample				
-	**+**	**-**	**-**	**Negative result** **The results are valid**

with " + ": Cp/Ct is specified **" – " Cp/Ct is not specified**

In the samples of human biological material with target viruses RNA, the detecting amplifier should register an increase in fluorescence on the corresponding detection channels (FAM/*Green*, ROX/*Orange*, Cy5/*Red*), see Tables 7, 8.
In the samples of human biological material **free** of target viruses RNA, the detecting amplifier should register an increase in fluorescence in the HEX/Yellow (Internal control sample) detection channel, the increase of fluorescence in the FAM/*Green*, ROX/*Orange*, and Cy5/*Red* channels must be absent.

The results are considered as unreliable (Invalid) if there is **no** exponential increase of fluorescence in the FAM/*Green*, ROX/*Orange*, and Cy5/*Red* channels (specific product) and in HEX/*Yellow* channel (Internal control sample).

Table 7: The interpretation of assay results for PCR

Detection Channel				Interpretation
FAM/*Green*	**HEX/*Yellow***	**ROX/*Orange***	**Cy5/*Red***	
Influenza A	RNA IC	SARS-CoV-2	Influenza B	
+	Not considered	**-**	**-**	**RNA of Influenza A virus is detected**
-	Not considered	**+**	**-**	**RNA of SARS-CoV-2 virus is detected**
-	Not considered	**-**	**+**	**RNA of Influenza B virus is detected**
-	**+**	**-**	**-**	Target viruses are not detected
-	**-**	**-**	**-**	**Unreliable result!** **Repeat NA extraction or PCR amplification or re-collect the clinical sample, performed sequentially**

with " + ": Cp is specified **" – " Cp is not specified**

RealLine SARS-CoV-2 / Influenza A+B

Table 8: The interpretation of assay results for PCR

Detection Channel				Interpretation
FAM/*Green*	**HEX/*Yellow***	**ROX/*Orange***	**Cy5/*Red***	
Influenza A	RNA IC	SARS-CoV-2	Influenza B	
+	Not considered	**+**	**-**	**RNA of Influenza A virus and SARS-CoV-2 virus is detected**
-	Not considered	**+**	**+**	**RNA of Influenza B virus and SARS-CoV-2 virus is detected**
+	Not considered	**-**	**+**	**RNA of Influenza A virus and Influenza B virus is detected**
+	Not considered	**+**	**+**	**RNA of SARS-CoV-2 virus , Influenza A virus and Influenza B virus is detected**

with " + ": Cp is specified " – " Cp is not specified

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 there is for the Positive Control (PC) no fluorescence signal measured in channels FAM/*Green*, ROX/*Orange*, or Cy5/*Red* - and **Cp/Ct cannot be specified -**, the results of the whole series are considered false. It is required to repeat the whole test.

If there is a fluorescence signal measured for the Negative Control (NC) in channels FAM/*Green*, ROX/*Orange*, or Cy5/*Red* - and **Cp/Ct can specified** -, the results of whole series are considered false. It is required to repeat the whole test and eliminate the contamination first.

⚠ A single negative test result, especially if it is a sample from the upper respiratory tract, does not exclude infection.

⚠ Negative results should not be used as the sole basis for making a decision about the treatment of patients.

RealLine SARS-CoV-2 / Influenza A+B

If in the samples of human biological material the detecting amplifier registers an increase in fluorescence for the specific product earlier than 25 cycle for Cp (Cp less than 25 or Ct less than 22), this indicates a high initial RNA concentration of the corresponding pathogen. In this case, it is possible to obtain a false negative result during mixed infection for a pathogen whose RNA is present in a low concentration.

To exclude false negative results, it is recommended to repeat RT-PCR for the extracted RNA preparation using the kit for individual detection of the corresponding virus. The issue of mixed infection has not been sufficiently studied, based on a small amount of published literature data, coinfection with Influenza (A or B) and SARS-CoV-2 may occur in 0.5 – 5 % of cases, increasing with age of patients.

The controls should be also considered to exclude false positive and false negative results (see Paragraph 11 of the current manual).

The cut-off Ct values for Rotor-Gene thermal cycler are 40 (specific product) and 33 (PC). The result characterized by Ct above this value should be considered doubtful and the whole assay should be repeated.
These cut-offs can be recommended for other cyclers, but depends on your complete analytical setup

RealLine SARS-CoV-2 / Influenza A+B

12. TRANSPORT AND STORAGE CONDITIONS

Expiry date - 12 months from the date of production.

- All components of **RealLine SARS-CoV-2** / **Influenza A+B** Kit, except the Enzyme Taq/RT, must be stored at temperatures from (2 – 8) °C during the storage period.
- The PCR-mix for amplification must be stored out of light at temperatures from (2 – 8) °C during the storage period.
- Excessive temperature and light can be detrimental to product performance. The Enzyme Taq/RT must be stored at temperatures from **(minus 18 – minus 22) °C** during the storage period.

The kit can be transported in thermal containers with icepacks by all types of roofed transport at temperatures corresponding to the storage conditions of the kit components over the transportation time. Transportation is allowed in thermal containers with icepacks by all types of covered transport at temperatures from (2 – 25) °C inside the container, but for no longer than 5 days.

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

- components of the kit, except the Enzyme Taq/RT, 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 **(minus 18 – minus 22) °C** during the storage period.

An expired **RealLine SARS-CoV-2** / **Influenza A+B** Kit, should not be used.
We strongly recommend to follow the given instructions in order to obtain accurate and reliable results.

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

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	invalid	PCR inhibition RNA extraction violation	Repeat RNA extraction Repeat whole test Resample

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

RealLine SARS-CoV-2 / Influenza A+B

BIORON Diagnostics GmbH
info@bioron.de - www.bioron.de