DNA-TECHNOLOGY

1032 2023-11-15

RUO

For research use only

Influenza A virus H1N1pdm09

REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

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REF R3-P454-S3/9EU
R3-P454-23/9EU
R3-P454-UA/9EU

VER 1032.2023.11.15

TABLE OF CONTENTS

1. INTENDED USE

The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – pathogen-detection-based product. The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** is designed to detect the RNA of Influenza A virus H1N1pdm09 subtype in human biological material (nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, phlegm) by reverse transcription (RT) and polymerase chain reaction (PCR).

Indications for the use:

- AVRI symptoms or contacts with AVRI infected;
- stay in the centers of infection (for the purpose of early detection of possible infection and prevention of its further spread).

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 REAL-TIME PCR Detection Kit.**

The **Influenza A virus H1N1pdm09 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

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

The principle of the method is based on 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 elongation of polynucleotide chains from these primers with Taq polymerase.

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

To increase the sensitivity and specificity of reaction, the use of a hot start is provided. Hot start is provided by Taq-polymerase blocked by antibodies. The polymerase chain reaction starts only after the Taq-polymerase and antibody complex temperature dissociating, which excludes the possibility of non-specific annealing of primers on the DNA target during the initial heating of the tube.

The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** includes the internal control (RNA-IC "A"), which is added into test samples during RNA extraction and is intended for efficiency assessment of all analysis stages.

DNA probes, each containing a fluorescent label and a fluorescence quencher, are introduced into the PCR-mix. 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 proportionally to the number of specific amplicons formed. The fluorescence level is measured at each amplification cycle in real time.

DNA probes used for the detection of the Influenza A virus H1N1pdm09 subtype cDNA products are labeled with fluorescent dye Fam.

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

Table 1. Detection channels of amplification products

Fam/Green	Hex/Vic/Yellow	Rox/Orange	Cy5/Red	Cy5.5/Crimson
Influenza A H1N1pdm09 virus	IC*	-	-	-

* - Internal control RNA-IC "A"

Automatic analysis of the results for **Influenza A virus H1N1pdm09 REAL-TIME-PCR Detection Kit** is available on "DNA-Technology" made DTprime and DTlite real-time thermal cyclers; the latest version of the software is available for download at https://www.dna-technology.com/software.

The **Influenza A virus H1N1pdm09 REAL-TIME-PCR Detection Kit** is also approved for use with Rotor-Gene Q (QIAGEN), CFX96 (Bio-Rad) and Applied Biosystems QuantStudio 5 (Life Technologies Holdings Pte. Ltd.) real-time thermal cyclers.

3. CONTENT

The detailed description of content is represented in Tables 2-4.

Table 2. The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** content, package S, strips, for R3-P454-S3/9EU

Reagent	Description	Nominal volume	Amount
Paraffin sealed PCR-mix	Colorless or pink transparent liquid under waxy white fraction	1.44 mL (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 "RNA-IC ''A'"" is allowed

** - marking as "C+" is allowed

Table 3. The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** content, package S, for R3-P454-23/9EU

Reagent	Description	Nominal volume	Amount
Paraffin sealed PCR-mix	Colorless or pink transparent liquid under waxy white fraction	1.44 mL (15 µL in each tube)	96 individual 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 "RNA-IC ''A''" is allowed

** - marking as "C+" is allowed

Table 4. The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** content, package U, for R3-P454-UA/9EU

Reagent	Description	Nominal volume	Amount
PCR-mix	Colorless or pink transparent liquid	600 µL	1 tube
RT-PCR-buffer "U"	Colorless transparent liquid	600 µL	1 tube
Enzyme Taq/RT	Colorless transparent viscous liquid	30 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 "RNA-IC ''A''" is allowed

** - marking as "C+" is allowed

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

The kit, package S, is designed for 96 tests (no more than 12 runs), including test samples, negative and positive controls.

The kit, package U, is designed for 96 tests provided at least 5 samples in each run (3 test samples, negative control and positive control).

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

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.

For NA extraction from phlegm (method 1):

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

For NA extraction from phlegm (method 2):

- Mucolysin.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Refrigerator with freezer;
- Vortex mixer;
- Vortex rotor for strips (in case of using package S, strips REF R3-P454-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;
- RNase and DNase free 1.5 mL microfuge tubes with caps;
- RNase and DNase free 0.2 mL amplification tubes or a 96-well PCR microplate[1];
- DTstream dosing instrument (version 12M1 or 15M1) manufactured by DNA-Technology R&P, LLC[2];
- RNase and DNase free 200 µL filter tips for DTstream *M1 dosing instrument or recommended for similar dosing instrument[2];
- Centrifuge (RCF(g) at least 100) with microplate adapter[3];
- DTpack plate sealing instrument manufactured by DNA-Technology R&P, LLC[3];
- Polymer thermal seal for microplates[3];
- 384-well microplate[2];
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;

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 REAL-TIME PCR Detection Kit**, except the Enzyme Taq/RT, shall be stored in a refrigerator or a freezer at the temperature from 2 °C to 8 °C throughout the shelf life of the kit. The Enzyme Taq/RT shall be stored in a freezer at the temperature from minus 18 °C to minus 22 °C throughout the shelf life of the kit.The PCR-mix shall be stored in a refrigerator or a freezer at the temperature from 2 °C to 8 °C and out of light throughout the shelf life of the kit.

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

Transportation of the reagent kit is carried out in thermocontainers 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 throughout the shelf life of the kit.

It is allowed to transport the kit, except the Enzyme Taq/RT, in thermocontainers 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 and store at temperatures from 2 °C to 8 °C immediately on receipt.

It is allowed to transport the Enzyme Taq/RT in thermocontainers 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 and store at temperatures from minus 18 °C to minus 22 °C immediately on receipt.

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

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[1] Package U, REF R3-P454-UA/9EU, manual dosing

[2] Package U, REF R3-P454-UA/9EU, automated dosing

[3] Package U, REF R3-P454-UA/9EU

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

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

The kit stored under undue regime should not be used.

An expired **Influenza A virus H1N1pdm09 REAL-TIME-PCR Detection Kit** shall not be used.

We strongly recommend to follow the given instructions in order to obtain accurate and reliable results.

Conformity of **Influenza A virus H1N1pdm09 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 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 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 correspond 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

7.1. Biomaterial for the analysis

The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** is designed to detect RNA extracted from the nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, endotracheal aspirate, nasopharyngeal aspirate, phlegm.

Method limitations[4]: local application of medications (sprays, drops, creams and ointments) less than 24 hours before the analysis. When using aerosols and other forms of inhalations in the treatment of bronchial asthma, the material for the analysis should not be taken earlier than three hours after inhalation.

7.2. General requirements

[4] if it does not contradict the requirements of the used NA extraction kits

1. RT-PCR analysis is a direct method of laboratory analysis, therefore human biomaterial sampling shall be carried out from the localization spot of the infectious process. The decision on the necessity of the analysis shall be made by professional based on the anamnesis and aspects of the disease.

2. To obtain correct results, quality of sampling, its storage, transport and pretreatment are of great importance.

3. Incorrect biomaterial sampling may lead to unreliable results and, therefore, to the need to repeat sampling.

4. 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.

5. 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.

6. To prevent contamination, always open 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.

7.3. Interfering substances

The presence of PCR inhibitors in a sample may cause unreliable (doubtful) results. The sign of full PCR inhibition is the simultaneous absence of internal control and specific products 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 PCR-inhibiting interfering substances, it is necessary to follow the rules of biological material sampling. 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 of inhibitors from the sample; express methods of nucleic acid extraction are not recommended.

7.4. Sample collection

WARNING! Biomaterial pretreatment may be necessary before RNA extraction (see 7.6).

Phlegm

Material is taken into single-use graduated sterile vials with wide neck and screw caps with a volume of at least 50 mL in an amount at least 1.0 mL.

After sample collection the vial is tightly closed and labeled.

Note: Phlegm must be pretreated before RNA extraction. The procedure of phlegm pretreatment with a solution of trisubstituted sodium phosphate (Na3PO4) or mucolysin is given in the instruction to the reagent kit “DNA/RNA extraction kit (PREP-NA/PREP-NA-PLUS)” produced by “DNA-Technology R&P”, LLC.

Bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate

Material is taken into single-use screw cap tubes with a volume of up to 50 mL. After the sample collection the tube 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 with transport medium intended by the manufacturer for transport and storage of biological material samples for PCR analyses, and rinse it thoroughly in liquid for 10-15 s, 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.

7.5. Transport and storage of samples

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 or up to 1 week.

ATTENTION! Avoid repeated freezing and thawing of samples.

Nasopharyngeal and oropharyngeal swabs

Transport and storage conditions for nasopharyngeal and oropharyngeal swabs are determined by the instructions for the recommended transport media and kits for NA extraction.

7.6. Human biomaterial preparation for RNA extraction

Biomaterial preparation (if it is necessary) is performed in accordance with instructions for use to the reagent kits/sets used for NA extraction (see 8.1).
If extracting RNA from phlegm using PREP-NA reagent kit, use mucolysin (see PREP-NA instruction for use, method 2).

WARNING! When preparing nasopharyngeal and oropharyngeal swabs collected into tube with transport medium, samples of bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate, preliminary centrifugation is not required.

8. PROCEDURE

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

To prevent cross-contamination in the laboratory, the following rules are recommended:

- it is necessary to conduct visual assessment of incoming biomaterial and cull all samples if there are any tubes with broken integrity;
- if possible, it is recommended to analyze samples of the people from inpatient facility with symptoms of acute infection separately from the rest of the samples (the biological material for screening of exposed individuals and individuals 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 controls, starting from the stage of RNA extraction, for each protocol;
- use tips with aerosol filters at all stages of the analysis;
- strictly follow the analysis procedure, open the Eppendorf test tubes with tweezers (do not touch the insides of tube cap with gloved hand); when introducing reagents, do not touch tube with the tip (if this happened, change the tip immediately).

Analysis procedure

8.1 RNA extraction

RNA extraction is performed in accordance with the instruction for an extraction kit.
Use **100 µL of sample** for extraction.

1. WARNING! The volume of the obtained RNA preparation shall not exceed 50 µL. In case of using PREP-MB-NA-S and PREP-MB DWP extraction kit the volume may increase to 100 µL.
2. The obtained RNA preparation must be used in the course of two hours for reverse transcription and polymerase chain reaction. It is allowed to freeze the RNA preparation once and store at minus 18 °C and lower for no longer than 7 days.

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

Reagent kit	Biomaterial
PREP-NA DNA/RNA Extraction Kit	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

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

8.1.1.1 Internal control sample

To exclude false negative results of the study and to control the quality of the study, it is necessary to use an internal control sample added to the clinical samples at the stage of nucleic acid extraction.

The internal control (RNA-IC “A”) from the **Influenza A virus H1N1pdm09 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) form the PREP-NA extraction kits are not used during the RNA isolation.

8.1.1.2 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). Package S

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

ATTENTION! When using package S (R3-P449-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.

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 considering "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! Open the tube, add DNA sample (or control sample), then close the tube before proceeding to the next DNA 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).

- Set the strips into the Real-time Thermal Cycler.
- Launch the operating software for DT instrument[5]. Add corresponding test[6], 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 8.1.10) and run PCR. See Table 5.

For use with Rotor-Gene Q (QIAGEN), CFX96 (Bio-Rad) and Applied Biosystems QuantStudio 5 (Life Technologies Holdings Pte. Ltd.) thermal cyclers consult user manuals for devices. See Tables 6-8.

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

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						

Table 6. Amplification program for Rotor-Gene Q thermal cycler

Cycling	Temperature	Hold time	Cycle repeats
Cycling	32 deg	1200 sec	1 time
Cycling 2	95 deg	300 sec	1 time
Cycling 3	94 deg	10 sec	50 times
	60 deg √	15 sec	
√ - optical measurement, set the fluorescence measurement (Acquiring) on the channels Green (Fam) and Yellow (Hex) at 60 °C			

Table 7. Amplification program for CFX 96 thermal cycler

Block No. (Step)	Temperature, °C	Time Min:sec	Number of cycles (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 required detection channels (Fam, Hex) at 64 °C			

Table 8. Amplification program for Applied Biosystems QuantStudio 5 thermal cycler

Stage	Step No.	Temperature, °C	Time Min:sec	Number of cycles (repeats)
Hold	1	35	20:00	1
	2	95	5:00	1
PCR	1	94	0:15	50
	2	64 **√**	0:20	
√- data collection for the required fluorophores (Fam, Vic (Hex)) is on				

For CITO diagnostics, it is allowed to use a shortened amplification program for the “DTprime” detection thermal cycler (Annex A, Table A.1).

WARNING! It is not allowed to use a shortened amplification program in routine analyses.

8.3 PCR with Reverse Transcription (RT-PCR). Package U, manual dosing

WARNING!

1. For amplification use single-use 0.2 mL amplification tubes or 96-well PCR microplates[7] sealed by thermal film. It is not recommended to use strip tubes as it may lead to post-amplification contamination.
2. Avoid direct sunlight on the tubes with PCR-mix!

8.3.1. Mark the necessary amount of single-use 0.2 mL amplification tubes or 96-well PCR microplate for test samples, negative control “C-” and positive control “C+”.

N o t e . At least 5 samples per run (3 test samples, negative and positive control) are recommended.

E x a m p l e :

To test 6 samples, mark 6 tubes/microplate wells for test samples; 1 tube/well for “C-” and 1 tube/well for “C+”. Total number of tubes/wells is 8.

8.3.2. Vortex the tube with PCR-mix for 3-5 seconds and spin on vortex for 1-3 seconds.

8.3.3. Add 6 µL of PCR-mix into each marked tube/microplate well (including “C-” and “C+”).

[7] - 96-well microplates are not used for DTlite and Rotor-Gene Q thermal cyclers.

8.3.4. Vortex tubes with RT-PCR-buffer “U” and Taq/RT enzyme for 3-5 seconds and spin on vortex for 1-3 seconds.

WARNING! Take Taq/RT enzyme out of the freezer immediately before use.

8.3.5. Prepare a mixture of RT-PCR-buffer “U” and Taq/RT enzyme. Mix in a separate tube:

- 6.0 x (N+1) µL of RT-PCR-buffer “U”;
- 0.3 x (N+1) µL of Taq/RT enzyme,

where N is the number of marked tubes/required microplate wells considering “C-” and “C+”.

E x a m p l e :

You have to test 6 samples. There are 8 marked tubes/necessary microplate wells. Prepare a mixture for 9 (8+1) tubes/wells, i.e. 54 µL of RT-PCR-buffer “U” + 2.7 µL of Taq/RT enzyme.

WARNING! When taking Taq/RT enzyme dip the tip no deeper than 1.0 mm and observe the rules of dosing for viscous liquids. Wash the leftovers of Taq/RT enzyme from the tip by pipetting at least 5 times.

8.3.6. Vortex the tube with the mixture of RT-PCR-buffer “U” and Taq/RT enzyme for 3-5 seconds and spin on vortex for 1-3 seconds.
It is only allowed to store the mixture for 1 hour at 2 °C to 8 °C.

8.3.7. Add 6.0 µL of mixture of RT-PCR-buffer “U” and Taq/RT enzyme into each tube/well with PCR-mix. Close the tubes.

WARNING! After adding mixture of RT-PCR-buffer “U” and Taq/RT enzyme into each tube/well with PCR-mix it is necessary to perform pp. 8.3.8 – 8.3.14 in the course of one hour.

8.3.8. Vortex the tube with positive control for 3-5 seconds and spin on vortex for 1-3 seconds.

WARNING!

1. Before adding RNA preparation and negative control into tubes/wells with reaction mixture, follow the recommendations from the instructions for an NA extraction kit.
2. When using PREP-NA or PREP-NA-S for RNA extraction, vortex the tubes with RNA preparation and negative control for 3-5 seconds and spin on vortex for 1-3 seconds.
3. When using PREP-MB-DBS DWP, spin the sealed deep-well plate with RNA preparation and negative control for 30 seconds at RCF(g) 100 to spin down the drops, then take off the seal.
4. To prevent contamination, only open caps of the tubes where the RNA sample is going to be introduced, and close it before proceeding to the next one. Use filter tips.

8.3.9. Add 6.0 µL of extracted RNA preparation into the corresponding marked tubes/necessary microplate wells. Do not add RNA preparation into tubes/wells marked as “C-“ and “C+”.

8.3.10. Add 6.0 µL of negative control that passed RNA extraction stage into the tube/microplate well marked “C-“.

8.3.11. Add 6.0 µL of positive control into the tube/microplate well marked “C+”.

8.3.12. In case of using 96-well microplate:

8.3.12.1. Place microplate carefully, without shaking, into the DTpack plate sealing device.
8.3.12.2. Seal the PCR microplate with polymer thermal film according to the DTpack operation manual.

8.3.12.3. Spin the PCR microplate for 30 seconds at RCF(g) 100.

Spin all the tubes on vortex for 3-5 seconds on vortex mixer (if using Rotor-Gene Q thermal cycler for RT-PCR spinning is not required).

8.3.13. Set all the tubes/microplate into the thermal cycler and perform RT-PCR (see 8.3.15, 8.3.16).

8.3.14. For DT thermal cyclers:

Launch thermal cycler software. For the first RT-PCR upload the required test[8]. For further runs create the corresponding analysis protocol: specify the number and IDs of samples, including positive and negative controls, mark the positioning of samples on the heat block matrix and run RT-PCR. When selecting test, a program from Table 9 must show up.

8.3.15. For Rotor-Gene Q, CFX96 and Applied Biosystems QuantStudio 5 thermal cyclers:

Run RT-PCR considering the 18 µL volume of reaction mixture according to programs shown in Tables 6 – 8.

Table 9 – DTprime, DTlite thermal cyclers amplification program (package U)

Block No.	Temperature, °C	min	sec	Number of cycles	Optical measurement	Block type
1	47	20	0	1		Cycle
2	95	5	0	1		Cycle
3	94	0	10	50		Cycle
	64	0	15		√	
4	80	0	1	1		Cycle
5	10	…	…	Hold		Hold
√- optical measurement						

8.4 PCR with Reverse Transcription (RT-PCR). Package U, DTstream (only for DTprime thermal cycler, modification DTprime *X*)

WARNING!

1. For amplification use 384-well PCR microplates sealed with thermal film.
2. Avoid direct sunlight on the tubes with PCR-mix!

Note - At least 5 samples per run (3 test samples, negative and positive control) are recommended.

8.4.1. Vortex the tube with PCR-mix for 3-5 seconds and spin on vortex for 1-3 seconds.

8.4.2. Vortex tubes with RT-PCR-buffer “U” and Taq/RT enzyme for 3-5 seconds and spin on vortex for 1-3 seconds.

WARNING! Take Taq/RT enzyme out of the freezer immediately before use.

[8] - Test for DT thermal cyclers is created by entering parameters or is provided by the kit manufacturer.

8.4.3. Follow the instruction from DTstream dosing instrument and prepare a mixture of RT-PCR-buffer "U" and Taq/RT enzyme in a separate tube.

WARNING! When taking Taq/RT enzyme dip the tip no deeper than 1.0 mm and observe the rules of dosing for viscous liquids. Wash the leftovers of Taq/RT enzyme from the tip by pipetting at least 5 times.

8.4.4. Vortex the tube with the mixture of RT-PCR-buffer "U" and Taq/RT enzyme for 3-5 seconds and spin on vortex for 1-3 seconds.

8.4.5. Vortex the tube with positive control for 3-5 seconds and spin on vortex for 1-3 seconds.

WARNING!

1. Before adding RNA preparation and negative control into tubes/wells with reaction mixture, follow the recommendations from the instructions for an NA extraction kit.
2. When using PREP-NA or PREP-NA-S for RNA extraction, vortex the tubes with RNA preparation and negative control for 3-5 seconds and spin on vortex for 1-3 seconds.

8.4.6. Set the tubes with PCR-mix, mixture of RT-PCR-buffer "U" and Taq/RT enzyme, RNA preparations, negative controls and positive controls, and PCR microplate on the DTstream working table and perform dosing of components according to operation manual.

8.4.7. Place microplate carefully, without shaking, into the DTpack plate sealing device after DTstream finishes dosing.

8.4.8. Seal the PCR microplate with polymer thermal film according to the DTpack operation manual.

8.4.9. Spin the PCR microplate for 30 seconds at RCF(g) 100.

8.4.10. Set the PCR microplate into the thermal cycler and perform RT-PCR.

Launch thermal cycler software. For the first RT-PCR upload the required test[9]. For further runs create the corresponding analysis protocol: specify the number and IDs of samples, including positive and negative controls, mark the positioning of samples on the heat block matrix and run RT-PCR. When selecting test, a program from Table 9 must show up.

9. CONTROLS

The **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** contains positive control sample. Positive control is a cloned part of the virus genome. It is produced with genetic engineering techniques and characterized by automatic sequencing. The kit includes the Internal control RNA-IC "A". RNA-IC "A" is intended to assess the quality of polymerase chain reaction. To reveal possible contamination a negative control is required.

WARNING! 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 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 **Influenza A virus H1N1pdm09 REAL-TIME PCR Detection Kit** the test result is considered invalid when

[9] - Test for DT thermal cyclers is created by entering parameters or is provided by the kit manufacturer.

the exponential growth of the fluorescence level for the specific product and for internal control is not observed.

If negative result is obtained for positive control (C+), it is necessary to repeat the whole test (from PCR stage). It may be caused by inhibitors, operation error or violation of storage and handling requirements.

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

10. DATA ANALYSIS

10.1. Registration of PCR results is held in automatic mode by the software delivered with thermal cycler.

10.2. When using CFX96 thermal cyclers, use regression type analysis (Cq Determination Mode: Regression); select “Baseline Subtraction Curve Fit” in the “Baseline Subtraction” tab.

10.3. Interpretation of the PCR results should be performed according to the Table 10. Run results are valid, if the conditions for interpretation of the results obtained for controls are fulfilled.

Table 10. Interpretation of RT-PCR results

Detection channel		**Result interpretation**
Fam/Green (the sought RNA), Cp/Cq/Ct	**Hex/Yellow/Vic (internal control), Cp/Cq/Ct**	
Samples		
Specified	Not considered	**Influenza A virus H1N1pdm09 RNA is detected**
Not specified	S**pecified**	Influenza A virus H1N1pdm09 RNA is not detected
Not specified	Not specified	Invalid (unreliable) result
Negative control sample		
Not specified	**Specified**	**Negative result**. The results of PCR run are valid.
Positive control sample		
Specified	Not specified	**Positive result**. The results of PCR run are valid.

10.4. Unreliable result can be caused by the presence of PCR inhibitors in NA sample obtained from biomaterial, mistakes at the preanalytical stage, incorrect analysis procedure, violation of the amplification temperature regime, etc. In this case either NA extraction and RT-PCR for this sample, or biomaterial sampling should be repeated (performed sequentially).

10.5. If a positive result is obtained for negative control, the results of the whole run are considered invalid. In this case special measures for detection and elimination of possible contamination are necessary.

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

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 the

infected.

11. SPECIFICATIONS

a. Analytical specificity

In the samples of human biomaterial containing Influenza A virus H1N1pdm09 RNA, thermal cycler software shall register positive amplification result of specific product (Influenza A virus genome fragment) on the corresponding detection channels.

In the samples of human biomaterial not containing Influenza A virus H1N1pdm09 RNA, thermal cycler software shall register negative amplification result of specific product (Influenza A virus genome fragment) and positive result of internal control amplification.

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 no non-specific positive results of amplification of RNA sample in the presence of RNA of Influenza virus A(H3N2), 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 of 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 trials showed the presence of specific amplification results when RNA extracted from 15 influenza A subtype A(H1N1)pdm09 strains of different epidemic seasons were present in the sample.

b. Analytical sensitivity

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

The analytical sensitivity was set 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	RNA extraction reagent kit	Obtained preparation volume, µL	Limit of detection, copies/mL of sample
Naso-/oropharyngeal swabs in 500 µL of transport medium;	PREP-NA	50	1000
	PREP-NA-S	50	1000
	PREP-MB-NA-S	100	2000
	PREP-MB DWP	100	2000
Bronchoalveolar lavage, endotracheal, nasopharyngeal aspirate	PREP-NA	50	1000
Phlegm (pretreated with mucolysin)	PREP-NA	50	5000

12. TROUBLESHOOTING

Table 8. Troubleshooting

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

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

Phone: +7(495)640.16.93

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

13. QUALITY CONTROL

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

- observation of quality management in manufacturing of 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 REAL-TIME PCR Detection Kit**.

Technical support:

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Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

http://www.dna-technology.com

14. KEY TO SYMBOLS

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

R3-P454-S3/9EU
R3-P454-23/9EU
R3-P454-UA/9EU

1032.2023.11.15

Annex A

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

WARNING!

1. Before work take the nucleic acid extraction kit from the refrigerator and make sure there is no precipitate in lysis solution. In case there is precipitate, warm up the solution at 65 °C until precipitate dissolves and mix by turning the vial upside down 5-10 times, avoiding foaming.

2. Caps may open during heating. Use tubes with lock caps (e.g. Eppendorf Safe-Lock Tubes) or programmable thermostats with clamp cover.

- Mark one 1.5 mL plastic for each test sample and negative control ("C-").
- Add 10 µL of preliminary vortexed internal control RNA-IC "A" from Influenza A virus H1N1pdm09 reagent kit 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 the marked 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 on vortex-mixer for 3-5 seconds.
- Add 400 µl of the precipitation buffer without touching the walls of the tube. Close the tubes tightly and vortex them for 3–5 seconds.
- Centrifuge the tubes at RCF(g) 12000 - 16000 for 10 minutes at room temperature (18-25 °C).
- Remove supernatant completely, avoid touching the precipitate. Use new tip for each sample.
- Add 500 µL of the wash solution №1 to the precipitate without touching the walls of the tube, 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, avoid touching 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, avoid touching the precipitate. Use new tip for each sample. It is allowed to leave the liquid covering the precipitate 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 dilution buffer to the precipitate, close the tubes.
- Vortex the tubes for 3-5 seconds and spin down the drops by centrifuging for 3-5 seconds.
- 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) to precipitate the condensate.

The RNA 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 study, 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 thawing before run.

Table A.1 – Shortened amplification program for the “DTprime” detection thermal cycler (only allowed for CITO diagnostics)

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						