DNA-TECHNOLOGY

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RUO

SARS-CoV-2/RSV/Influenza A virus/Influenza B virus

Multiplex REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

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1. INTENDED USE

The **SARS-CoV-2/RSV/Influenza A virus/Influenza B 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 A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** is designed to detect RNA of coronavirus SARS-CoV-2, human respiratory syncytial virus (RSV), influenza A virus and influenza B virus in human biological samples (nasopharyngeal swabs, oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, phlegm) by reverse transcription and real-time polymerase chain reaction (RT-PCR).

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 A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit.**

The **SARS-CoV-2/RSV/Influenza A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** can be used in research practice.

Potential users: qualified personnel trained in molecular research methods and rules of work in the laboratory.

It is necessary to apply the kit only as directed in this instruction for use.

2. METHOD

Method: reverse RNA transcription with subsequent amplification of cDNA fragments by real-time polymerase chain reaction (RT-PCR); qualitative multiplex analysis.

The RNA reverse transcription and PCR amplification of cDNA are performed in one test tube, which increases the sensitivity of the method, reduces the likelihood of contamination and the hands-on time.

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 only starts when paraffin is melted or thermal dissociation of a complex of Taq polymerase and antibodies has happened. It excludes non-specific annealing of primers to targets DNA in the initial heating of the tube.

The kit includes the internal control RNA-IC “A”, which is added into test sample during RNA extraction and is intended for the assessment of RNA extraction and quality of reverse transcription and polymerase chain reaction.

DNA probes are introduced into the PCR-mix; each probe contains a fluorescent dye and a fluorescence quencher. When the specific product is formed, DNA probe is destroyed and the effect of the fluorescence quencher on the fluorescent dye terminate, which leads to the rise of fluorescence level. The number of destroyed probes (and hence the fluorescence level) rises in proportion with the number of specific amplicons. The fluorescence level is measured at each real-time amplification stage.

DNA probe used for the detection of the Human respiratory syncytial virus (RSV) amplification product includes fluorescent dye Fam.

DNA probe used for the detection of the SARS-CoV-2 (E, N – genes) amplification product includes fluorescent dye Rox.

DNA probe used for the detection of the Influenza B virus amplification product includes fluorescent dye Cy5.

DNA probe used for the detection of the Influenza A virus amplification product includes fluorescent dye Cy5.5.

DNA probe used for the detection of the internal control amplification product includes the fluorescent dye Hex.

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 B virus	Influenza A 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 A virus/Influenza B 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 A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** content is represented in Table 2.

Table 2. The **SARS-CoV-2/RSV/Influenza A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** content, package S (standard) for R3-P457-S3/9EU and R3-P457-23/9EU

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless or pink transparent liquid under waxy white fraction	1440 µL (15 µL in each)	tubes, 12 strips of 8 or 96 individual tubes
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 caps [1]	12 strips of 8		

* - 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 A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** is intended for single use and designed for 96 tests (up to 12 runs), including the analysis of test samples, positive and negative controls.

[1] - for package S, strips REF R3- P457-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 12,000) 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 with caps;
- 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 0.2-1,000 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, **PREP-MB DWP** REF P-119-A/9EU and **PREP-MB-RAPID II** REF P-122-A/9EU, P-122-N/9EU, P-122-P/9EU, P-124-P/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 with freezer;
- Vortex mixer;
- Vortex rotor for 0.2 mL 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 2.0-1,000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL; 200 µL; 1,000 µL);

– Pipette stand;

– 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. TRANSPORT AND STORAGE CONDITIONS

Expiry date – 12 months from the date of production.

5.1. Transport and storage conditions

– All components of the reagent kit, except the Enzyme Taq/RT, must be stored in a refrigerator or a cooling chamber at the temperature from 2 °C to 8 °C throughout the shelf life of the kit.

– Paraffin sealed PCR-mix must be kept away from light.

– The Enzyme Taq/RT must be stored in a freezer at the temperature from minus 22 °C to minus 18 °C throughout the shelf life of the kit.

– Transport of the reagent kit is carried out in thermoboxes with ice packs by all types of roofed transport at the temperature inside the container corresponding to the storage conditions of the components included in the kit.

– It is allowed to transport the kit in thermoboxes with ice packs by all types of roofed transport at the temperature inside the container from 2 °C to 25 °C for no longer than 5 days.

– It is allowed to transport the Enzyme Taq/RT in thermoboxes with ice packs by all types of roofed transport at the temperature inside the container up to 25 °C for no longer than 5 days.

WARNING! Reagent kits transported with violation of temperature conditions must not be used.

5.2. Shelf-life of the kit following the first opening of the primary container

- All components of the kit, except the Enzyme Taq/RT, must be stored in a refrigerator or a cooling chamber at the temperature temperatures from 2 °C to 8 °C over the storage period.

- Paraffin sealed PCR-mix must be stored at temperatures from 2 °C to 8 °C and out of light over the storage period.

- Enzyme Taq/RT must be stored in a freezer at the temperature from minus 22 °C to minus 18 °C over the storage period.

WARNING! The kits stored under undue regime must not be used.

An expired **SARS-CoV-2/RSV/Influenza A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** must 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 A virus/Influenza B 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.

6. WARNINGS AND PRECAUTIONS

WARNING! 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.

Handle and dispose all biological samples, reagents and materials used to carry out the analysis 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 analysis. 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 A virus/Influenza B 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.

Interfering substances

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

PCR inhibitors are:

- endogenous substances (whole blood, leukocytes, mucus);
- exogenous substances (added to samples in the course of sample preparation (isopropyl alcohol, methyl acetate); topical medications).

The maximum concentration of interfering substances, which do not affect the amplification are: hemoglobin – 0.35 mg/mL of RNA sample, isopropyl alcohol – 100 µL/mL of mRNA sample, methyl acetate – 100 µL/mL of RNA sample.

Interfering effects of substances such as leukocytes, mucus, topical medications that may be contained in biomaterial samples in clinically relevant concentrations were not observed.

To reduce the amount of PCR-inhibiting interfering substances, it is necessary to follow the rules of biological material collection. If a large number of PCR inhibitors are suspected in the sample, it is recommended to choose nucleic acid extraction methods that allow their maximum removal from the sample; express methods of nucleic acid extraction are not recommended.

The features of biomaterial sampling

WARNING! Sample pretreatment may be needed before RNA extraction!

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

Sample preparation

Sample preparation (if necessary) is performed in accordance with the instructions to the NA extraction reagent kits.

If **PREP-NA** extraction kit is used for RNA extraction from phlegm, mucolysin should be used for pretreatment (see instruction to **PREP-NA**, method 2).

WARNING! For nasopharyngeal and oropharyngeal swabs collected into transport medium, bronchoalveolar lavage, endotracheal and nasopharyngeal aspirate, no preliminary centrifugation is required.

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.

WARNING! Avoid repeated freezing and thawing of samples.

8. PROCEDURE

WARNING! 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 with symptoms of acute infection separately from the rest of the samples (the biological material for screening exposed individuals). 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 controls, starting from the RNA extraction in each protocol;
- use tips with aerosol filters at all stages of the analysis;
- strictly follow the procedure, open the Eppendorf test tubes with tweezers or a special opener (do not touch inside the tube cap with the gloved hand); when adding 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 use extraction kits designed for RNA extraction for further RT-PCR (e.g. **PREP-NA**, **PREP-NA-S**, **PREP-MB-NA-S**, **PREP-MB DWP**, **PREP-MB-RAPID II** (see Table 3).

RNA extraction shall be performed in accordance with the instruction to the NA extraction kit.

For RNA extraction, **100 µL of biomaterial** are used.

WARNING!

- The volume of the resulting RNA preparation should not exceed 50 µL. In case of using **PREP-MB-NA-S**, **PREP-MB DWP** and **PREP-MB-RAPID II** kits for RNA extraction. The preparation volume may increase to 100 µL.
- 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 22 °C to minus 18 °C for no longer than a week with a single defrost before reverse transcription.

Table 3. The reagent kits validated for RNA extraction and further study with the **SARS-CoV-2/RSV/Influenza A virus/Influenza B 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
PREP-MB-RAPID II	nasopharyngeal and oropharyngeal swabs

8.2. The use of controls at the stage of nucleic acid extraction

Internal control

To exclude false negative results of the analysis and to control the quality of the analysis, it is necessary to add an internal control to test samples for nucleic acid extraction.

The internal control RNA-IC "A" from the **SARS-CoV-2/RSV/Influenza A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** should be used as an internal control.

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

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

Negative control

To exclude false positive results of the analysis and to control the quality of the analysis, it is necessary to use a negative control 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 or a negative control that included in the corresponding extraction kit can be used as a negative control in volumes as indicated in the instructions for use of extraction kits.

8.3. Reverse transcription and PCR

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

WARNING! When using package S, strips (R3-P457-S3/9EU), strictly observe the completeness of the strips and caps to them. Do not use the caps for the strips from the other kits!

8.3.1. Mark the required number of the tubes or strips with paraffin sealed PCR-mix according to the number of test samples, 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.

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

WARNING! Take Enzyme Taq/RT from the freezer immediately prior to use.

8.3.3. 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,

where 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.

WARNING! 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.

8.3.4. Vortex the tube with the mixture of RT-PCR-buffer and Enzyme Taq/RT thoroughly, then spin for 1-3 seconds. The mixture can be stored at 2 °C - 8 °C for up to 1 hour.

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

WARNING! After adding the mix of RT-PCR-buffer and Enzyme Taq/RT into the tubes with PCR mix, proceed immediately to 8.3.6 - 8.3.12.

8.3.6. Vortex the tubes with positive control for 3-5 seconds and spin down the drops for 1-3 seconds.

WARNING!

- Before introducing RNA preparation and negative control into the tubes with PCR-mix, fulfill the RNA preparation use recommendations listed in the instruction to NA extraction kit.
- If using **PREP-NA** and **PREP-NA-S** kits for RNA extraction, shake the tubes with RNA preparation and negative control on vortex for 3-5 seconds and spin on vortex for 1-3 seconds.
- If using **PREP-MB DWP** kit for RNA extraction, spin the sealed deep-well plate with RNA preparation at RCF(g) 100 for 30 seconds to precipitate the condensate, then take off the film.
- To prevent contamination, only open the tube where the RNA preparation is going to be introduced, and close it before proceeding to the next. If using strips, close the strip cap after adding samples before proceeding to the next. Use filter tips.

8.3.7. Add 10 µL of the RNA sample into corresponding tubes. Avoid paraffin layer break.

8.3.8. Add 10 µL of negative control (C-), which passed whole RNA extraction procedures into corresponding tube.

8.3.9. Add 10 µL of positive control sample (C+) into corresponding tube. Avoid paraffin layer break.

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

8.3.11. Set the tubes/strips into the real-time thermal cycler.

8.3.12. Launch the operating software for DT instrument[2]. Add corresponding test[3], specify the number and IDs of the samples, positive and negative controls. 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	15	0	1		Cycle
2	92	0	30	1		Cycle
3	92	0	10	8		Cycle
	64	0	15		v	
4	90	0	5	40		Cycle
	64	0	15		v	
5	64	0	5	1		Cycle
6	25[1]	…	…	Holding		Holding
√ - optical measurement [1] – holding at 10°C is allowed						

[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 A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit** contains positive control. 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.

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

For **SARS-CoV-2/RSV/Influenza A virus/Influenza B 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 A virus/Influenza B 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 RT-PCR results

Detection channel					**Interpretation**
Fam, Cp	Hex, Cp	Rox, Cp	Cy5, Cp	Cy5.5, Cp	
Test samples					
Specified	Not considered	Not specified	Not specified	Not specified	**RNA of respiratory syncytial virus (RSV) is detected**
Not specified	Not considered	**Specified**	Not specified	Not specified	**RNA of SARS-CoV-2 is detected**
Not specified	Not considered	Not specified	**Specified**	Not specified	**RNA of Influenza B virus is detected**
Not specified	Not considered	Not specified	Not specified	**Specified**	**RNA of Influenza A virus is detected**
Not specified	**Specified**	Not specified	Not specified	Not specified	No viral RNA is detected
Not specified	Not specified	Not specified	Not specified	Not specified	Unreliable result

Negative control					
Not specified	**Specified**	Not specified	Not specified	Not specified	Negative result The results are valid
Positive control					
Specified	Not considered	**Specified**	**Specified**	**Specified**	Positive result The results are valid

Unreliable results may be caused by the presence of inhibitors in the nucleic acid preparation obtained from the clinical material; 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.

If mixed infecting occurred, different combinations of AVRI agents are possible.

If a positive result is obtained for the negative control, the whole run is considered invalid. In this case, special measures to detect and eliminate a possible contamination are required.

If a negative result is obtained for the positive control, the whole run is considered invalid. In this case, a repeated amplification of the whole batch of samples 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.

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

WARNING! Negative results should not be used as the sole basis for decision-making on further actions.

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. Analytical specificity

Since it is impossible to exclude the occurrence of new mutations in the genome of the SARS-CoV-2 coronavirus, two genome regions were selected as targets to improve the reliability of analysis: the N and E genes regions.

In the samples of human biological material with the RNA of target viruses, the thermal cycler software should register exponential growth of fluorescence on the corresponding detection channels.

In the samples of human biological material not containing RNA of target viruses, the thermal cycler software should register the absence of fluorescence growth on the corresponding detection channels and a positive amplification result for internal control on Hex detection channel.

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 no non-specific positive results of amplification of RNA sample in the presence of *Human*

Coronavirus HKU-1, Human Coronavirus NL-63, Human Coronavirus 229E, Human Coronavirus OC-43, Human Rhinovirus, Human Parainfluenza virus type 1, Human Parainfluenza virus type 2, Human Parainfluenza virus type 3, Human Parainfluenza virus type 4, Human Metapneumovirus, MERS-CoV, ДНК Human Adenovirus, Mycoplasma pneumoniae, Streptococcus pneumoniae, Chlamydophila pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Moraxella catarrhalis, Bordetella pertussis, Bordetella parapertussis, MRSA, Legionella pneumophila, as well as human DNA in concentrations up to 1.0×10^8 copies/mL of the sample.

The validation tests showed specific amplification results in the presence of RNA extracted from SARS-CoV-2 and Respiratory syncytial virus samples obtained under the international QCMD (Quality Control for Molecular Diagnostics) system, UK, as well as RNA extracted from 15 influenza A strains of subtypes A(H1N1)pdm09 and A(H3N2) and 11 influenza B strains of different epidemic seasons in the sample.

b. Analytical sensitivity

Analytical sensitivity is 10 copies of nucleic acid per amplification tube. Sensitivity is determined by the analysis of serial dilutions of the laboratory control (LC).

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 (preparation 50 µL)**	**PREP-NA-S (preparation 50 µL)**	**PREP-MB-NA-S (preparation 100 µL)**	**PREP-MB DWP (preparation 100 µL)**	**PREP-MB-RAPID II (preparation 100 µL)**
Nasopharyngeal and oropharyngeal swabs in 500 µL of transport medium	1,000 copies /mL of sample	1,000 copies /mL of sample	2,000 copies/mL of sample	2,000 copies/mL of sample	2,000 copies/mL of sample
Bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate	1,000 copies /mL sample	not used	not used	not used	not used
Phlegm (pretreated with mucolysin)	5,000 copies /mL sample	not used	not used	not used	not used

c. Diagnostic characteristics

Biomaterial type	**Diagnostic sensitivity**	**Diagnostic specificity**
Respiratory syncytial virus (RSV)		
Nasopharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Oropharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Bronchoalveolar lavage	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Endotracheal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Nasopharyngeal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Phlegm	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Total	100 % (95 % CI: 98.77 % – 100 %)	100 % (95 % ДИ: 98.77 % – 100 %)

Biomaterial type	Diagnostic sensitivity	Diagnostic specificity
Influenza A virus		
Nasopharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Oropharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Bronchoalveolar lavage	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Endotracheal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Nasopharyngeal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Phlegm	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Total	100 % (95 % CI: 98.77 % – 100 %)	100 % (95 % ДИ: 98.77 % – 100 %)
Influenza B virus		
Nasopharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Oropharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Bronchoalveolar lavage	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Endotracheal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Nasopharyngeal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Phlegm	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Total	100 % (95 % CI: 98.77 % – 100 %)	100 % (95 % ДИ: 98.77 % – 100 %)
SARS-CoV-2		
Nasopharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Oropharyngeal swab	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Bronchoalveolar lavage	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Endotracheal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Nasopharyngeal aspirate	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Phlegm	100 % (95 % CI: 92.88 % – 100 %)	100 % (95 % CI: 92.88 % – 100 %)
Total	100 % (95 % CI: 98.77 % – 100 %)	100 % (95 % ДИ: 98.77 % – 100 %)

d. Precision and repeatability

Precision amounts to 100%.
Repeatability amounts to 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 of the current batch
C-	+	Contamination	Dispose of the 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

The quality control procedures performed in accordance with ISO 9001:2015 and ISO 13485:2016:

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

Contact our customer service with quality issues of the **SARS-CoV-2/RSV/Influenza A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit**.

Technical support:

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

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https://www.dna-technology.com

14. KEY TO SYMBOLS

	Date of manufacture		Consult instructions for use
	Temperature limit		Catalogue number
	Contains sufficient for <n> tests		Manufacturer
	Use-by date		Keep away from sunlight
	Batch code		For research use only
	Version		Non-sterile

 R3-P457-S3/9EU
R3-P457-23/9EU

 1030.2025.01.30

Annex A

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

WARNING! The lysis solution can form the precipitate. Take the kit out of the fridge and make sure there is no precipitate in the solution. In case there is, dissolve it at 65 °C for 15 minutes prior to use, then mix the solution by turning the vial upside down 5-10 times, avoiding foaming.

WARNING! Caps may open during heating! Use the tubes with locking caps (Eppendorf Safe-Lock Tubes) or programmable thermostat with clamp lid (e.g. solid-state programmable thermostat "TT-1-DNA-Tech" by DNA-Technology).

1. Mark the required number of 1.5 mL plastic tubes (with locking caps, if necessary) according to the number of test samples and negative control (C-).
2. Vortex the tube with RNA-IC "A" (from **SARS-CoV-2/RSV/Influenza A virus/Influenza B virus Multiplex REAL-TIME PCR Detection Kit)** and add 10 µL of the RNA-IC "A" into each tube.
3. Add 300 µL of the lysis solution into each tube avoiding contact of the pipette tip with an edge of the tube.
4. Add 100 µL of the sample into corresponding tubes.
5. Add 100 µL of negative control into the tube marked "C-".
6. Close the tubes tightly. Vortex the tubes for 3-5 seconds.
7. Thermostate the tubes for 5 minutes at 65 °C.
8. Spin the tubes for 3-5 seconds.
9. Add 400 µL of the precipitation buffer. Close the tubes tightly and vortex for 3–5 seconds.
10. Centrifuge the tubes at RCF(g) 12,000 – 16,000 for 10 minutes at room temperature (18-25 °C).
11. Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use new tip for each sample.
12. Add 500 µL of wash solution No.1. to the precipitate, close tubes and mix by inverting the tube 3-5 times.
13. Centrifuge the tubes at RCF(g) 12,000 – 16,000 for 1 minute at room temperature (18-25 °C).
14. Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use new tip for each sample.
15. Add 300 µL of the wash solution No. 2 to the precipitate, close the tubes and mix by inverting the tube 3-5 times.
16. Centrifuge the tubes at RCF(g) 12,000 – 16,000 for 1 minute at room temperature (18-25 °C).
17. 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.
18. Open the tubes and dry the precipitate at 65 °C for 5 minutes.
19. Add **50 µL** of the dilution buffer to the precipitate. Vortex the tubes for 3-5 seconds and spin down the drops for 3-5 seconds.
20. Thermostate the tubes for 5 minutes at 65 °C. Vortex the tubes for 3-5 seconds.
21. Centrifuge the tubes at RCF(g) 12,000 – 16,000 for 30 seconds at room temperature (18-25 °C) to precipitate the condensate.

RNA preparation is ready to be introduced to RT-PCR mix.

The NA preparation is ready for RT-PCR.

The resulting RNA preparation must be used for RT-PCR within 2 hours. For a repeated RT-PCR, immediately put the rest of the RNA into the freezer and store at a temperature up to minus 18 °C for no longer than 7 days. Do not thaw the preparation before PCR.

Before using RNA preparation for RT-PCR, thaw it at room temperature (18 °C to 25 °C) or at a temperature from 2 °C to 8 °C, then shake the RNA preparation and negative control on vortex for 3-5 seconds and spin for 1-3 seconds.

RNA preparation is ready to be introduced to RT-PCR mix.

WARNING! Only one thawing is allowed for RNA preparation!