Diagnostic kit rTEST COVID-19/FLU qPCR kit / 400 reactions

User Information

Ver. 1.0 (en), 10/2020

NOTICE!
This kit is intended for *in vitro* diagnostic purposes.

1. Explanations and abbreviations

Baseline: basic background fluorescence or so-called „noise"
BHQ-1: Black Hole Quencher-1, a non-fluorescent quencher for the FAM fluorescent reporter dye
BHQ-2: Black Hole Quencher-2, a non-fluorescent quencher for the YY fluorescent reporter dye
BSL: bio-safety level
COVID-19: disease caused by the SARS-CoV-2 virus (COronaVIrus Disease 2019)
Ct: threshold cycle, the cycle in which the fluorescence signal of the reaction exceeds the set fluorescence threshold
Cy5: cyanine 5, a fluorescent reporter dye
DNA: deoxyribonucleic acid
E gene: a gene encoding the small membrane envelope protein of the SARS-CoV-2 virus
FAM: 6-carboxyfluorescein, a fluorescent reporter dye
IAV: Influenza A Virus
IBV: Influenza B Virus
NTC: No Template Control
PA gene: a gene located on segment 3 encoding a polymerase subunit of IBV responsible for protease activity
PB1 gene: a gene located on segment 2 encoding a polymerase subunit of IAV responsible for RNA elongation and endonuclease activity
PC: Positive Control
qPCR: quantitative Polymerase Chain Reaction
RdRP gene: a gene encoding the RNA-dependent RNA polymerase of the SARS-CoV-2 virus
RNA: ribonucleic acid
RNase P gene: a gene encoding human nuclear ribonuclease P
ROX: 6-carboxy-X-rhodamine, a fluorescent reference dye used to normalize the reporter dye signal
RT-qPCR: reverse transcription qPCR
SARS-CoV-2: Severe Acute Respiratory Syndrome CoronaVirus 2
Threshold: the point at which the fluorescence reporter signal significantly exceeds background fluorescence
rTEST: room TEmperature STable
YY: Yakima Yellow®, a fluorescence reporter dye developed by Epoch Biosciences

2. Intended use

The rTEST COVID-19/FLU qPCR kit is a qualitative in vitro diagnostic test designed to detect the presence of SARS-CoV-2, Influenza A Virus (IAV) and Influenza B Virus (IBV) genetic material in biological samples obtained primarily from the human upper airways (nose and nasopharynx). The kit is intended exclusively for use in a diagnostic laboratory with the appropriate equipment, safety standards and properly trained personnel.

3. Test principle

The rTEST COVID-19/FLU qPCR kit is the first fully room temperature stable diagnostic kit to detect genomic RNA of SARS-CoV-2, IAV and IBV. It can be stored at room temperature for at least one month enabling transportation without dry ice and easier handling of the kit upon arrival. The room temperature stability is allowed by lyophilized mixes of SARS-CoV-2, IAV- and IBV-specific primers/probes, internal positive control (full genomic viral RNA of SARS-CoV-2, IAV and IBV spiked with human RNA), and proprietary, room temperature stable 1-step RT-qPCR reagents from Solis Biodyne.

The rTEST COVID-19/FLU qPCR kit is an innovative, improved, and re-designed version of the WHO-recommended Charité, Berlin protocol, along with a newly designed differential test distinguishing between SARS-CoV-2, IAV and IBV. The kit contains one set of primers and hydrolysis probes (TaqMan®) targeting either the SARS-CoV-2 specific E gene or part of segment 2, encoding the PB1 subunit of IAV. The second set of primers and hydrolysis probes (TaqMan®) is designed to detect the SARS-CoV-2 specific RdRP gene and a part of segment 3, encoding the PA subunit of IBV. In addition, both sets allow the detection of the human RNase P gene. The TaqMan® probes for E and RdRP gene are conjugated to FAM, the TaqMan® probes for IAV and IBV are conjugated to YY, and the TaqMan® probe for RNase P is conjugated to Cy5. This enables multiplexed detection of SARS-CoV-2, IAV, IBV and human RNase P, which serves as an internal control to validate proper sample collection, RNA extraction, and performance of the test. **For E and RdRP genes, we developed proprietary dual TaqMan® probes to increase the sensitivity and specificity of our 1-Step RT-qPCR method.** The full genomic viral RNA of SARS-CoV-2, IAV and IBV spiked with human RNA provided by the Biomedical Center of the Slovak Academy of Sciences is included as an internal positive control. The 5X One-step Probe CoV Mix (ROX) reagent includes ROX passive reference dye as an inert additive providing a constant fluorescent signal for sample normalization in the real-time PCR assay. Due to the unique molecular structure of the supplied ROX dye, the solution is compatible with all ROX-dependent (both high-ROX and low-ROX) and ROX-independent real-time PCR cyclers.

The RT and qPCR reactions are conducted in a one tube, 1-step fashion. The recommended workflow consists of an initial first-line multiplex diagnostic assay for the SARS-CoV-2_E gene, IAV and human RNase P gene followed by a multiplex assay for the SARS-CoV-2 specific RdRP gene, IBV and human RNase P gene. Multiplexing allows co-detection of and distinguishing between SARS-CoV-2, IAV and IBV, and detection of human RNase P, which can be used for secondary confirmation of negative samples, as well as to validate sample collection, RNA extraction, and performance of the qPCR reaction.

One package of the kit is sufficient for 400 testing reactions. This kit offers flexibility in testing by permitting either screening using two sets of primers and probes (200 screening tests for SARS-CoV-2_E/IAV/RNase P and 200 tests for SARS-CoV-2_RdRP/IBV/RNase P detection) or can be exclusively used for 400 general screening tests detecting SARS-CoV-2 and either IAV or IBV. This option is recommended when many symptomatic patients have to be screened at once and is ensured by the compatibility of both primer/probe sets in one reaction. For detailed information about the preparation of general diagnostic assays, see section 7.5. Preparation of the reaction mixture.
The human RNase P internal positive control assay in multiplex mode with the E gene and the IAV or RdRP gene and IBV serves to verify the quality of the swab and RNA extraction, to determine the presence of human RNA within the extracted sample, while eliminating false negative results.

NOTICE!

- **Read all instructions in this user manual, the outer packaging, and the component labels before starting work.**

CAUTION!

SARS-CoV-2, IAV, and IAB are dangerous pathogens so follow all applicable regulations and recommendations for BSL2+ or BSL3 class laboratories.

4. Kit composition

- 1x lyophilized primers/probes mix for SARS-CoV-2_E, IAV, and human RNase P, labelled as E gene/IAV/RNase P Mix (must be dissolved in 800 µl of PCR water)
- 1x lyophilized primers/probes mix for SARS-CoV-2_RdRP, IBV, and human RNase P, labelled as RdRP gene/IBV/RNase P Mix (must be dissolved in 800 µl of PCR water)
- 1x lyophilized PC4.01 (must be dissolved in 200 µl of PCR water)
- 2x 800 µl 5X One-step Probe CoV Mix (ROX)
- 1x 200 µl 40X One-step SOLIScript® CoV Mix
- 2x 5 ml PCR water
- 1x Instructions for use

5. Storage and shelf life:

All kit components can be transported and stored at room temperature (15-25 °C) up to 1 month. The kit can be routinely stored at −20 °C. The 5X One-step Probe CoV Mix (ROX) contains ROX as a reference dye that is photo-sensitive and should be protected from light whenever possible. The shelf life of the kit is a maximum of 12 months from the date of manufacture. The exact expiration date of the kit is indicated on the outer box. The exact expiration date of the individual components of the kit is indicated on the inner packaging/vials.

CAUTION!

Do not use the kit after the expiry date, which is stated on the outer box.

The kit and its individual components are designed to perform 400 reactions.
For users who perform fewer reactions within one run, we recommend that you aliquot all kit components according to internal procedures and the standard number of reactions per run. Aliquoting the kit components minimizes the need to reopen individual tubes and thus minimizes the risk of contamination of the kit components. Aliquoting the components

of the kit will also prevent repeated thawing and freezing of the individual components, which may lead to reduced efficiency. Before aliquoting, it is necessary to completely thaw the individual components of the kit. Thawing is optimally performed gently, by incubation in a refrigerator (at 4 °C) for approx. 2 hours. Immediately before aliquoting, it is necessary to mix the contents of the tubes thoroughly, but at the same time carefully, avoiding the formation of bubbles, until the mixture is completely homogeneous. In the case of tubes containing enzymes (5X One-step Probe CoV Mix (ROX), 40X One-step SOLIScript® CoV Mix), we recommend mixing by rotating the tube several times, pulse vortexing, or short vortexing for no longer than 5 seconds without generating any bubbles. Pipette the enzymes carefully and slowly; otherwise, the viscosity of the buffer may lead to pipetting errors.

NOTICE!

- **Wear suitable protective clothing, gloves, and eye/face protection.**
- **Never pipette by mouth.**
- **Never eat, drink, or smoke in the laboratory and do not use any cosmetics.**
- **Always wash your hands thoroughly when handling specimens and reagents.**

6. Consumables and equipment not included in the kit

- Real-time PCR equipment: the rTEST COVID-19/Flu qPCR kit has been validated and tested on Agilent devices - Mx3005P® and AriaMx®, Thermo Fisher Scientific - QuantStudio™ 5, BioRad - CFX96™, Analytik Jena - qTOWER3
- Laminar flow box
- Vortex mixer
- Mini centrifuge
- Centrifuge with rotor for plates
- Personal protective equipment: powder-free disposable laboratory gloves, goggles, protective shield, FFP3 respirator, protective clothing
- Laboratory plasticware certified sterile and free of DNA, RNA, DNases and RNases: reagent tubes, PCR tubes, PCR strips, PCR plates, PCR foils, sterile pipette tips with filter
- Autoclavable adjustable micropipettes
- Bio-waste container
- Autoclavable test tube racks
- PCR tube/plate cooler

7. Workflow

CAUTION!

Any work with the kit must be performed by qualified personnel.

NOTICE!

- **Workspaces must be arranged in such a way that there are separate, dedicated rooms (zones), laboratory equipment, and consumables for each step in the workflow: nucleic acid isolation, preparation of amplification reactions, and amplification and detection of amplification products. The amplified products must never come into contact with space, equipment, and consumables intended for the isolation of nucleic acids or for the preparation of amplification mixtures.**
- **Use separate, designated lab coats, gloves, and all other personal protective equipment for each step in the workflow. Never use the same personal protective equipment in different rooms (zones).**
- **Always handle all biological samples as potentially infectious material and avoid direct contact with biological material. Avoid spilling samples and reagents and generating aerosols.**
- **After sample preparation, it is advised to avoid excessive delay before starting the reaction in a thermocycler.**
- **Follow the enclosed instructions for use thoroughly.**

7.1. Sampling and RNA extraction

Improper sample collection, transport, and storage procedures as well as RNA extraction may result in incorrect test results. Users should refer to established guidelines for collecting, transporting, and storing samples and should adhere to manufacturer instructions for specimen collection. Sterile swabs with a plastic or aluminum shaft and a synthetic swab must be used for sampling. Swabs with a wooden shaft and / or a cotton swab must not be used. Following sample collection, immerse swabs immediately into sterile tubes containing 2-3 ml of viral transport media.

RNA extraction from the samples should be performed promptly after collection according to the manufacturer's instructions. If a delay in extraction is expected, samples must be stored at 4 °C for up to 12 hours after collection or at −70 °C for long-term sample storage. Avoid repeated thawing and freezing of samples.

The rTEST COVID-19/FLU qPCR kit was validated and tested on RNA samples obtained using the following extraction kits:

- Zymo Research, Quick-RNA Viral 96 Kit (Catalog # R1040, R1041)
- Cytiva (formerly GE Healthcare Life Sciences), RNAspin 96 Kit (Catalog # 25050075)
- RNAdvance Viral Genomic Reagent (magnetic beads, Catalog # C59543, C63510)

CAUTION!

During the workflow, always wear personal protective equipment and work in a laminar flow box. Viral RNA can also cause infection. Thus, handle all samples with caution and treat as potentially infectious material.

NOTICE!

- **The samples analyzed are intended solely for this type of analysis.**
- **Strictly follow sample processing guidelines to avoid degradation of nucleic acids.**
- **Do not open different samples at the same time to avoid possible cross contamination.**
- **Vortex and centrifuge the samples in a laminar flow box to prevent aerosol contamination.**
- **Use pipettes designated exclusively for handling specimens and use disposable filter tips that are certified sterile and free of DNA, RNA, DNases and RNases.**

7.2. RT-qPCR

The routine rTEST COVID-19/FLU qPCR kit workflow begins with a multiplex diagnostic test to determine the presence of viral RNA for the SARS CoV-2 E gene and IAV or SARS CoV-2 RdRP gene and IBV (order is based on user preference). Next, a multiplex diagnostic test is conducted to determine the presence of viral RNA for SARS CoV-2 RdRP gene and IBV or SARS CoV-2 E gene and IAV (order is based on user preference). Moreover, an internal control test for human RNase P is included in each multiplex assay with E gene/IAV or RdRP gene/IBV to confirm swab and RNA extraction efficiency and assay performance.

NOTICE!

- **RNase P is a human transcript, therefore the primers and probe mix designed for RNase P can detect both RNA and genomic DNA if present in RNA extract.**

An alternative approach involves combining both sets of primers and probes into a single reaction, ensuring the detection of SARS CoV-2, IAV, IBV, and human RNase P in one reaction, without the possibility of distinguishing between IAV and IBV.

7.3. Workspace preparation

Before starting the protocol, first clean the working space of the laminar flow box and adjacent surfaces with a 10% solution of sodium hypochlorite (bleach) and then with a 70% solution of ethanol to remove residual bleach. Use the UV decontamination cycle before and after working in the laminar flow box.

7.4. Preparation of reagents

Remove the necessary kit components from the freezer and thaw completely in the refrigerator (4 °C), on ice, or in a refrigerated tube cooling rack. Once thawed, mix their contents thoroughly and gently until completely homogeneous.
We recommend mixing tubes containing enzymes (5X One-step Probe CoV Mix (ROX), 40X One-step SOLIScript® CoV Mix) by rotating the tube several times, pulse vortexing, or short vortexing for no longer than 5 seconds without generating any bubbles. Then centrifuge the tubes briefly to remove droplets from the cap and ensure all liquid is at the bottom of the tubes. Pipette enzyme mixes carefully and slowly as the viscosity of the buffer may lead to pipetting errors. Mixtures of primers and TaqMan® probes designated SARS-CoV-2_E, SARS-CoV-2_RdRP, IAV, IBV and Human RNase P (provided as E gene/IAV/RNase P Mix or RdRP/IBV/RNase P Mix) as well as the PC4.01 spiked with human RNA are supplied in lyophilized form to increase stability. It is therefore necessary to dissolve mixtures of primers and probes and the positive control in PCR water when the kit is used for the first time.

CAUTION!

Since the colored oligo pellet can become dislodged during shipping, it is crucial to briefly centrifuge every tube before opening. Failure to do so could result in yield loss, because oligo pellets that are not at the bottom of the tube could fly out of the tube when the cap is opened.

Add 800 µl of water from the PCR Water tube to the tube labeled as E gene/IAV/RNase P Mix (400 reactions) and 800 µl of water from the PCR Water tube to the tube labeled as RdRP gene/IBV/RNase P Mix (400 reactions). In addition, add 200 µl of water from the PCR Water tube to the tube containing positive control PC4.01.

Thoroughly vortex the contents of the tubes. Then briefly centrifuge the tubes to remove droplets from the cap and ensure all liquid is at the bottom of the tubes.
Prepare the reaction mixture as soon as possible after mixing the contents of the individual components of the kit. If necessary, vortex and centrifuge the contents of the tubes once more just before preparing the reaction mixture.
After use, store kit components in the freezer

(−20 °C). Avoid repeated thawing and freezing of kit components. If any kit components will be reused within 2 hours, store at 4 °C.

CAUTION!

Always handle reagents in a laminar flow box. Always prepare reagents for amplification separately, preparing reagents exclusively for one analysis at a time. Use pipettes designed exclusively for preparation of reagents and use disposable filter tips. The tips used must be sterile and free of DNA, RNA, DNases and RNases.

NOTICE!

- **Use only reagents contained in this kit and reagents recommended by the manufacturer.**
- **Do not combine or mix reagents from different lots.**
- **Do not combine reagents from kits from different manufacturers.**

7.5. Preparation of the reaction mixture

The recommended total volume of one reaction is 20 µl. To prepare the reaction mixture, the individual components of the kit must be mixed in the following order and ratio:

Table 1. **Reaction mixture setup**

Kit component	Component volume per reaction		
	E gene/IAV/ RNase P	**RdRP gene/IBV/ RNase P**	**E gene/IAV/RNase P + RdRP/IBV/RNase P**
PCR water	8,5 µl	8,5 µl	7,5 µl
5X One-step Probe CoV Mix (ROX)	4 µl	4 µl	4 µl
40X One-step SOLIScript® CoV Mix	0,5 µl	0,5 µl	0,5 µl
E gene/IAV/RNase P Mix **and/or** RdRP gene/IBV/RNase P Mix	2 µl	2 µl	**1 µl** E gene/IAV/RNase P Mix **+ 2 µl** RdRP gene/ IBV/RNase P Mix
Total volume	**15 µl**	**15 µl**	**15 µl**

Table 2. **Calculated volumes for a given number of reactions**

Number of reactions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**96**
5X One-step Probe CoV Mix (ROX)	4 µl	8 µl	12 µl	16 µl	20 µl	24 µl	28 µl	32 µl	36 µl	40 µl	384 µl
40X One-step SOLIScript® CoV Mix	0,5 µl	1 µl	1,5 µl	2 µl	2,5 µl	3 µl	3,5 µl	4 µl	4,5 µl	5 µl	48 µl
E gene/RNase P Mix **or** RdRP gene/RNase P Mix	2 µl	4 µl	6 µl	8 µl	10 µl	12 µl	14 µl	16 µl	18 µl	20 µl	192 µl
PCR water	8,5 µl	17 µl	25,5 µl	34 µl	42,5 µl	51 µl	59,5 µl	68 µl	76,5 µl	85 µl	816 µl
Total volume	**15 µl**	**30 µl**	**45 µl**	**60 µl**	**75 µl**	**90 µl**	**105 µl**	**120 µl**	**135 µl**	**150 µl**	**1440 µl**

Table 3. **Calculated volumes for a given number of reactions when using both sets of primers/probes in one reaction**

Number of reactions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**96**
5X One-step Probe CoV Mix (ROX)	4 µl	8 µl	12 µl	16 µl	20 µl	24 µl	28 µl	32 µl	36 µl	40 µl	384 µl
40X One-step SOLIScript® CoV Mix	0,5 µl	1 µl	1,5 µl	2 µl	2,5 µl	3 µl	3,5 µl	4 µl	4,5 µl	5 µl	48 µl
E gene/IAV/RNase P Mix **and** RdRP gene/IBV/ RNase P Mix	1 µl + 2 µl	2 µl + 4 µl	3 µl + 6 µl	4 µl + 8 µl	5 µl + 10 µl	6 µl + 12 µl	7 µl + 14 µl	8 µl + 16 µl	9 µl + 18 µl	10 µl + 20 µl	96 µl + 192 µl
PCR water	7,5 µl	15 µl	22,5 µl	30 µl	37,5 µl	45 µl	52,5 µl	60 µl	67,5 µl	75 µl	720 µl
Total volume	**15 µl**	**30 µl**	**45 µl**	**60 µl**	**75 µl**	**90 µl**	**105 µl**	**120 µl**	**135 µl**	**150 µl**	**1440 µl**

NOTICE!

- **The reaction mixture has limited stability, use it as soon as possible after preparation. If the reaction mixture cannot be used immediately, store it in a refrigerator at 4 °C.**
- **When preparing multiple reactions, it is recommended to make 5 - 10% extra reaction mixture to account for pipetting errors.**

7.6. Plate preparation and inspection

Add 5 µl of sample to each prepared 15 µl reaction mixture, resulting in a 20 µl total reaction volume.

Prepare the required number of clean PCR tubes, PCR strips, or PCR plates and place them in a refrigerated cooling rack. Mix the prepared reaction mixture thoroughly but at the same time gently by turning the tube several times, pulse vortexing, or short vortexing for no longer than 5 seconds without generating any bubbles. Then centrifuge it briefly to remove droplets from the cap and ensure all the liquid is at the bottom of the tube. Pipette 15 µl of the prepared reaction mixture into individual PCR tubes or wells of a PCR plate in accordance with the required number and position of reactions. Pipette the reaction mixture carefully and slowly as the viscosity of the buffer may lead to pipetting errors. Then transfer the PCR tubes or PCR plate with the pipetted reaction mixture from the laminar flow box for preparation of the reaction mixture to the laminar flow box for finalization of plate preparation. Add 5 µl of sample, or 5 µl of positive control (PC4.01), or 5 µl of PCR water (NTC) into appropriate PCR tubes or PCR plate wells. Then tightly seal the individual PCR tubes with the lids or wells of the PCR plate with optical foil. Centrifuge the PCR tubes or PCR plate briefly so that all fluid is at the bottom of the tubes/wells and insert into the real-time PCR instrument.

A minimum of one negative control must be included in each analysis to verify the presence of contamination. A no template control (NTC) containing PCR water is used as a negative control instead of an unknown sample. A separate NTC for each set of primers/probes (E gene/IAV/RNase P, RdRP gene/IBV/RNase P) tested must be included.
A minimum of one positive control (PC) must be included in each analysis to validate the workflow of the analysis and the functionality of the kit components. A reaction containing the positive control (PC4.01) is used instead of an unknown sample. A separate PC for each set of primers/probes (E gene/IAV/RNase P, RdRP gene/IBV/RNase P) tested must be included.

The PC4.01 consists of isolated viral genomic RNA of SARS-CoV-2, IAV, and IBV spiked with human RNA. The PC4.01 will yield a positive result with all primer and probe sets (SARS CoV-2 primers/probes for E and RdRP genes; IAV primers/probe detecting PB1 gene; IBV primers/probe detecting PA gene; and primers/probe for human RNase P).

7.7. Real-time PCR instrument settings

Follow the instructions below to set the assay conditions for the reaction volume, temperature conditions, and optical channels.

Reaction volume:
- **20 µl**

Thermocycling conditions:
- **Reverse transcription: 55 °C, 10 min**
- **Initial denaturation: 95 °C, 10 min**
- **Cycling, 45 cycles:**
  - **» Denaturation: 95 °C, 15 s**
  - **» Annealing/extension: 60 °C, 30 s**

Optical channels used:

- Optical channel for FAM label: blue or green channel according to the real-time PCR device - excitation maximum 495 nm, emission maximum 520 nm
- Optical channel for YY label: yellow channel (HEX, JOE or VIC) according to the real-time PCR device - excitation maximum 525 nm, emission maximum 550 nm
- Optical channel for ROX dye: orange or red channel according to the real-time PCR device - excitation maximum 575 nm, emission maximum 605 nm
- Optical channel for Cy5 label: red channel - excitation maximum 650 nm, emission maximum 670 nm

If the real-time PCR device supports normalization to the ROX passive reference dye, we recommend performing the analysis with this function enabled. The 5X One-step Probe CoV Mix (ROX) contains ROX with a unique molecular structure as a reference dye providing a constant fluorescent signal for sample normalization of the assay. The fluorescent signal of the ROX dye does not interfere with the detection channel of the FAM fluorescent label. As a result, the rTEST COVID-19/FLU qPCR kit is fully compatible with all ROX-dependent and ROX-independent real-time PCR cyclers.

Follow the real-time PCR equipment manufacturer's manual and your internal procedures for this type of assay when setting the analysis conditions for the number and type of samples, the distribution of samples on the plate, and the type of plasticware used (tubes, strips, plates).

CAUTION!

Do not modify or change the recommended protocols for PCR analyses.

CAUTION!

Handle amplification products with extreme care to avoid dispersal into the laboratory area and possible contamination of new test specimens. Use pipettes designated exclusively for handling amplification products and use disposable filter tips that are certified sterile and free of DNA, RNA, DNases, and RNases.

7.8. Analysis of the obtained data

To set the baseline and threshold for each reaction, follow the manufacturer's manual for the real-time PCR instrument in accordance with your internal procedures for this type of assay.

8. Interpretation of results

8.1. Interpretation of Results and reporting (clinical samples)

- **Extraction and Positive Control Results and Interpretation**
- **No Template Control (NTC)**
  The NTC consists of using nuclease-free water (PCR water) in the RT-qPCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence amplification curves that cross the threshold line. If any of the NTC reactions exhibit an amplification curve that crosses the cycle threshold line, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.
- **Positive control (PC4.01)**
  The PC4.01 consists of lyophilized isolated genomic RNA of SARS-CoV-2, IAV, and IBV spiked with human RNA and co-precipitant (such as salmon sperm DNA or Baker's yeast tRNA to increase stability). The PC4.01 will yield a positive result with all primer and probe sets (i.e., SARS-CoV-2 E and RdRP genes, IAV PB1 gene, IBV PA gene, and RNase P gene). Standard Ct values for the PC4.01 positive control should exhibit a Ct lower than 35.00 for all tested markers. The signal level (i.e., the relative fluorescence without normalization with the ROX dye) should also result in values above 1000 RFU to be considered valid. Indications of an error or failure in the workflow or analysis of the experiment include: the complete absence of a signal, the presence of an amplified signal but with higher Ct values than usual for a given control material, or the presence of a low-level signal. In the case of a negative result in the positive control, it is not possible to unambiguously determine the correctness of other positive/negative results obtained in the given analysis and to distinguish between negative and false negative results. Therefore, the output of such an analysis cannot be evaluated.

Table 4. **Expected performance of controls included in the rTEST COVID-19/FLU qPCR kit**

Control Type	External Control Name	SARS--CoV-2 E	SARS--CoV-2 RdRP	Human RNa-se P	IAV PB1	IBV PA	Expected Ct values	Possible causes of the unexpected results
Positive	PC4.01	+	+	+	+	+	Ct < 35.00	Substantial reagent failure including primer and probe integrity
Negative	NTC	–	–	–	–	–	None detected	Reagent and/or environmental contamination

If both primer and probe sets are used in one reaction, the expected profile of the controls included in the rTEST COVID-19/FLU qPCR assay is as follows:

Table 5. **Expected performance of controls included in rTEST COVID-19/FLU qPCR kit when both primer/probes sets are included in a single reaction**

Control Type	External Control Name	SARS--CoV-2 E + RdRP	IAV PB1 + IBV PA	Human RNase P	Expected Ct values	Possible causes of the unexpected results
Positive	PC4.01	+	+	+	Ct < 35.00	Substantial reagent failure including primer and probe integrity
Negative	NTC	–	–	–	None detected	Reagent and/or environmental contamination

Deviation from the expected performance of the controls suggests improper assay set up and/or execution, or failure/malfunction of reagents and/or equipment could have occurred. Invalidate the run and re-test.

- **RNase P (Extraction Control)**
  All clinical samples should exhibit fluorescence amplification curves in the RNase P reaction that cross the threshold line within 35.00 cycles (Ct < 35.00), thus indicating the presence of the human RNase P gene in the RNA sample. Failure to detect RNase P in any clinical specimens may indicate:
  - Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
  - Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
  - Improper assay set up and execution.
  - Reagent or equipment malfunction.
- If the RNase P assay does not produce a positive result for human clinical specimens, interpret as follows:
  - If the SARS-CoV-2 E and RdRP assays and/or IAV and IBV assay are positive even in the absence of a positive RNase P, the result should be considered valid. It is possible that some samples may fail to exhibit RNase P amplification curves due to low cell numbers in the original clinical sample. A negative RNase P signal does not preclude the presence of SARS-CoV-2, IAV or IBV RNA in a clinical specimen.
  - If all SARS-CoV-2 markers, IAV, IBV **and** RNase P are negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the RNA extraction procedure and repeat the test. If all markers remain negative after re-testing, report the results as invalid and a new specimen should be collected if possible.
- **SARS-CoV-2 markers (E and RdRP)**
  » When all controls exhibit the expected performance, a specimen is considered **negative** if the amplification curves for both SARS-CoV-2 genes (E, RdRP) **do not** cross the threshold line within 40.00 cycles (Ct > 40.00) **and** the RNase P amplification curve **does** cross the threshold line within 35.00 cycles (Ct < 35.00).
  » When all controls exhibit the expected performance, a specimen is considered **positive** if the amplification curves for both SARS-CoV-2

genes (E, RdRP) cross the threshold line within 40.00 cycles (Ct < 40.00). The RNase P may or may not be positive as described above, but the SARS-CoV-2 result is still valid.

» When all controls exhibit the expected performance and the amplification curves for both SARS-CoV-2 genes (E, RdRP) **and** the RNase P marker **do not** cross the threshold line within 40.00 cycles (Ct < 40.00) **and** 35.00 cycles (Ct < 35.00), respectively, the result is considered **invalid**. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is negative for all markers and RNase P, the result is invalid and collection of a new specimen from the patient should be considered.

» When all controls exhibit the expected performance and the amplification curve for one of the SARS-CoV-2 specific genes (E or RdRP but **not both**) crosses the threshold line within 40.00 cycles (Ct < 40.00) the result is considered **inconclusive**. The extracted RNA should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained, report the inconclusive result. Consult with your public health authority, as appropriate, to request guidance and/or to coordinate transfer of the specimen for additional analysis.

- **Influenza A (PB1) and influenza B (PA) markers**

» When all controls exhibit the expected performance, a specimen is considered Influenza **negative** if the amplification curves for both IAV and IBV **do not** cross the threshold line within 40.00 cycles (Ct > 40.00) **and** the RNase P amplification curve **does** cross the threshold line within 35.00 cycles (Ct < 35.00).

» When all controls exhibit the expected performance, a specimen is considered **IAV and/or IBV positive** if the amplification curves for **IAV and/or IBV** cross the threshold line within 40.00 cycles (Ct < 40.00). The RNase P may or may not be positive as described above, but the IAV and/or IBV result is still valid.

» When all controls exhibit the expected performance and the amplification curves for both IAV and IBV **and** the RNase P marker **do not** cross the threshold line within 40.00 cycles (Ct < 40.00) **and** 35.00 cycles (Ct < 35.00), respectively, the result is considered **invalid**. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is negative for all markers and RNase P, the result is invalid and collection of a new specimen from the patient should be considered.

- **rTEST COVID-19/FLU qPCR diagnostic test results interpretation guide**

» The table below lists the expected results for the rTEST COVID-19/FLU qPCR diagnostic test. If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please consult your public health authority.

NOTE

- **To set the fluorescence threshold, we recommend following the manual included with the thermal cycler used or the CDC recommendations.**

NOTICE!

- **Before using the rTEST COVID-19/FLU qPCR kit, we recommend that you calibrate the real-time PCR instrument. Follow the user´s instructions of your real-time PCR instrument.**
- **The rTEST COVID-19/FLU qPCR kit is designed for use by qualified and trained laboratory personnel with sufficient experience in real-time RT-qPCR testing techniques.**

Table 6. **Interpretation of SARS-CoV-2 test results and corresponding actions**

SARS-CoV-2 E	SARS-CoV-2 RdRP	Human RNase P	Result Interpretation[a]	Report	Actions
+	+	+/–	SARS-CoV-2 detected	SARS-CoV-2 positive	Evaluate the results from the IAV/IBV testing as well, then report the results to sender.
+	–	+/–	Inconclusive result	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat RT-qPCR. If the repeated result remains inconclusive, contact your public health authorities for further instructions or guidance.
–	+	+/–			
–	–	+	SARS-CoV-2 not detected	Not detected	Evaluate the results from the IAV/IBV testing as well, then report the results to sender[b].
–	–	–	Invalid result	Invalid	Repeat extraction and RT-qPCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

Table 7. **Interpretation of Influenza A and Influenza B test results and corresponding actions**

IAV PB1	IBV PA	Human RNase P	Result interpretation[a]	Report	Actions
+	+	+/–	IAV + IBV coinfecti-on detected	IAV and IBV positive	Evaluate the results from SARS-CoV-2 testing as well, then report the results to sender.
+	–	+/–	IAV detected	IAV positive	Evaluate the results from SARS-CoV-2 testing as well, then report the results to sender.
–	+	+/–	IBV detected	IBV positive	
–	–	+	IAV/IBV not detected	Not detected	Evaluate the results from SARS-CoV-2 testing as well, then report the results to sender.
–	–	–	Invalid result	Invalid	Repeat extraction and RT-qPCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

Table 8. **Interpretation of SARS-CoV-2, Influenza A and Influenza B test results and corresponding actions when both sets of primers and probes are used in a single reaction**

SARS-CoV-2 E + RdRP	IAV PB1 + IBV PA	Human RNase P	Result interpretation[a]	Report	Actions
+	+	+/–	SARS-CoV-2 IAV/IBV coinfection detected	SARS CoV-2 and IAV/IBV positive	Report results to sender.
+	–	+/–	SARS CoV-2 detected	SARS CoV-2 positive	Report results to sender.
–	+	+/–	IAV/IBV detected	IAV/IBV positive	
–	–	+	SARS CoV-2 and IAV/IBV not detected	Not detected	Report results to sender.
–	–	–	Invalid result	Invalid	Repeat extraction and RT-qPCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

[a] Laboratories should report their diagnostic results as appropriate and in compliance with their specific reporting system.

[b] Optimum specimen types and timing for peak viral levels during infections caused by SARS-CoV-2 have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest COVID-19 disease while diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If SARS-CoV-2 infection is still suspected, re-testing should be considered in consultation with public health authorities.

9. Functional characteristics

9.1. Limit of detection

Evaluation of analytical sensitivity (detection limit) was performed on the combinations of primers/probes used for multiplexed detection of SARS-CoV-2_E/IAV/RNase P and SARS-CoV-2_RdRP/IBV/RNase P. The test was performed using the positive control „SARS-CoV-2 Standard" (Exact Diagnostics), which in the undiluted state contains 200 copies of synthetic template per 1 µl. „AMPLIRUN® INFLUENZA A H3 RNA CONTROL" (Vircell Microbiologists) containing the complete IAV genome, diluted to 200 copies of the template per 1 µl, was used as a control template for IAV detection. Viral RNA isolated from a MDCK cell line infected with Influenza B 17/381 was diluted to 200 copies per 1 µl and used as a template for IBV detection.
Dilutions were prepared by serial dilutions of the stock standard, resulting in samples with concentrations of 8 copies/µl (= 40 copies/reaction), 2 copies/µl (= 10 copies/reaction), 0.8 copies/µl (= 4 copies/reaction), 0.4 copies/µl (= 2 copies/reaction) and 0.2 copies/µl (= 1 copy/reaction) that were used in the analytical sensitivity test. A synthetic matrix "SARS-CoV-2 Negative" (Exact Diagnostics) containing genomic DNA at a concentration of 75,000 copies/ml was used to dilute the control material. The assay was performed in 8 replicates for each prepared dilution.
The test confirmed the high sensitivity of the rTEST COVID-19/FLU qPCR kit. Reliable template detection of SARS-CoV-2 E and RdRP genes, IAV PB1 gene, and IBV PA gene was demonstrated down to 2 copies per reaction (0.4 copies/µl), which was also confirmed in an extensive LoD experiment where 22 out of 24 replicates were positive for E gene and IAV PB1 gene, 23 replicates were positive for IBV PA gene, and all 24 replicated were positive for RdRP gene*.

Table 9. **Limit of detection of SARS-CoV-2 E and RdRP tests**

	E gene/RNase P gene			RdRP gene/RNase P gene		
	Total number of replicates	Number of reactions with positive results	Detection success (%)	Total number of replicates	Number of reactions with positive results	Detection success (%)
40 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
10 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
4 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
2 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
1 copy/ reaction	8	2/8	25/100	8/8	2/8	25/100
2 copies/ reaction*	24	22/24	92/100	24/24	24/24	100/100

* Extensive LoD experiment for 2 copies/reaction

Table 10. **Limit of detection of IAV PB1 and IBV PA tests**

	IAV/RNase P gene			IBV/RNase P gene		
	Total number of replicates	Number of reactions with positive results	Detection success (%)	Total number of replicates	Number of reactions with positive results	Detection success (%)
40 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
10 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
4 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
2 copies/ reaction	8	8/8	100/100	8/8	8/8	100/100
1 copy/ reaction	8	2/8	25/100	8/8	1/8	12,5/100
2 copies/ reaction*	24	22/24	92/100	24/24	23/24	96/100

* Extensive LoD experiment for 2 copies/reaction

Evaluation of the analytical sensitivity (detection limit) was also performed for the combination of both sets of primers/probes enabling simultaneous detection of SARS-CoV-2_E/IAV/RNase P + SARS CoV-2_RdRP/IBV/RNase P in one reaction. The assay was performed in a similar manner and using control templates as described above for the analytical sensitivity assessment. Dilutions with concentrations of 8 copies/µl (= 40 copies/reaction), 2 copies/µl (= 10 copies/reaction), 0.8 copies/µl (= 4 copies/reaction) and 0.4 copies//µl (= 2 copies/reaction) were prepared by serial dilution. A synthetic matrix "SARS-CoV-2 Negative" (Exact Diagnostics) containing genomic DNA at a concentration of 75,000 copies/ml was used to dilute the control material. The assay was performed in 8 replicates for each dilution prepared.

Table 11. **Limit of detection of IAV PB1 and IBV PA tests**

	SARS-CoV-2/IAV/RNase P gene			SARS-CoV-2/IBV/RNase P gene		
	Total number of replicates	Number of reactions with positive results	Detection success (%)	Total number of replicates	Number of reactions with positive results	Detection success (%)
40 copies/ reaction	8	8/8/8	100/100/100	8/8/8	8/8/8	100/100/100
10 copies/ reaction	8	8/8/8	100/100/100	8/8/8	8/8/8	100/100/100
4 copies/ reaction	8	8/8/8	100/100/100	8/8/8	8/8/8	100/100/100
2 copies/ reaction	8	3/5/8	38/63/100	8/8/8	3/5/8	38/63/100
4 copies/ reaction	24	24/24/24	100/100/100	24/24/24	24/24/24	100/100/100

* Extensive LoD experiment for 4 copies/reaction

The test confirmed the high sensitivity of the rTEST COVID-19/FLU qPCR kit. Reliable template detection of SARS-CoV-2 E and RdRP genes, IAV PB1 gene, and IBV PA gene was demonstrated down to 4 copies per reaction (0.8 copies/µl) and subsequently confirmed by an extended LoD test, where all 24 replicates were positive for SARS CoV-2, IAV, and IBV*.

9.2. Test specificity

Evaluation of specificity (cross-reactivity to other coronaviruses and respiratory viruses) was performed for the SARS-CoV-2 E and RdRP genes as well as for IAV PB1 gene and IBV PA gene. The test was performed using the control material "Coronavirus RNA specificity panel" (EVAg, European Virus Archive - Global), which contains RNA viruses HCoV-229E, HCoV-OC43, HCoV-NI63, SARS-CoV HKU39849, and MERS-CoV, each in a separate tube. A set of respiratory viruses (Vircell microbiologists) containing RNA of Influenza A H1N1, Novel Influenza A H1N1, Influenza A H3N2, Influenza A H5N1, Novel Influenza B, Human parainfluenza, Respiratory syncytial virus and Human rhinovirus, each provided in a separate tube, were used to assess cross-reactivity to respiratory viruses. The assay was performed in 3 replicates for each of the indicated viruses.

The test confirmed the high specificity of the rTEST COVID-19/FLU qPCR kit. A positive result was recorded exclusively in reactions containing SARS-CoV-2 RNA (Exact Diagnostics) in the presence of primers/probe sets for the E and RdRP genes. Likewise, positive results for reactions containing IAV and IBV RNA were detected only in reactions containing primers and probes designed to detect IAV (Novel Influenza A H1N1, Influenza A H3N2) and IBV (Novel Influenza B), respectively.
The occurrence of contamination by synthetic positive controls in various commercially available products used to perform RT-qPCR diagnosis of COVID-19 disease, e.g., primers, probes, or RT-qPCR mixtures is a global problem. The mixture of primers and probes for SARS-CoV-2_E in the rTEST COVID-19/FLU qPCR kit was designed not to amplify the most commonly used synthetic positive controls at all or only with low efficiency. Thus, the rTEST COVID-19/FLU qPCR kit can also be used effectively in workplaces that have a problem with contamination when testing for the presence of the SARS-CoV-2 E gene. For IAV and IBV, only those IAV and IBV strains that were predicted by the WHO as circulating in the population in the 2020/2021 influenza season were included in the bioinformatics analysis and primer design.

Evaluation of chemical stability (e.g., degradation of probes during thermocycling) was performed

on the SARS-CoV-2 E gene and RdRP genes. The test was performed as an analysis of multiple no template controls (NTC), including 20 replicates for the E gene/IAV and 40 replicates for the RdRP gene/IBV.

The assay confirmed the high chemical stability of the oligonucleotides contained in the rTEST COVID-19/FLU qPCR kit. In each of the analyzed NTCs, a negative result was recorded with no indication of an increase in signal or the presence of amplification.

9.3. Clinical performance evaluation

Evaluation of the clinical performance of the rTEST COVID-19/FLU qPCR kit was performed for the SARS-CoV-2 E and RdRP genes as well as the IAV PB1 gene and IBV PA gene. For SARS-CoV-2, the evaluation was performed on a selected set of 38 positive and 54 negative clinical samples of patients, which were confirmed by a reference method used for routine testing by regional public health authorities of the Slovak Republic; In regards to IAV and IAB, the evaluation was performed on a selected set of 52 and 37 clinical samples of patients with IAV and IBV, respectively. One sample was negative for both, IAV and IBV. All influenza samples were notably collected during the last three years and confirmed by the reference methods used for routine testing by regional public health authorities of the Slovak Republic. Influenza samples were thawed once before RNA isolation.

Testing of this selected set of samples was performed blinded in an external laboratory that is a member of the External Quality Assessment (EQA) scheme organized by institutions such as the European Centre of Disease Prevention and Control (ECDC), Institute of Virology, Charité, Berlin, Germany, and National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands.

The clinical evaluation of the SARS-CoV-2 samples uniformly confirmed the results of the primary testing for all samples, except 3 samples that were exposed to one freeze/thaw cycle and undetectable by either the reference method or with the rTEST COVID-19/FLU qPCR kit. The undetected samples, when analyzed in primary testing, expressed Ct values higher than the recommended values for retrospective validation; therefore, these samples were excluded in the final calculations for sensitivity and specificity of the kit. The analysis demonstrated high reliability and reproducibility of the results obtained with the rTEST COVID-19/FLU qPCR kit with 100% diagnostic sensitivity and 100% diagnostic specificity (see Tables 11 and 12 below).

The clinical evaluation of the influenza samples uniformly confirmed the results of the reference method for all evaluated samples and demonstrated the high reliability and reproducibility of the results obtained with the rTEST COVID-19/FLU qPCR kit with 100% diagnostic sensitivity and 100% diagnostic specificity for both IAV and IBV (see Tables 11 and 12 below).

Detection of RNase P gene showed high homogeneity in all analyzed samples, confirming the suitability of this assay as an internal control for collection and RNA extraction from a clinical sample.

10. Disposal

NOTICE!

- **Decontaminate any material that has come into contact with biological samples with 3% sodium hypochlorite for a minimum of 30 minutes or autoclave at 121 °C for a minimum of 60 minutes before disposing.**
- **All used equipment, tips, tubes, work materials, and protective clothing should be considered potentially contaminated and disposed of in accordance with applicable infectious waste disposal regulations.**
- **Dispose of remaining reagents and material in accordance with applicable safety regulations.**

11. Troubleshooting and safety reporting (Medical device vigilance)

In case of any problems contact:

MultiplexDX, s. r. o.
Manufacturer

Address: Ilkovičova 8
841 04 Bratislava

Tel.: +421 2 902 68 310
Email: vigilance@multiplexdx.com

Table 12. **Clinical performance of SARS-CoV-2 E gene, SARS-CoV-2 RdRP gene and RNase P gene detection**

	Reference method			rTEST COVID-19/FLU qPCR		
	E gene	RdRP gene	RNase P gene	E gene	RdRP gene	RNase P gene
Number of correctly identified positive samples	37	33	33	38	38	92
Number of false positive samples	0	0	0	0	0	0
Number of correctly identified negative samples	54	54	54	54	54	0
Number of false - negative samples	1	5	5	0	0	0

* These samples, when analyzed in primary testing, expressed Ct values higher than the recommended values for retrospective validation; therefore, these samples were excluded in the final calculations for sensitivity and specificity of the kit.

Table 13. **Clinical performance of IAV PB1 gene, IBV PA gene and RNase P gene detection**

	Reference method			rTEST COVID-19/FLU qPCR		
	IAV PB1 gene	IBV PA gene	RNase P gene	IAV PB1 gene	IBV PA gene	RNase P gene
Number of correctly identified positive samples	52	37	89	52	37	89
Number of false positive samples	0	0	0	0	0	0
Number of correctly identified negative samples	1	1	2	1	1	2
Number of false - negative samples	0	0	0	0	0	0

12. Symbols

	Manufacturer
	Batch number
	Recommended storage temperature
	Package size
	This product complies with the requirements of European Directive 98/79/EC on *in vitro* diagnostic medical devices
	Date of manufacturing
	In vitro diagnostic medical devices
	Attention, follow the safety instructions in the operating instructions that came with this product

Registration code: P 1751A

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