DNA-TECHNOLOGY

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For research use only

SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

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R3-P440-23/9ER

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1. INTENDED USE

The **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – pathogen-detection-based product. The **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit** is designed to detect coronavirus SARS-CoV-2, Influenza A virus and Influenza B virus in human biological samples with an aid of reverse transcription and real-time polymerase chain reaction (RT-PCR). Samples are human biological materials: nasopharyngeal swabs, oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, sputum.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit.**

The **SARS-CoV-2/Influenza 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

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 analysis.

To increase the sensitivity and specificity of the amplification reaction, the use of a hot-start is provided. Hot-start is provided by reaction mixture preparation consisting of two layers separated by a layer of paraffin. The polymerase chain reaction starts only when paraffin is melted. It excludes non-specific annealing of primers to targets DNA in the initial heating of the tube.

The **SARS-CoV-2/Influenza 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/Influenza 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 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/Green	Hex/Yellow	Rox/Orange	Cy5/Red	Cy5.5/Crimson
Influenza A virus	IC*	SARS-CoV-2 coronavirus, E, N - genes	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/Influenza 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.

The **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit** is also approved for use with Rotor-Gene Q (Qiagen) real-time thermal cycler.

For the use of detecting thermal cyclers other than those listed above, please consult the reagent kit manufacturer for consultation.

3. CONTENT

The **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit** contains PCR-mix, RT-PCR-buffer, Enzyme Taq/RT, internal control RNA-IC "A" and positive control. The detailed description of content is represented in Table 2.

Table 2. The **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit** content, package S (standard) for R3-P440-S3/9ER and R3-P440-23/9ER

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
RT-PCR-buffer	Colorless transparent liquid	1620 µL (810 µL in each tube)	2 tubes
Enzyme Taq/RT	Colorless transparent viscous liquid	55 µL	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		

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

The kit is designed for 96 tests (94 defined samples, one positive control and one negative control) for **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit.** It is recommended to perform no more than 12 performances.

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

4.1. Specimen collection

- Sterile single use swabs, single-use sterile flasks and sterile containers to collect biological material;
- Sterile tubes containing transport media: "DNA-Technology" made **STOR-F** (REF P-901-1/1EU, P-901-N/1EU, P-901-R/1EU) or equivalent or sterile physiological saline solution for the transportation of the sample.

4.2. RNA extraction and PCR

Preamplification-specimen and control preparation area:

- Biological safety cabinet class II-III;

[1] - for detection kit packaged in strips REF R3-P440-S3/9ER

- Refrigerator;
- Vortex mixer;
- High speed centrifuge (RCF(g) no less than 16000) 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) and **PREP-NA-S** (REF P-007-N/1EU) extraction kits are recommended);
- Physiological saline solution 0.9% NaCl (Sterile) (if needed);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Refrigerator;
- Vortex mixer;
- Vortex rotor for strips (using detection kit packaged in strips REF R3-P440-S3/9ER);
- Tube rack for 1.5 mL tubes;
- 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);
- DTstream M1 dosage instrument (only for automated dosing);
- 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.

All components of **SARS-CoV-2/Influenza 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 Enzyme Taq/RT must be stored at temperatures from minus 18 °C to minus 22 °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 excessive temperature and light can be detrimental to product performance.

The kit has to 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 termobox with ice packs by all types of roofed transport at temperatures from 2 °C to 25 °C but 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 termobox with ice packs by all types of roofed transport at temperatures up to 25 °C but no more than 5 days and should be stored at temperatures from minus 18 °C to minus 22 °C immediately on receipt.

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

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 the **SARS-CoV-2/Influenza 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/Influenza 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

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 corresponding 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 analysis are allowed to work with the kit of reagents.

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 instruction.

7. SAMPLES

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

Interfering substances

The presence of PCR inhibitors in a sample may cause controversial (uncertain) results. The sign of PCR inhibition is the simultaneous absence of 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 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.

Transportation and storage of the samples in accordance with guidance

Type of the sample	Collecting material requirements	Transportation	Storage conditions before transportation	Comments
Nasopharynx and oropharynx swabs	Plastic test tubes and probes for swabs**	4 °C	≤5 days: 4 °C >5 days *: -70 °C	Nasopharyngeal and oropharyngeal probes 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	
Phlegm	Sterile container	4 °C	≤48 hours: 4 °C >48 hours *: -70 °C	Make sure that the material is from the lower respiratory tract

* if it is not possible to store samples at minus 70 °C, store samples at minus 20 °C.
** Use a transport medium for storage and transportation of the respiratory swabs or saline solution (if transportation to the laboratory no more than 24 hours after taking the sample) or a dry probe (if transportation to the laboratory no more than 4 hours after taking the sample).

It is recommended to use transport media containing preservatives, for example, **STOR-F** produced by DNA-Technology or similar registered in the established order, intended for further analysis 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 Influenza and SARS-CoV-2 viral load can vary widely. In this regard, when performing research in a 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;
- it is desirable to work with the supposed high-copy samples in a separate box or after working with the supposed low-copy samples;
- 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;
- 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 from the nasopharynx and oropharynx swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, sputum, RNA extraction kits are used (see Table 3).

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

Reagent kit	Biomaterial
PREP-NA DNA/RNA Extraction Kit, produced by "DNA-Technology"	nasopharynx and oropharynx swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, sputum (or nasopharynx and oropharynx swabs if shortened method in accordance with Annex A is used)
PREP-NA-S DNA/RNA Extraction Kit, produced by "DNA-Technology"	nasopharynx and oropharynx 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.

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.

8.2. The features of biomaterial preparation

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

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

8.3. 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 use an internal control to the test samples at the stage of nucleic acid extraction.

The internal control RNA-IC "A" from the **SARS-CoV-2/Influenza 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.

ATTENTION! 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.

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

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

8.4. PCR with Reverse Transcription (RT-PCR)

ATTENTION!

1. The reagents and tubes should be kept away from direct sun light.
2. When using package S (R3-P440-S3/9ER), strips, strictly observe the completeness of the strips and caps to them. Do not use the caps to the strips of 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! Open the tube, add RNA sample (or control), then close the tube before proceeding to the next RNA sample to prevent contamination. In case of using tubes in strips, close the strip before proceeding to the next 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 (C-), which passed whole RNA extraction procedures into corresponding tube. Add 10 µL of positive control (C+) into corresponding tube. Avoid paraffin layer break.

8.4.9. Spin down the tubes/strips for 3-5 seconds to collect drops (when using the Rotor-Gene Q thermal cycler, spin is not required).

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 controls. Specify the position of the tubes/strips in the thermal unit (8.4.10) and run PCR. See Table 4.

For use with Rotor-Gene Q real-time thermal cyclers consult user manual for devices. See Table 5.

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[1]	…	…	Holding		Holding

√ - optical measurement
[1] – holding at 25°C is allowed

Table 5. The PCR program for Rotor-Gene Q thermal cycler

Cycling	Temperature	Hold time, sec	Cycle repeats
Cycling	32 deg	1200	1 time
Cycling 2	95 deg	300	1 time
Cycling 3	94 deg	10	50 times
	60 deg*	15	

* optical measurement, set the fluorescence measurement (Acquiring) on the channels Green (Fam), Yellow (Hex), Orange (Rox) and Red (Cy5) at 60 °C.

[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.

8.5. PCR with Reverse Transcription (RT-PCR) using DTStream (only for detection kit packaged in strips)

ATTENTION!

1. The reagents and tubes should be kept away from direct sun light.
2. Strictly observe the completeness of the strips and caps to them. Do not use the caps to the strips of the other kits!

8.5.1 Mark the required number of the 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.

8.5.2 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.

8.5.3 Prepare the mixture of RT-PCR-buffer and Enzyme Taq/RT. Follow the DTStream instructions or mix in a separate tube:

- 15.0 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+".

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.

8.5.4 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.

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

8.5.6 Set tubes with mixture of RT-PCR-buffer and Enzyme Taq/RT, analyzed samples, positive and negative controls and strip tubes with PCR-mix to the DTstream and dispense the components according to the instruction manual.

8.5.7 Close the caps of the strips.

8.5.8 Spin the strips for 3-5 seconds.

8.5.9 Set strips to the real-time PCR thermal cycler.

8.5.10 Launch the operating software for DT instrument[4]. Add corresponding test[5], specify the number and ID's of the samples, positive and negative controls. Specify the position of the strips in the thermal unit (8.5.9) and run PCR. See Table 4.

[4] - Please, apply to Operation Manual for DTprime and DTlite Real-Time PCR instruments PART II.

[5] - 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/Influenza 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 the 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 should go through all stages of RNA extraction. Physiological saline solution can be used as a negative control in volumes indicated in supplied instructions.

For **SARS-CoV-2/Influenza 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 absence and for internal control is present.

For **SARS-CoV-2/Influenza 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 6.

Table 6. The interpretation of RT-PCR results for controls

Detection channel				**Interpretation**
Fam/Green	Hex/Yellow	Rox/Orange	Cy5/Red	
Positive control				
Cp/Ct is specified	Cp/Ct is not specified	**Cp/Ct is specified**	**Cp/Ct is specified**	Positive result The results are valid
Negative control				
Cp/Ct is not specified	**Cp/Ct is specified**	Cp/Ct is not specified	Cp/Ct 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/Green, Rox/Orange or Cy5/Red), see Tables 7, 8.

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/Yellow (Internal control) detection channel, the increase in fluorescence on the Fam/Green, Rox/Orange, and Cy5/Red channels should be absent.

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

Table 7. The interpretation of RT-PCR results

Detection channel				**Interpretation**
Fam/Green	Hex/Yellow	Rox/Orange	Cy5/Red	
Analyzed samples				
Cp/Ct is specified	Is not considered	Cp/Ct is not specified	Cp/Ct is not specified	**Influenza A virus RNA is detected**
Cp/Ct is not specified	Is not considered	**Cp/Ct is specified**	Cp/Ct is not specified	**SARS-CoV-2 RNA is detected**
Cp/Ct is not specified	Is not considered	Cp/Ct is not specified	**Cp/Ct is specified**	**Influenza B virus RNA is detected**
Cp/Ct is not specified	**Cp/Ct is specified**	Cp/Ct is not specified	Cp/Ct is not specified	Target viruses RNA is not detected
Cp/Ct is not specified	Cp/Ct is not specified	Cp/Ct is not specified	Cp/Ct is not specified	Unreliable result. Repeat PCR amplification or NA extraction or re-collect of a test sample, performed sequentially

Table 8. Other possible results

Detection channel				**Interpretation**
Fam/Green	Hex/Yellow	Rox/Orange	Cy5/Red	
Analyzed samples				
Cp/Ct is specified	Is not considered	**Cp/Ct is specified**	Cp/Ct is not specified	**Influenza A virus RNA is detected, SARS-CoV-2 RNA is detected**
Cp/Ct is not specified	Is not considered	**Cp/Ct is specified**	**Cp/Ct is specified**	**Influenza B virus RNA is detected, SARS-CoV-2 RNA is detected**
Cp/Ct is specified	Is not considered	Cp/Ct is not specified	**Cp/Ct is specified**	**Influenza A virus RNA is detected, Influenza B virus RNA is detected,**
Cp/Ct is specified	Is not considered	**Cp/Ct is specified**	**Cp/Ct is specified**	**Influenza A virus RNA is detected, Influenza B virus RNA is detected, SARS-CoV-2 RNA is detected**

Unreliable results may be caused by the presence of inhibitors in the nucleic acid preparation obtained from the biological 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 biological material (performed sequentially) is required.

When the expressed growing fluorescence (Cp/Ct is not specified) through the Fam/Green, Rox/Orange, or Cy5/Red 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/Ct is specified) through the Fam/Green, Rox/Orange, or Cy5/Red 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.

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 (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.

11. SPECIFICATIONS

a. The analytical specificity of the **SARS-CoV-2/Influenza 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 analysis: 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.

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 HKU-1, NL-63, OC-43, 229E, Human parainfluenza viruses type 1-4, Human respiratory syncytial virus, Human metapneumovirus, Human rhinovirus, DNA of Human adenovirus, Human bocavirus, *Mycoplasma pneumoniae, Streptococcus pneumoniae, Chlamydophila pneumoniae, Haemophilus influenzae, 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.

b. Analytical sensitivity is 10 copies of RNA 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 DNA/RNA Extraction Kit (at elution in 50 µL)**	**PREP-NA-S DNA/RNA Extraction Kit (at elution in 50 µL)**
Nasopharynx and oropharynx swabs in 500 µL of transport medium	1000 copies /mL sample	1000 copies /mL sample
Bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate	1000 copies /mL sample	Not used
Sputum	2000 - 5000 copies /mL sample depending on the preprocessing method	Not used

c. Diagnostic characteristics

The diagnostic sensitivity and specificity were established for each virus of the ARVI pathogen which RNA is detected using the **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection 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%)

ATTENTION! The claimed specifications are guaranteed when RNA extraction is performed with **PREP-NA** and **PREP-NA-S** extraction kits.

12. TROUBLESHOOTING

Table 9. 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 issueswith the kit:

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com/support

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 **SARS-CoV-2/Influenza Multiplex REAL-TIME PCR Detection Kit**.

Technical support:

E-mail: hotline@dna-technology.ru

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14. KEY TO SYMBOLS

	For research use only		Date of manufacture
	Temperature limit		Consult instructions for use
	Contains sufficient for <n> tests		Catalogue number
	Use-by date		Manufacturer
	Batch code		Keep away from sunlight
	Version		Do not reuse
	Caution		

Annex A

Shortened method for the RNA extraction from the tested material (nasopharynx and oropharynx 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/Influenza 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 wash 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 wash 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.