DNA-TECHNOLOGY

921 2023-01-27

RUO

For research use only

Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

"DNA-Technology Research & Production", LLC,

142281, Russia,

Moscow Region, Protvino,

Zheleznodorozhnaya Street, 20

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

Customer service department

E-mail: hotline@dna-technology.ru

REF R3-P433-S3/9EU
R3-P433-23/9EU

VER 921.2023.01.27

TABLE OF CONTENTS

1. INTENDED USE

The **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – pathogen-detection-based product. The **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** is designed to detect the Influenza A virus subtypes (Influenza A(H1N1)pdm09 virus and Influenza A(H3N2) virus) RNA in human biological material (nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, phlegm) by Reverse Transcription (RT) and Polymerase Chain Reaction (PCR) methods.

Indications for the use:

- presence of symptoms and contact with influenza patients;
- stay in the centers of infection (for the purpose of early detection of possible infection and prevention of further spread of infection);
- influenza differential diagnostics.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit.**

The **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** can be used in 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 (RT-PCR); qualitative analysis.

The principle of the method is based on the use of the process of reverse transcription of RNA and subsequent amplification of cDNA, which consists of repeated cycles of temperature denaturation of DNA, annealing of primers with complementary sequences, and subsequent extension of polynucleotide chains from these primers with Taq polymerase.

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

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 Taq-polymerase blocked by antibodies. The polymerase chain reaction starts only when paraffin melts or after the Taq-polymerase or antibody complex temperature dissociating, which excludes the possibility of non-specific annealing of primers on the DNA target during the initial warm-up of the test tube.

The **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** includes the Internal control (RNA-IC "A"), which is intended to assess the quality of the RNA extraction, reverse transcription and polymerase chain reaction.

DNA probes, each containing a fluorescent label and a fluorescence quencher, are introduced into the reaction mixture for amplification. When a specific product is formed, the DNA probe is destroyed and the quencher stops affecting the fluorescent label, which leads to an increase in the fluorescence level. The number of destroyed probes (and hence the fluorescence level) increases in proportion to the number of specific amplicons formed. The fluorescence level is measured at each amplification cycle in real time.

DNA probe used for the detection of the Influenza A(H1N1)pdm09 virus cDNA products is labeled with fluorescent reporter dye Fam.

DNA probe used for the detection of the Influenza A(H3N2) virus cDNA products is labeled with fluorescent reporter dye Cy5.

DNA probe used for the detection of the internal control (RNA-IC "A") amplification product is labeled with the fluorescent reporter dye Hex. Table 1 shows the detection channels of amplification products.

Table 1. Detection channels of amplification products

Fam	Hex	Rox	Cy5	Cy5.5
Influenza A(H1N1)pdm09 virus	IC*	-	Influenza A(H3N2) virus	-

* - Internal control RNA-IC "A"

The automatic analysis for **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME-PCR Detection Kit** is available on "DNA-Technology" made DTprime[1] and DTlite[2] REAL-TIME Thermal Cyclers; the latest version of the software is available for download at https://www.dna-technology.com/software.

The **Influenza A/B virus Multiplex REAL-TIME-PCR Detection Kit** is also approved for use with CFX96 (Bio-Rad) real-time thermal cycler.

3. CONTENT

The detailed description of content is represented in Tables 2-3.

Table 2. The **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** content, package S, strips for R3-P433-S3/9EU

Reagent	Description	Nominal volume	Amount
Paraffin sealed PCR-mix	Colorless or pink transparent liquid under waxy white fraction	1440 µL (15 µL in each tube)	tubes, 12 strips of 8
RT-PCR-buffer	Colorless transparent liquid	1.62 mL	1 tube
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 caps	12 strips of 8		

* - marking as C+ is allowed

[1] - supported by 5M1, 5M3, 5M6, 6M1, 6M3, 6M6 instruments.
[2] - supported by 4S1, 4S2, 5S1, 5S2, 6S1, 6S2 instruments.

Table 3. The **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** content, package S, tubes for R3-P433-23/9EU

Reagent	Description	Nominal volume	Amount
Paraffin sealed PCR-mix	Colorless or pink transparent liquid under waxy white fraction	1440 µL (15 µL in each tube)	96 tubes
RT-PCR-buffer	Colorless transparent liquid	1.62 mL	1 tube
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

* - marking as C+ is allowed

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

The kit is intended for single use and designed for 96 tests (including one positive control and one negative control in each run). It is recommended to perform no more than 12 runs.

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) or **STOR-M** (REF P-910-1/1EU) (only nasopharyngeal and oropharyngeal swabs) or physiological saline solution (Sterile) 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) no less than 16000);
- Solid-state thermostat (temperature range 40-95 °C);
- Tube rack for 1.5 mL tubes;
- DNase and RNase free 1.5 mL microcentrifuge tubes with caps;
- 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);
- Pipette stand;

- Nucleic acid extraction kit ("DNA-Technology" made **PREP-NA** REF P-002/1EU, **PREP-NA-S** REF P-007-N/1EU, **PREP-MB DWP** REF P-119-A/9EU, REF P-119-N/9EU, REF P-119-P/9EU, **PREP-MB-NA-S** REF P-117-A/9EU, REF P-118-A/9EU DNA/RNA extraction kits are recommended);
- Physiological saline solution 0.9% NaCl (Sterile);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

When extracting NA from phlegm (method 1):

- 10% trisodium phosphate x $12H_2O$;
- 1M HCl solution;
- 5.0% chloramines solution;
- Distilled water.

When extracting NA from phlegm (method 2):

- Mucolysin.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Refrigerator;
- Freezing chamber;
- Vortex mixer;
- Vortex rotor for strips (in case of using package S, strips REF R3-P449-S3/9EU);
- 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);
- Pipette stand;
- Tube rack for 1.5 mL tubes;
- 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 **Influenza A virus H1N1pdm09/H3N2 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 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 thermobox 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 thermobox 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.

The kit transported under undue regime should 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 under undue regime should not be used.

An expired the **Influenza A virus H1N1pdm09/H3N2 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 **Influenza A virus H1N1pdm09/H3N2 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

Only personnel trained in the methods of molecular diagnostics and the rules of work in the clinical and diagnostic laboratory are allowed to work with the kit.

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 cannot be used more than once in amplification reactions. Plastic tubes do not contain phthalates. Do not breathe gas/fumes/vapor/spray produced by the components of the kit. Do not eat/drink components of the kit. Avoid contact with eyes. Only use the reagents provided in the kit and those recommended by manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits.

All laboratory equipment, including 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 **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** is designed to detect RNA extracted from nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, phlegm.

Method limitations: local application of medications (sprays, drops, creams and ointments) – less than 24 hours before the assay. When using aerosols and other form of medications in the treatment of bronchial asthma, the material for the assay should not be taken earlier than three hours after inhalation or meal.

General requirements

1. Use RNase- and DNase-free disposable tips (with filter, except for the supernatant extraction step using an aspirator) during the biomaterial preparation and nucleic acid extraction steps.

2. When adding solution to a tube containing biological material, carefully introduce liquids without touching the walls of the tubes. If you touch the wall of the tube, change the tip. The tip should be changed each time the solution is removed from the sample.

3. To prevent contamination, always open only the cap of the tube you are working with and close it afterwards. It is not allowed to work with several tubes with open caps at the same time.

Interfering substances

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

PCR-inhibiting interfering substances include:

- endogenous substances (whole blood, leukocytes, mucus);
- exogenous (substances added to biomaterial samples during sample preparation (isopropyl alcohol and methyl acetate), topical medications).

The maximum concentrations of interfering agents at which no effect on amplification was observed were: hemoglobin, 0.35 mg/mL of RNA sample; isopropyl alcohol, 100 µL/mL of RNA sample; methyl acetate, 100 µL/mL of RNA sample.

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

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

Sample collection

Phlegm

Material is taken into single-use graduated sterile vials with wide neck and screw caps with a volume of no less than 50 mL in an amount no less than 1.0 mL.

After the sample collection the vial is tightly closed and labeled.

NOTE:

Phlegm must be pretreated. The procedure for phlegm pretreatment with a solution of trisubstituted sodium phosphate (Na3PO4) or mucolysin is given in the instructions to the reagent kit (**PREP-NA DNA/RNA Extraction Kit**, "DNA-Technology R&P", LLC).

Bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate

Material is taken into single-use graduated sterile vials with wide neck and screw caps with a volume of up to 50 mL. After the sample collection the vial is tightly closed and labeled.

Nasopharyngeal and oropharyngeal swabs

Material collection is performed using specific medical devices depending on the source of the biological material according to the established procedure.

After collecting the material, transfer the probe to a tube of transport medium intended by the manufacturer for transporting and storing biological material samples for PCR assays, and rinse it thoroughly in liquid for 10-15 seconds, avoiding splashing the liquid.

Remove the probe from the solution and, rotating it against the inner wall of the tube above the level of the solution, squeeze out the excess liquid. Completely remove the probe from the tube and discard.

Close the lid of the tube tightly and label the tube.

If biomaterial from more than one biotope needs to be collected, repeat the procedure, each time taking material with a new probe into a new tube.

Transportation and storage of the samples in accordance with guidance

Phlegm

Phlegm samples are allowed to be stored:

at room temperature (18 °C – 25 °C) for up to 6 hours;

at 2 °C – 8 °C for up to 3 days.

Bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate

Bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate are allowed to be stored:

at 2 °C – 8 °C for up to 24 hours;

at minus 18 °C to minus 20 °C for up to 1 week.

ATTENTION! Avoid repeated freezing and thawing of samples.

Nasopharyngeal and oropharyngeal swabs

The transportation and storage conditions of nasopharyngeal and oropharyngeal swabs are determined by the instructions for the recommended transport media and kits for RNA isolation.

Further processing of the above types of biological material is performed according to the instructions for the used kits for RNA isolation.

8. PROCEDURE

ATTENTION! The range of influenza viral load can vary widely. In clinical laboratory practice the risk of cross-contamination between samples remains a serious danger, especially during aliquoting and RNA extracting. Cross-contamination with high-copy nucleic acids can lead to sporadic false-positive results.

To prevent cross-contamination 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).

Assay procedure

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 4).

Table 4. The reagent kits validated for RNA extraction and further study with the **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit.**

Reagent kit	Biomaterial
PREP-NA	nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, phlegm
PREP-NA (shortened method in accordance with Annex A)	nasopharyngeal and oropharyngeal swabs
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.

The resulting RNA preparation must be used in the course of 2 hours 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.

8.1.1 **The features of biomaterial preparation for Influenza A virus H1N1pdm09/H3N2 RNA testing**

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

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

8.1.2 **The use of control samples at the stage of nucleic acid extraction**

8.1.2.1 Internal control sample

To exclude false negative results of the assay and to control the quality of the assay, 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 **Influenza A virus H1N1pdm09/H3N2 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**.

NOTE – Internal controls (RNA-IC and DNA-IC) from the **PREP-NA** extraction kits are not used during the RNA isolation.

8.1.2.2 Negative control sample

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

In the nucleic acid isolation step, be sure to prepare a negative control and run it through all isolation steps simultaneously with RNA isolation from clinical samples.

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

8.2 PCR with Reverse Transcription (RT-PCR)

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

ATTENTION! When using package S (R3-P433-S3/9EU), strips, strictly observe the completeness of the strips and caps to them. Do not use the caps to the strips of the other kits!

8.2.1 Mark the required number of the tubes/ stripped tubes 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.

8.2.2 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.

The amount of reagents is calculated for no more than 12 tests, assuming a variable number of unknown samples, 1 negative control and 1 positive control in each run.

8.2.3 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 into 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.

8.2.4 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 can be stored at 2 °C – 8 °C for up to 1 hour.

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

8.2.6 Vortex the tubes with "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. The RNA samples must stand in a magnetic rack while taking out RNA. 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 tube, add RNA sample (or control sample), 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.

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

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

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

8.2.10 Spin down the strips for 3-5 seconds to collect drops (when using the Rotor-Gene Q for PCR, centrifugation is not necessary).

8.2.11 Set the strips into the Real-time Thermal Cycler.

8.2.12 Launch the operating software for DT instrument[3]. Add corresponding test[4], specify the number and ID's of the samples, positive and negative control samples. Specify the position of the strips in the thermal unit (see 8.2.11) and run PCR. See Table 5.

For use with CFX96 (Bio-Rad) thermal cycler consult user manual for devices. See Table 6.

Table 5. The PCR program for DTprime and DTlite 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		√	
4	90	0	5	40		Cycle
	64	0	15		√	
5	64	0	5	1		Cycle
6	10	…	…	Holding		Holding
√ - optical measurement						

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

Table 6. Amplification program for CFX96 (Bio-Rad) thermal cycler

Step	Temperature, °C	Time, min:sec	Cycle repeats
1	35	20:00	1
2	95	5:00	1
3	94	0:15	50
4	64 **√**	0:20	
√ - optical measurement (Plate Read), set the fluorescence measurement on the Fam, Hex and Cy5 channels at 64 °C			

9. CONTROLS

The **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** contains positive control sample. Positive control is a cloned part of the virus genome. It is produced by 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 RNA extraction, reverse transcription 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 can be used as a negative control sample.

For **Influenza A virus H1N1pdm09/H3N2 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 taken into account;
- the exponential growth of the fluorescence level is absent for the specific product and is present for internal control.

For **Influenza A virus H1N1pdm09/H3N2 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 have positive result, it is necessary to repeat the whole test. It may be caused by inhibitors, operation error or violation of storage and handling requirements.

If negative control (C-) has positive result, all results of current PCR run are considered false. The retesting and decontamination are required.

10. DATA ANALYSIS

Registration of the PCR results is held in automatic mode. Interpretation of the PCR results should be performed in accordance with the Table 7-8.

Table 7. Control assay result interpretation

Detection channel			**Result interpretation**
Fam	Hex	Cy5	
Negative control sample			
Cp/Cq is not specified	**Cp/Cq** **is specified**	Cp/Cq is not specified	Negative result. The results of PCR run are valid.
Positive control sample			
Cp/Cq **is specified**	Cp/Cq is not specified	**Cp/Cq** **is specified**	Positive result. The results of PCR run are valid.

Table 8. Interpretation of PCR results

Detection channel			**Result interpretation**
Fam	Hex	Cy5	
Samples			
Cp/Cq **is specified**	Is not considered	Cp/Cq is not specified	**Influenza A(H1N1)pdm09 virus RNA** **is detected**
Cp/Cq is not specified	Is not considered	**Cp/Cq** **is specified**	**Influenza A(H3N2) virus RNA is detected**
Cp/Cq **is specified**	Is not considered	**Cp/Cq** **is specified**	**Influenza A(H1N1)pdm09 virus RNA** **is detected** **Influenza A(H3N2) virus RNA** **is detected**
Cp/Cq is not specified	**Cp/Cq** **is specified**	Cp/Cq is not specified	RNA of **Influenza A(H1N1)pdm09** and **Influenza A(H3N2)** viruses are not detected
Cp/Cq is not specified	Cp/Cq is not specified	Cp/Cq is not specified	Invalid result

When using CFX96 (Bio-Rad) detection thermal cyclers, use regression type analysis (Cq Determination Mode: Regression).

10.1 The results of the run are valid if the conditions for control result interpretation are met (Table 7).

10.2 In biological samples containing RNA of viruses detected by the kit, the detection thermal cycler registers an exponential increase in the fluorescence level on the corresponding detection channel ((Fam or Cy5), Table 8)

10.3 In biological samples containing no RNA of viruses detected by the reagent kit and in the negative control sample, the detection thermal cycler registers an exponential increase in the fluorescence level on the Hex channel (internal control sample), there is no exponential increase in fluorescence on the Fam and Cy5 channels.

10.4 The result is considered by the software to be invalid if there is no exponential increase in the fluorescence level for the specific product (Fam and Cy5 channels) and for the internal control sample (Hex channel).

10.5 Unreliable result can be related to the presence of PCR inhibitors in RNA sample, incorrect analysis procedure, violation of the amplification temperature regimen, etc.

In case of suspicion for the presence of inhibitors in sample, it is necessary to repeat nucleic acid extraction from sample, or repeat sampling (performed sequentially).

Warnings

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

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

If an increase in fluorescence for a specific product is detected before cycle 25 of Cp (Cp less than 25) in a biological sample, this indicates a high initial RNA concentration of the corresponding pathogen. In this case, it is possible to obtain a false-negative result for a pathogen which RNA is present at a low concentration in the mixture. To rule out false negatives, it is recommended to repeat the RT-PCR for the isolated RNA preparation using a kit for the individual detection of the corresponding virus.

11. SPECIFICATIONS

a. The analytical specificity of the **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit** was assessed by bioinformatic 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.

The absence of cross-reactivity of each of the oligonucleotide systems included in the kit with respect to viruses determined by the other systems has been shown.

There are not non-specific positive results of amplification of RNA sample in the presence Influenza virus A(H7N9), Influenza B virus, Human Coronavirus 229E, Human Coronavirus HKU-1, Human Coronavirus NL-63, Human Coronavirus OC-43, Human Metapneumovirus, Human Parainfluenza virus type 1, Human Parainfluenza virus type 2, Human Parainfluenza virus type 3, Human Parainfluenza virus type 4, Human Rhinovirus, MERS-CoV, Respiratory syncytial virus, SARS-CoV-2, DNA Human Adenovirus, Human Bocavirus, *Bordetella parapertussis*, *Bordetella pertussis*, *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.

The validation tests showed specific amplification results in the presence of RNA extracted from 17 influenza A subtypes A(H1N1)pdm09 and A(H3N2) strains of different epidemic seasons in the sample.

b. Analytical sensitivity. LOD (limit of detection) is 10 NA copies per amplification tube.

The analytical sensitivity was determined by analyzing serial dilutions of two series of a laboratory control (LC).

The RNA analytical sensitivity in a biomaterial sample depends on the sample preparation method and the final volume of isolated RNA (elution volume).

The detection limit of 10 nucleic acid copies per amplification tube corresponds to the following values of RNA concentration in the sample when nucleic acid extraction kits are used:

Biomaterial	**PREP-NA** (obtained preparation volume 50 µL)	**PREP-NA-S** (obtained preparation volume 50 µL)	**PREP-MB-NA-S; PREP-MB DWP** (obtained preparation volume 100 µL)
Naso-/oropharyngeal swabs in 500 µL of transport medium	1000 copies/mL of sample	1000 copies/mL of sample	2000 copies/mL of sample
Bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate	1000 copies/mL of sample	Not applicable	Not applicable
Phlegm (pretreated with Na_3PO_4)	2000 copies/mL of sample	Not applicable	Not applicable
Phlegm (pretreated with mucolysin)	5000 copies/mL of sample	Not applicable	Not applicable

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

Contact our customer service with quality issues of **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit**.

Technical support:

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

Manufacturer: "DNA-Technology Research & Production", LLC,

142281, Russia, Moscow Region,

Protvino, Zheleznodorozhnaya Street, 20

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

Seller: "DNA-Technology" LLC,

117587, Russia, Moscow,

int. ter. Municipal District Chertanovo Severnoye,

Varshavskoye shosse, 125 Zh, building 5, floor 1, office 12;

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

http://www.dna-technology.com

14. KEY TO SYMBOLS

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

REF R3-P433-S3/9EU
R3-P433-23/9EU

VER 921.2023.01.27

Annex A

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

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

1. Mark the required number of 1.5 mL tubes according to the number of test samples and negative control ("C-").
2. Vortex the tube with RNA-IC "A" (from **Influenza A virus H1N1pdm09/H3N2 Multiplex REAL-TIME PCR Detection Kit**). Add 10 µL of the RNA-IC "A" into each tube.
3. Add 300 µL of the lysis buffer into each tube avoiding contact of the pipette tip with an edge of the tube.
4. Add 100 µL of sample into marked tubes. Add 100 µL of negative control into the tube marked "C-".
5. Close the tubes tightly. Vortex the tubes for 3-5 seconds.
6. Incubate the tubes for 5 minutes at 65 °C.
7. Vortex the tubes for 3-5 seconds.
8. Add 400 µl of the precipitation buffer. Close the tubes tightly and vortex them for 3–5 seconds.
9. Centrifuge the tubes at RCF(g) 12000 - 16000 for 10 minutes at room temperature (18-25 °C).
10. Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use a new tip for each sample.
11. Add 500 µL of the wash solution №1 to the precipitate, close tubes and mix by inverting the tube 3-5 times.
12. Centrifuge the tubes at RCF(g) 12000 - 16000 for 1 minute at room temperature (18-25 °C).
13. Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use a new tip for each sample.
14. Add 300 µL of the wash solution №2 to the precipitate, close tubes and mix by inverting the tube 3-5 times.
15. Centrifuge the tubes at RCF(g) 12000 - 16000 for 1 minute at room temperature (18-25 °C).
16. Remove supernatant completely avoiding contact of the pipette tip with the precipitate. Use a 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.
17. Open the tubes and dry the precipitate at 65 °C for 5 minutes.
18. 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.
19. Incubate the tubes for 5 minutes at 65 °C. Vortex the tubes for 3-5 seconds.
20. 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.

The obtained RNA preparation should be used for reverse transcription and polymerase chain reaction within two hours. To be able to repeat the assay, the remaining RNA should be immediately placed in a freezer and stored at no more than minus 18 °C for no more than 7 days without defrosting before the run.