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For professional use only

SARS-CoV-2/RSV/Influenza AB virus

Multiplex REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

EC REP

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1. INTENDED USE

The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** is intended for research and diagnostic applications. The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – pathogen-detection-based product. The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** is designed to detect Coronavirus SARS-CoV-2, Human respiratory syncytial virus (RSV), Influenza A virus and Influenza B virus in human biological samples with an aid of Polymerase Chain Reaction (PCR) method. Samples are human biological materials: nasopharyngeal swabs, oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, phlegm.

Indications for use:

- AVRI symptoms;
- contact with COVID-19 or Influenza infected, regardless of their age;
- staying in the 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 use of the **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit.**

The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR 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 instruction for use.

2. 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, 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 or the use of Taq-polymerase blocked by antibodies. The polymerase chain reaction starts only when paraffin is melted or thermal dissociation of a complex of Taq polymerase and antibodies is happened. It excludes non-specific annealing of primers to targets DNA in the initial heating of the tube.

The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR 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 **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR 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 Human respiratory syncytial virus (RSV) 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 A virus and 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	Cy5.5
RSV	IC*	SARS-CoV-2 coronavirus, E, N - genes	Influenza A virus, Influenza B virus	-

* - Internal control (RNA-IC "A")

The automatic analysis is available on "DNA-Technology" made instruments: DTlite or DTprime REAL-TIME Thermal Cyclers for **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** (see the catalogue at https://www.dna-technology.com to see available supply options). The current version of the software is available for download at https://www.dna-technology.com/software.

3. CONTENT

The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** content is represented in Tables 2-4.

Table 2. The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** content, package S (standard) for R3-P448-S3/9EU and R3-P448-23/9EU

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless transparent liquid under waxy white fraction	1440 µL (15 µL in each tube)	96 tubes or 12 8-tube strips
Enzyme Taq/RT	Colorless transparent viscous liquid	55 µL	1 tube
RT-PCR-buffer	Colorless transparent liquid	1.62 mL	1 tube
Internal control RNA-IC "A"	Colorless transparent liquid	1.0 mL	1 tube
Positive control*	Colorless transparent liquid	130 µL	1 tube
Strip's caps [1]	12 8-caps		

* - marking as C+ is allowed

All components are ready to use and do not require additional preparation for operation.

The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** is intended for single use and designed for 96 tests (it is not recommended to carry out more than 12 performances), including the analysis of the unknown samples, positive control and negative control.

[1] - for detection kit packaged in strips REF R3-P448-S3/9EU

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

4.1. Specimen collection

- Sterile single use swabs, single use sterile containers to collect clinical material;
- Sterile tubes containing transport medium: “DNA-Technology” made **STOR-F** (REF P-901-1/1EU, P-901-N/1EU, P-901-R/1EU) or **STOR-M** (REF P-910-1/1EU) or equivalent for the transportation of the sample.

4.2. RNA extraction and PCR

Preamplification-specimen and control preparation area:

- Biological safety cabinet class II;
- Refrigerator;
- Vortex mixer;
- High speed centrifuge (RCF(g) at least 12000) for 1.5 mL tubes;
- Solid-state thermostat (temperature range 24-65°C) or solid-state thermostat with timer (“DNA-Technology” made TT-2 “Thermit” thermostat or equivalent are recommended);
- Tube rack for 1.5 mL tubes;
- 1.5 mL tubes;
- Electric laboratory aspirator with trap flask for the removal of supernatant;
- RNase and DNase free pipette tips for aspirator with trap flask;
- Single channel pipettes (dispensers covering 0.2-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1000 µL);
- Nucleic acid extraction kit (“DNA-Technology” made **PREP-NA** (REF P-002/1EU), **PREP-NA-S** (REF P-007-N/1EU), **PREP-MB-NA-S** REF P-117-A/9EU and **PREP-MB DWP** REF P-119-A/9EU, DNA/RNA extraction kits are recommended);
- Physiological saline solution 0.9% NaCl (Sterile) (if needed);
- Container for used pipette tips and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Refrigerator;
- Vortex mixer;
- Vortex rotor for strips (in case of using package in strips);
- Tube rack for 1.5 mL tubes;
- PCR tube rack for 0.2 mL tubes or strips;
- Single channel pipettes (dispensers covering 2.0-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1000 µL);
- Container for used pipette tips, tubes and other consumables;

- Powder-free surgical gloves;
- Disinfectant solution.

Post-Amplification – Amplification detection area:

- Real-time PCR thermal cycler.

Software:

The most recent version of the DT thermal cyclers software can be downloaded from https://www.dna-technology.com/software.

The OS supported: all versions of Windows starting from 7.

5. STORAGE AND HANDLING REQUIREMENTS

Expiry date – 12 months from the date of production.

All components of **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit**, except the Enzyme Taq/RT, must be stored at temperatures from 2 °C to 8 °C during the storage period. The PCR-mix for amplification must be stored out of light at temperatures from 2 °C to 8 °C during the storage period. The Enzyme Taq/RT must be stored at temperatures from minus 18 °C to minus 22 °C during the storage period.

The excessive temperature and light can be detrimental to product performance.

The kit must be transported in thermoboxes with ice packs by all types of roofed transport at temperatures corresponding to storage conditions of the kit components.

Transportation of the kit, except the Enzyme Taq/RT, is allowed in thermoboxes with ice packs by all types of roofed transport at temperatures from 2 °C to 25 °C but for no more than 5 days and should be stored at temperatures from 2 °C to 8 °C immediately on receipt.

It is allowed to transport the Enzyme Taq/RT in thermoboxes with ice packs by all types of roofed transport at temperatures up to 25 °C but for no more than 5 days and should be stored at temperatures from minus 18 °C to minus 22 °C immediately on receipt.

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

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

The kit stored in under undue regime should not be used.

An expired **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection 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 **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

Contact our official representative in EU by quality issues of the **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit**.

6. WARNINGS AND PRECAUTIONS

ATTENTION! The SARS-CoV-2 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 specially 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. Wear powder-free surgical gloves. Wear protective clothing (work clothes and personal protective equipment) working with microorganisms classified as particularly pathogenic. The protective clothing and personal protective equipment must comply with the work to be performed and health and safety requirements. Avoid producing spills or aerosol. Any material being exposed to biological samples must be treated for at least 30 minutes with disinfecting solution or autoclaved for 1 hour at 121 °C before disposal.

Molecular biology procedures, such as nucleic acids extraction, reverse transcription, PCR-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 can not 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 pipettes, 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. 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. Remove waste materials (tubes, tips) only in a special closed container containing a disinfectant solution. Work surfaces, as well as rooms where NA extraction and PCR are performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work.

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 PCR-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:

- When the transportation and storage conditions are breached;
- When the reagents' appearance does not respond to the kit passport;
- When the kit components packaging is breached;
- 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. SAMPLES

The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** is designed to detect RNA extracted from the nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, phlegm, depending on professional prescription.

Interfering substances

The presence of PCR inhibitors in a sample may cause controversial (unreliable) results. The sign of PCR inhibition is the simultaneous absence of internal control and specific product of amplification.

PCR inhibitors are the presence of hemoglobin 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 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: hemoglobin – 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

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

Sample taking is made with special sterile single-use tools – probes, cytobrushes, and swabs depending on the source of biological material according to established procedure.

Nasopharyngeal swabs

Take the swab with a dry sterile disposable probe into 1.5 mL plastic tubes with transport medium.

Order of taking:

1. Insert the probe carefully along the outer wall of the nose to a depth of 2-3 cm to the lower shell. Then lower the probe down slightly, insert into the lower nasal passage under the lower nasal conch, after a rotational movement remove along the outer wall of the nose.
2. Open the tube.
3. Put the probe into the tube with transport medium, rotate the probe for 10-15 seconds and rinse it thoroughly. Avoid spraying of solution.
4. Remove the probe from the solution and, by rotating it against the wall of the test tube above the level of the solution, squeeze out the excess liquid. Dispose the used probe.
5. Close the tube tightly and mark it.

Oropharyngeal swabs

Take the swab with a dry sterile disposable probe into 1.5 mL plastic tubes with transport medium.

Order of taking:

1. Take the swab with a probe with a rotational movement from the surface of the tonsils, palatine arches and the back wall of the pharynx.
2. Open the tube.
3. Put the probe into the tube with transport medium, rotate the probe for 10-15 seconds and rinse it thoroughly. Avoid spraying of solution.
4. Remove the probe from the solution and, by rotating it against the wall of the test tube above the level of the solution, squeeze out the excess liquid. Dispose the used probe.
5. Close the tube tightly and mark it.

Bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, phlegm

Samples are collected in sterile plastic containers. Transfer 1.0 mL of the sample to a 1.5 mL plastic test tubes, close the test tube and mark.

NOTE:

Phlegm must be pretreated. The procedure for phlegm pretreatment with a solution of trisubstituted sodium phosphate (Na_3PO_4) or mucolysin is given in the instructions to the reagent kit (**PREP-NA DNA/RNA Extraction Kit**, “DNA-Technology”, LLC).

ATTENTION! For RNA extraction, 100 µL of the sample is used. Do not perform centrifugation as a pretreatment of nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate.

Transportation and storage of the samples in accordance with guidance

Type of the sample	Collecting material requirements	Transportation	Storage conditions before testing	Comments
Nasopharyngeal and oropharyngeal swabs	Plastic test tubes and tampons for swabs **	4 °C	≤5 days: 4 °C >5 days *: minus 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 *: minus 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 *: minus 70 °C	
Phlegm	Sterile container	4 °C	≤48 hours: 4 °C >48 hours *: minus 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, for example, **STOR-F** or **STOR-M** produced by “DNA-Technology” or similar registered in the established order, intended for further study of samples by PCR.

ATTENTION! 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.

8. PROCEDURE

ATTENTION! The range of causative agents of AVRI 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 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 or a special opener (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).

8.1. RNA extraction

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

Table 3. The reagent kits validated for RNA extraction and further study with the **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit**

Reagent kit	Biomaterial
PREP-NA	nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, phlegm (nasopharyngeal and oropharyngeal swabs if shortened method in accordance with Annex A is used)
PREP-NA-S	nasopharyngeal and oropharyngeal swabs
PREP-MB-NA-S	nasopharyngeal and oropharyngeal swabs
PREP-MB DWP	nasopharyngeal and oropharyngeal swabs

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

ATTENTION! The volume of the resulting RNA preparation should not exceed 50 µL.

If the **PREP-MB-NA-S** and **PREP-MB DWP** kits are used for RNA isolation, the volume can be increased up to 100 µL.

ATTENTION! The resulting RNA preparation must be used for RT-PCR within 2 hours. 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.

8.2. The features of biomaterial preparation

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

ATTENTION! For RNA extraction, 100 µL of the sample is used.

8.3. 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 **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** should be used as an internal control sample.

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

ATTENTION! The internal control (RNA-IC) and internal control (DNA-IC) from the **PREP-NA DNA/RNA Extraction Kit** are not used.

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.

ATTENTION! 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 included in the corresponding extraction kit.

8.4. PCR with Reverse Transcription (RT-PCR) for package S

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

ATTENTION! When using package S (R3-P448-S3/9EU), strips, strictly observe the completeness of the strips and caps to them. Do not use the caps for the strips from the other kits!

8.4.1. Mark the required number of the tubes or strips with paraffin sealed PCR-mix according to the number of samples to be analyzed, 1 tube for negative control (C-) and 1 tube for positive control (C+).

Example: to test 6 samples, mark 6 tubes (one for each sample), one for "C-" and one for "C+". The resulting number of tubes is 8.

8.4.2. Vortex the RT-PCR-buffer and Enzyme Taq/RT thoroughly for 3-5 seconds, then spin for 1-3 seconds.

ATTENTION! Enzyme Taq/RT should be got out from the freezer immediately prior to use.

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

- 15 x (N+1) µL of RT-PCR-buffer;
- 0.5 x (N+1) µL of Enzyme Taq/RT,

N is a quantity of the samples to be tested taking to account "C-", "C+".

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.

ATTENTION! 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.

8.4.4. Vortex the tube with the mixture of RT-PCR-buffer and Enzyme Taq/RT thoroughly for 3-5 seconds, then spin for 1-3 seconds.

ATTENTION! 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 °C to 8 °C but for no longer than one hour.

8.4.5. Add 15 µL of the RT-PCR-buffer and Enzyme Taq/RT mixture into each tube. Avoid paraffin layer break. Close the tubes/strips.

8.4.6. Vortex the tubes with samples, "C-" and "C+" for 3-5 seconds and spin down drops for 1-3 seconds.

ATTENTION! In case of using **PREP-MB NA-S and PREP-MB DWP** extraction kits, after vortexing put the tubes with the RNA preparation in magnetic rack. If, after isolation, the supernatant containing the isolated RNA was transferred to new tubes, centrifugation is carried out for 3-5 seconds in a vortex mixer.

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. Close the tubes/strips tightly. Use filter tips.

8.4.7. Add 10 µL of the RNA sample into corresponding tubes. Do not add RNA into the "C-", "C+" tubes. Avoid paraffin layer break.

8.4.8. Add 10 µL of negative control sample (C-), which passed whole RNA extraction procedures into corresponding tube. Add 10 µL of positive control sample (C+) into corresponding tube. Avoid paraffin layer break.

8.4.9. Spin down the tubes/strips for 3-5 seconds to collect drops.

8.4.10. Set the tubes/strips into the Real-time Thermal Cycler.

8.4.11. Launch the operating software for DT instrument[2]. Add corresponding test[3], specify the number and ID's of the samples, positive and negative control samples. Specify the position of the tubes/strips in the thermal unit (see 8.4.10) and run PCR. See Table 4.

Table 4. The PCR program for DTlite and DTprime Thermal Cyclers

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	35	20	0	1		Cycle
2	95	5	0	1		Cycle
3	94	0	10	5		Cycle
	64	0	10		v	
4	94	0	5	45		Cycle
	64	0	10		v	
5	80	0	1	1		Cycle
6	10	…	…	Holding		Holding
√ - optical measurement						

[2] Please, apply to Operation Manual for DTprime and DTlite Real-Time PCR instruments PART II.
[3] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

9. CONTROLS

The **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** contains positive control sample. 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 an internal control RNA-IC "A". RNA-IC "A" is intended to assess the quality of the RNA extraction and polymerase chain reaction. To reveal possible contamination a negative control is required.

ATTENTION! A negative control sample should go through all stages of RNA extraction. Physiological saline solution or negative control sample from an extraction kit can be used as a negative control sample in volumes as indicated.

For **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** the test result is considered valid when:

- the exponential growth of the fluorescence level for the specific product is present, in this case the internal control is not considered;
- the exponential growth of the fluorescence level for the specific product is absent and for internal control is present.

For **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit** the test result is considered invalid when the exponential growth of the fluorescence level for the specific product and for internal control is not observed.

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

10. DATA ANALYSIS

In case of using DNA-Technology made Real-Time PCR Thermal 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. The interpretation should be performed in accordance with Table 5.

Table 5. The interpretation of assay results for control samples

Detection channel				**Interpretation**
Fam	Hex	Rox	Cy5	
Positive control sample				
Cp is specified	Cp is not specified	**Cp is specified**	**Cp is specified**	Positive result The results are valid
Negative control sample				
Cp is not specified	**Cp is specified**	Cp is not specified	Cp is not specified	Negative result The results are valid

In the samples of human biological material with target viruses' RNA, the Real-time PCR thermal cycler should register an increase in fluorescence on the corresponding detection channels (Fam, Rox or Cy5), see Tables 6,7.

In the samples of human biological material free of target viruses' RNA, the Real-time PCR thermal cycler should register an increase in fluorescence on the Hex (Internal control sample) detection channel, the increase in fluorescence on the Fam, Rox, and Cy5 channels should be absent.

The results are considered as unreliable (Invalid) if there is no exponential increase in fluorescence on the Fam, Rox and Cy5 channels (specific product) and on the Hex channel (Internal control sample).

Table 6. The interpretation of assay results for PCR

Detection channel				**Interpretation**
Fam	Hex	Rox	Cy5	
Analyzed samples				
Cp is specified	Is not considered	Cp is not specified	Cp is not specified	**Human respiratory syncytial virus (RSV) RNA is detected**
Cp is not specified	Is not considered	**Cp is specified**	Cp is not specified	**SARS-CoV-2 RNA is detected**
Cp is not specified	Is not considered	Cp is not specified	**Cp is specified**	**Influenza A virus / Influenza B virus RNA is detected**
Cp is not specified	**Cp is specified**	Cp is not specified	Cp is not specified	Target viruses' RNA is not detected
Cp is not specified	Cp is not specified	Cp is not specified	Cp is not specified	Unreliable result. Repeat PCR amplification or NA extraction or re-collect of a clinical sample, performed sequentially

Table 7. Other possible results

Detection channel				**Interpretation**
Fam	Hex	Rox	Cy5	
Analyzed samples				
Cp is specified	Is not considered	**Cp is specified**	Cp is not specified	**Human respiratory syncytial virus (RSV) RNA is detected, SARS-CoV-2 RNA is detected**
Cp is not specified	Is not considered	**Cp is specified**	**Cp is specified**	**SARS-CoV-2 RNA is detected, Influenza A virus / Influenza B virus RNA is detected**
Cp is specified	Is not considered	Cp is not specified	**Cp is specified**	**Human respiratory syncytial virus (RSV) RNA is detected, Influenza A virus / Influenza B virus RNA is detected**
Cp is specified	Is not considered	**Cp is specified**	**Cp is specified**	**Human respiratory syncytial virus (RSV) RNA is detected, SARS-CoV-2 RNA is detected, Influenza A virus / Influenza B virus RNA is detected**

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 repeated run of reverse transcription and polymerase chain reaction, or re-extraction of the nucleic acid preparation, or re-collection of clinical material (performed sequentially) is required.

When the expressed growing fluorescence (Cp is not specified) through the Fam, Rox or Cy5 channels is not expressed for positive control (C+), the results of whole series are considered false. It is required to repeat the whole test.

When the expressed growing fluorescence (Cp is specified) through the Fam, Rox or Cy5 channels is expressed for negative control (C-), the results of whole series are considered false. It is required to eliminate contamination.

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

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

If in the samples of human biological material the Real-time PCR thermal cycler registers an increase in fluorescence for the specific product earlier than 25 cycle for Cp, 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.

11. SPECIFICATIONS

a. The analytical specificity of the **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR 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, 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 detection channel, the increase in fluorescence on the Fam, Rox and Cy5 channels should be absent.

There are not cross-nonspecific reactions of each of the oligonucleotide systems included in the kit in relation to viruses determined by other systems.

There are not non-specific positive results of amplification of RNA sample in the presence of Human coronaviruses 229E, HKU-1, NL-63, OC-43, Human metapneumovirus, Human parainfluenza viruses type 1-4, Human rhinovirus, MERS-CoV, DNA of Human adenovirus, Human bocavirus, *Bordetella pertussis, Bordetella parapertussis*, *Chlamydophila pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Legionella pneumophila, Moraxella catarrhalis, Mycoplasma pneumoniae, Staphylococcus aureus (methicillin-resistant), Streptococcus pneumoniae,* , as well as human DNA in concentrations up to 1.0×10^8 copies/mL of the sample.

b. 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 DNA Technology:

Biomaterial	**PREP-NA DNA/RNA Extraction Kit** (**at elution in 50 µL**)	**PREP-NA-S DNA/RNA Extraction Kit** (**at elution in 50 µL**)	**PREP-MB-NA-S; PREP-MB DWP** (obtained preparation volume 100 µL)
Nasopharyngeal and oropharyngeal swabs in 500 µL of transport medium	1000 copies /mL sample	1000 copies /mL sample	2000 copies /mL sample
Bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate	1000 copies /mL sample	not used	not used
Phlegm (pretreated with Na_3PO_4)	2000 copies /mL sample	not used	not used
Phlegm (pretreated with mucolysin)	5000 copies /mL sample	not used	not used

c. Diagnostic characteristics

Biomaterial type	Virus	Diagnostic sensitivity	Diagnostic specificity
Nasopharyngeal and oropharyngeal swabs	SARS-CoV-2	100% (91.59-100)	100% (96.19-100)
	RSV	100% (87.66-100)	100% (96.67-100)
	Influenza A virus / Influenza B virus	100% (89.72-100)	100% (96.48-100)
Bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate	SARS-CoV-2	100% (90.00-100)	100% (94.56-100)
	RSV	100% (88.78-100)	100% (94.87-100)
	Influenza A virus / Influenza B virus	100% (91.40-100)	100% (94.04-100)
Phlegm	SARS-CoV-2	100% (86.28-100)	100% (94.22-100)
	RSV	100% (87.66-100)	100% (93.94-100)
	Influenza A virus / Influenza B virus	100% (88.06-100)	100% (93.84-100)
All biomaterial	**SARS-CoV-2**	**100% (96.45-100)**	**100% (98.36-100)**
	RSV	**100% (95.85-100)**	**100% (98.46-100)**
	Influenza A virus / Influenza B virus	**100% (96.52-100)**	**100% (98.34-100)**

12. TROUBLESHOOTING

Table 8. Troubleshooting

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

If you face to any undescribed issues contact our customer service department regarding quality issues with the kit:

Phone: +7(495)640.16.93,

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

13. QUALITY CONTROL

“DNA-Technology Research&Production”, LLC declares that the abovementioned products meet the provision of the Council Directive 98/79/EC for *in vitro* Diagnostic Medical Devices. The quality control procedures performed in accordance with ISO 9001:2015 and ISO 13485:2016:

- observation of quality management in manufacturing of IVDD products;
- creation of values for customers;
- maintenance of the best service quality and customer management.

Contact our official representative in EU by quality issues of the **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit**.

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14. KEY TO SYMBOLS

IVD	*In vitro* diagnostic medical device		Date of manufacture
	Temperature limit		Consult instructions for use
	Contains sufficient for <n> tests	REF	Catalogue number
	Use-by date		Manufacturer
LOT	Batch code		Keep away from sunlight
VER	Version	CONTROL +	Positive control
EC REP	Authorized representative in the European Community		Caution

REF R3-P448-S3/9EU
R3-P448-23/9EU

VER 827-2.2023.04.07

Annex A

Shortened method for the RNA extraction from the tested material (nasopharyngeal and oropharyngeal swabs) using PREP-NA DNA/RNA Extraction Kit

ATTENTION! The lysis buffer can form the precipitate. Dissolve it at 65 °C for 10 minutes prior to use.

- Mark the required number of 1.5 mL tubes according to the number of samples to be analyzed and negative control (C-).
- Vortex the tube with RNA-IC "A" (from **SARS-CoV-2/RSV/Influenza AB virus Multiplex REAL-TIME PCR Detection Kit),** spin the tube for 3-5 seconds. Add 10 µL of the RNA-IC "A" into each tube.
- Add 300 µL of the lysis buffer into each tube avoiding contact of the pipette tip with an edge of the tube.
- Add 100 µL of the sample into corresponding tubes. Add 100 µL negative control into the tube marked "C-".
- Close the tubes tightly. Vortex the tubes for 3-5 seconds.
- Incubate the tubes for 5 minutes at 65 °C.
- Spin the tubes for 3-5 seconds.
- Add 400 µL of the precipitation buffer. Close the tubes tightly and vortex for 3–5 seconds.
- Centrifuge the tubes at RCF(g) 12000 - 16000 for 10 minutes at room temperature (18-25 °C).
- Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use new tip for each sample.
- Add 500 µL of the washout solution №1 to the precipitate, close tubes and mix by inverting the tube 3-5 times.
- Centrifuge the tubes at RCF(g) 12000 - 16000 for 1 minute at room temperature (18-25 °C).
- Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use new tip for each sample.
- Add 300 µL of the washout solution №2 to the precipitate, close tubes and mix by inverting the tube 3-5 times.
- Centrifuge the tubes at RCF(g) 12000 - 16000 for 1 minute at room temperature (18-25 °C).
- Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use new tip for each sample. It is allowed to leave the precipitate covering with liquid in the volume of no more than 20-30 µL.
- Open the tubes and dry the precipitate at 65 °C for 5 minutes.
- Add 50 µL of the dilution buffer to the precipitate. Vortex the tubes for 3-5 seconds and spin down the drops by centrifuging for 3-5 seconds.

ATTENTION! Dilution buffer differs for **PREP-NA** and **PREP-NA PLUS** DNA/RNA extraction kits. Using of dilution buffer from another kit is not allowed. Only dilution buffer from **PREP-NA DNA/RNA Extraction Kit** can be used.

- Incubate the tubes for 5 minutes at 65 °C. Vortex the tubes for 3-5 seconds.
- Centrifuge the tubes at RCF(g) 12000 - 16000 for 30 seconds at room temperature (18-25 °C).

The NA preparation is ready for RT-PCR.

ATTENTION! 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.

Table A.1 – Shortened amplification program for the “DTprime” detection thermal cycler

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	35	15	0	1		Cycle
2	92	0	30	1		Cycle
3	92	0	10	8		Cycle
	64	0	15		√	
4	90	0	5	40		Cycle
	64	0	15		√	
5	64	0	5	1		Cycle
6	10	…	…	Holding		Holding
√- optical measurement						