Updates with respect to the previous version are marked in grey.

EURORealTime SARS-CoV-2/Influenza A/B

Instructions for use

For in vitro diagnostic use IVD

ORDER NO.	PARAMETER	FORMAT
MP 2606-0125-20	SARS-CoV-2/Influenza A/B	25 reactions
MP 2606-0225-20		50 reactions
MP 2606-0425-20		100 reactions
MP 2606-0100-20		100 reactions
MP 2606-0200-20		200 reactions
MP 2606-1000-20		1000 reactions

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Intended use

This multiplex real-time RT-PCR test provides qualitative molecular diagnostic in vitro detection of RNA of the coronavirus SARS-CoV-2 and influenza virus types A and B from throat swabs. It supports the diagnosis of infections with these viruses and allows their differentiation. The product is designed to be used exclusively as IVD by qualified laboratory personnel only. The test is to be performed manually.

Clinical significance

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, previously called 2019-nCoV) belongs to the family of coronaviruses and, like SARS-CoV, is classified in the genus Betacoronavirus [1]. At the end of 2019, SARS-CoV-19 was identified as the causative pathogen of clustered cases of pneumonia of unclear origin. The virus caused an infection wave which quickly spread worldwide and was declared a pandemic by the WHO at the beginning of 2020 [2-5]. In February 2020, the disease caused by SARS-CoV-2 was named COVID-19 by the WHO.

Influenza viruses (flu viruses) belong to the family of orthomyxoviruses. They are classified into types A to D. Influenza A viruses are divided into 18 haemagglutinin subtypes (H1–18) and 11 neuraminidase subtypes (N1–11). As to influenza B viruses, there are two genetically different lines circulating worldwide: Yamagata and Victoria. Influenza A viruses occur in humans and in farm stock such as pigs, horses and poultry, as well as in wild birds. Influenza B and C viruses are only found in humans; type D in pigs and cattle [6-9]. Influenza virus types A and B cause seasonal epidemics, infections with types C and D cause insignificant clinical symptoms in humans. Pandemics are caused by influenza A viruses of zoonotic origin [6, 7, 10]. Seasonal flu outbreaks mostly occur in the winter months. They spread quickly, 5 to 10% of the adults and 20 to 30% of children worldwide are infected every year [6-10].

In the early disease stage, COVID-19 and influenza cannot be distinguished based on the clinical symptoms. In the same way, infections with influenza viruses A and B cannot be clinically delimited [10, 11]. In the following table [11, supplemented], the clinical characteristics of infections with SARS-CoV-2 and influenza viruses A and B are summarised:

	SARS-CoV-2	Seasonal influenza viruses
Transmission through	Droplets, also aerosols and smear infection [2, 4, 7]	
Highest infectiousness	Usually shortly before onset of symptoms	After onset of symptoms
Incubation period	2 – 14 days	1 – 4 days
Risk factors for a severe course	Risk increases with increasing age Adiposis, high blood pressure, chronic diseases	Younger than two years and older than 65 years of age Immunosuppression, pregnancy, adiposis, chronic disease
Most frequent disease symptoms	Fever, chills, headache, muscle pain, dry cough, shortness of breath, fatigue, olfactory loss [12].	Fever, chills, headache, muscle pain, cough, sputum, stuffed nose, sore throat, fatigue [12].
Peak of the disease	2nd or 3rd week	Within 3 to 7 days

Coinfections with influenza A and B viruses are not common and mainly nosocomial [13]. Coinfections with influenza viruses and SARS-CoV-2 are rarely observed [11].

The diagnosis is made after identification of the pathogen by nucleic acid or antigen detection in samples from the respiratory tract [11].

Test principle

The test is based on a one-tube reaction comprising reverse transcription (RT) to convert viral RNA into complementary DNA (cDNA) followed by PCR amplification and fluorescence-based real-time detection of two defined sections within the ORF1ab- and N-genes of the SARS-CoV-2 genome as well as one

defined section each in the genomes of influenza virus types A and B. Reverse transcription, amplification and detection of cDNA of SARS-CoV-2 and influenza virus types A and B are carried out by means of specific primers and probes. The test contains an internal amplification control which serves as an inhibition control and can additionally be used as an extraction control. The test kit includes a SARS-CoV-2/influenza A/B positive control that is used as an external control in every test run.
The EURORealTime Analysis software in connection with a compatible real-time PCR cycler (see below) enables fully automated and standardised evaluation and documentation of results, including all control results. Furthermore, the software provides full guidance through the individual work steps, thus ensuring a simple and error-free test procedure. Alternatively, the software of the real-time-PCR cycler can be used for evaluation.

Contents of the test kit

(MP 2606-####-20)

Component	0125	0225	0425	0100	0200	1000	Symbol
1. PCR Mix A SARS-CoV-2/Influenza A/B (green cap), ready for use	1 x 150 µl	2 x 150 µl	4 x 150 µl	1 x 600 µl	1 x1.2 ml	5 x 1.2 ml	PCR MIX A
2. PCR Mix B SARS-CoV-2/Influenza A/B (yellow cap), ready for use	1 x 150 µl	2 x 150 µl	4 x 150 µl	1 x 600 µl	1 x 1.2 ml	5 x 1.2 ml	PCR MIX B
3. Positive control SARS-CoV-2/Influenza A/B (purple cap), ready for use	1 x 400 µl	1 x 400 µl	1 x 400 µl	1 x 400 µl	1 x 400 µl	2 x 400 µl	POS CONTROL
4. RNA internal control (white cap), ready for use	1 x 1.2 ml	1 x 1.2 ml	1 x 1.2 ml	1 x 1.2 ml	1 x 1.8 ml	6 x 1.8 ml	INT CONTROL
5. Instructions for use	1 piece	1 piece	1 piece	1 piece	1 piece	1 piece	-

Test kit materials for re-ordering

The following materials can be re-ordered. They should always be used according to the instructions for use of the test kit.

Material	Order number	Format
Positive control SARS-CoV-2/Influenza A/B	MK 2606-0108-20	1 x 400 µl
RNA internal control	MK 0003-0112	1 x 1.2 ml

Additional materials and equipment (not supplied in the test kit)

Software (optional)

- EURORealTime Analysis software (at least version 1.2.3; for qTOWER³ support at least version 1.3.0), EUROIMMUN order no. YG 0661-0101

Pre-PCR and sample preparation area:

- Mini centrifuge for 0.2 ml and 1.5 ml reaction vessels, EUROIMMUN order no. YG 0612-0101 or similar
- Centrifuge for PCR plates, e.g. VWR, item no. 521-1648 or similar
- Laboratory shaker for reaction vessels (“vortex”), EUROIMMUN order no. YG 0641-0101 or similar
- PCR cooling rack for 0.2 ml reaction vessels (“IsoFreeze Tube-Rack”) EUROIMMUN order no. ZG 0617-0101 or similar
- Pre-PCR rack for 1.5 ml reaction vessels
- Pre-PCR cooling rack for 1.5 ml reaction vessels (“IsoPack and IsoRack”), EUROIMMUN order no. ZG 0618-0101 or similar

- Pipettes (volume-adjustable) and pipette tips with filter, 10, 20, 200 and 1000 µl, DNA and DNase-free
- Reaction vessels 1.5 ml, DNA and DNase-free (recommended: Micro tube 1.5 ml SafeSeal, Sarstedt, item no. 72.706.400)
- Disposable gloves
- PCR reaction vessels:

  For 7500 Fast Real-Time PCR Instrument (Applied Biosystems):
  MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1 ml), Fisher Scientific (Applied Biosystems), item no. 4346906
  MicroAmp® Fast Optical 96-Well Reaction Plate (0.1 ml), Fisher Scientific (Applied Biosystems), item no. 4346907
  MicroAmp® Optical Adhesive Film, Fisher Scientific (Applied Biosystems), item no. 4311971
  For CFX 96 Touch (Bio-Rad):
  Multiplate™ 96-Well PCR Plates, low profile, unskirted, clear, Bio-Rad, item no. MLL9601
  Microseal 'B' PCR Plate Sealing Film, adhesive, optical, Bio-Rad, item no. MSB1001
  For qTOWER³ (Analytik Jena):
  96-well PCR plate (0.2 ml; LP), full-skirted, white, Analytik Jena, item no. 844-70038-0
  Optical sealing foil (77 x 140 mm), adhesive, transparent, peelable, Analytik Jena, item no. 844-70045-0

Amplification area:

- One of the following real-time PCR cyclers is recommended:
  7500 Fast Real-Time PCR Instrument (Applied Biosystems)
  CFX 96 Touch (Bio-Rad)
  qTOWER³ (Analytik Jena)

Storage and stability

- **PCR Mix A (green cap) and B (yellow cap) SARS-CoV-2/Influenza A/B:** Ready for use. Protect from light and store in **pre-PCR area** at -18°C to -25°C. Before use, thaw no more than five times at +2°C to +8°C for a short period (do not exceed 60 minutes), mix by multiple inversion (**do not vortex!**) and centrifuge to collect the solutions at the bottom.
- **Positive control SARS-CoV-2/Influenza A/B (purple cap):** Ready for use. Store in the sample preparation area at -18°C to -25°C. Before use, thaw no more than five times at +2°C to +8°C for a short period (do not exceed 60 minutes), mix and centrifuge to collect the solution at the bottom.
- **RNA internal control (white cap):** Ready for use. Store in **pre-PCR area** at -18°C to -25°C. Before use, thaw no more than five times at +2°C to +8°C for a short period (do not exceed 60 minutes), mix and centrifuge to collect the solution at the bottom.

In-use stability following the first opening

After opening, the reagents are stable until the indicated expiry date when stored under the specified conditions and protected from contamination, unless stated otherwise herein. **EUROIMMUN recommends thawing the reagents no more than five times and, if required, making aliquots.**

Warnings and precautions

- The test systems may only be processed by qualified personnel with fundamental knowledge of Good Laboratory Practice (GLP) as described by the U.S. Food and Drug Administration (FDA) or the Organisation for Economic Cooperation and Development (OECD). Especially the recommendations for the performance of molecular amplification tests must be observed [14].
- When handling the samples and reagents, laboratory coat, disposable gloves and, if required, further personal protective equipment should be used. The valid recommendations for the SARS-CoV-2 and influenza virus testing, such as those from the German Committee for Biological Work Substances (ABAS) or the World Health Organization (WHO) should be observed.
- Heated lid and incubation block of the real-time PCR cycler may reach temperatures of up to 110°C. Risk of skin burns. Please observe the operating instructions with respect to the instrument.
- If the packed reagents are visibly damaged, do not use the test kit.
- Before using the product, read the instructions for use carefully. Only the valid version is to be used.
- Do not substitute or mix the EUROIMMUN reagents with reagents from other manufacturers.
- Due to the high analytical sensitivity of a PCR, extreme caution should be exercised to ensure the purity of all test kit components and samples. During PCR, sections of the nucleic acid which is present in the sample and is used for pathogen detection are amplified million- to billionfold. The smallest amounts of PCR products can lead to incorrect results, when they contaminate, e.g. via aerosols, the sample material, the reagents for RNA extraction or the PCR reagents of this test kit. In order to detect such possible contamination, it is important to always include negative controls during sample extraction and PCR.
- Change disposable gloves frequently and after (suspected) contamination with reagents or sample material.
- Use filter tips for pipetting in order to avoid cross contamination via aerosols.

Notes on the test performance and safety

- To prevent contamination, the individual steps of the test procedure should ideally be carried out in three separate rooms (pre-PCR, sample preparation, amplification area), as described in the following [15]. Store and use all instruments, pipettes, reagents and materials as well as protective equipment (laboratory coat, protective goggles, disposable gloves) only in the room where they belong.

  Room 1 – Pre-PCR area
  The pre-PCR area is used for storage and handling of PCR reagents. Do not bring samples, PCR products or RNA positive controls into this room.

  Room 2 – Sample preparation
  This room is used for isolation of RNA from the samples and for pipetting of extracted samples into the PCR mix. Do not bring PCR products into this room.

  Room 3 – Amplification area
  PCR amplification in the real-time PCR cycler is carried out in this area. After PCR, the fragments of the target nucleic acid to be detected are present in million- or billionfold amplified copies. Even the smallest carry-over into the pre-PCR and sample preparation rooms via clothing, instruments, materials or aerosols can lead to false results in samples tested thereafter and should be absolutely avoided.

- All work surfaces, devices and tools, e.g. racks, should be cleaned with DNA-/RNA-degrading agents, e.g. diluted hypochlorite solution [16] on a regular basis, ideally after each use. Note: Some disinfectants, e.g. diluted ethanol, are not suited to degrade nucleic acids. Please contact EUROIMMUN for further information on this matter.

Preparation and stability of samples

- **Sample material:** The starting material for this test is purified RNA from appropriate samples. A good quality of the RNA sample is an important prerequisite for the validity of the test system. Incorrect sampling, RNA preparation and RNA storage can lead to invalid or even false results.

  For validation/evaluation of the EURORealTime SARS-CoV-2/Influenza A/B test with clinical samples, RNA was used which had been isolated from throat swabs using the QIAamp® Viral RNA Mini Kit (Qiagen) and the CMG-2017 Prepito Viral DNA/RNA300 Kit (Chemagen) according to the manufacturers' instructions.

- **Sample collection and transport:** The products that are used for sample collection, storage and transport should generally render the appropriate sample amount and quality as required for the use in this test system.

  Depending on the type of sample and the transport medium used, specific storage and, for RNA isolation, pretreatment of the sample may be required. Please observe the instructions provided by the manufacturer.

- **Preparation of RNA from patient samples:** Commonly used products for RNA preparation, e.g. QIAamp® Viral RNA Mini Kit (Qiagen) or the CMG-2017 Prepito Viral DNA/RNA300 Kit (Chemagen), generally provide a sufficient RNA quantity and quality for the use with this test system, provided that the sample material used is suitable and has been validated.

  The RNA purified from the sample may consist of a mixture of human nucleic acids and viral RNA, depending on the sample material. Since the amount of human nucleic acids is generally significantly higher than the amount of viral RNA, the measured RNA concentration in a sample does not reflect the amount of viral RNA present. No adverse effects on the detection limit were observed with samples containing an amount of up to 20 ng nucleic acid/µl. A larger amount of nucleic acid may lead to invalid results or, in extreme cases, to false results.

  Note: EUROIMMUN recommends using the RNA internal control (IC) to control the efficiency of RNA preparation. For this purpose the IC should be added to the lysis buffer or the mixture of sample and lysis buffer. It must not be added directly to the sample material before addition of lysis buffer! The added volume of IC should generally be 1/10th of the elution volume. Example: If the nucleic acid elution is carried out in 60 µl, 6 µl of IC should be added to the mixture of sample and lysis buffer.

- **Storage of RNA samples:** Store RNA samples exclusively in the sample preparation area and according to the recommendations provided in the RNA preparation system. EUROIMMUN recommends storing the extracted RNA samples at -18°C to -25°C, ideally at -80°C. Short-term storage at a temperature of +4°C to +8°C is generally possible.

Note: Inappropriate storage of RNA samples as well as frequent thawing and re-freezing can damage the RNA and should be avoided; otherwise false negative results may occur.

Preparation of reagents

Preparation of reagents is not required. All kit components are ready for use.

Waste disposal

Samples, controls and used reaction vessels should be handled as potentially infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Quality control

To ensure correctness of results, parallel testing of a negative control (not provided in the test kit) and a positive control (included in the test kit) is crucial in every test run. Furthermore, the test system contains an RNA internal control (IC), which supports recognition of a potential real-time PCR inhibition or a faulty extraction. The IC should be detected especially in negative samples.

Assay procedure

Overview:

Please read the chapter **"Notes on the test performance and safety"** before starting.

1. RNA preparation	- Prepare RNA according to the instructions provided by the manufacturer of the preparation system used
⇩	
2. Create run in EURORealTime Analysis software[1]	- Enter the samples and allocate the tests (or import from LIMS or csv file) - Pipetting scheme and plate layout are generated - Export the plate layout
⇩	
3. Real-time PCR	- Prepare PCR master mix from PCR Mixes A and B - Pipette master mix into PCR plate - Add samples and controls - Import the plate layout generated by the EURORealTime Analysis software and the cycler-specific temperature profile[1] - Incubate in the real-time PCR cycler - Export PCR results (raw data)
⇩	
4. Evaluation using EURORealTime Analysis software[1]	- Import PCR results (raw data) into EURORealTime Analysis software - Start evaluation process in EURORealTime Analysis software - Check and validate results - Print result documents and/or transfer results to LIMS

[1] If the EURORealTime Analysis software is not used, enter the following information into the software of the real-time PCR cycler directly:
- Position and name of each sample and control
- Assign all four detection filters to each sample and control
- Cycler-specific temperature profile

After run has been completed:
- Start evaluation process in the real-time PCR cycler software
- Check and validate the results

Detailed work instructions

Controls

Internal control:
To identify a potential real-time PCR inhibition or extraction failure the test kit includes an RNA internal control (IC) containing an artificial sequence with no homology to any other known sequence. The IC must be added to each reaction. Without the IC, negative results will be classified as “invalid” by the EURORealTime Analysis software. If the evaluation is performed using the software of the real-time PCR cycler, negative results should then also be classified as “invalid”.

Negative control:
In order to exclude contamination of the test components with RNA/DNA that can be amplified, in each test series a negative control must be performed using a nucleic acid-free solution (e.g. DNase/RNase-free water or TE buffer) as the sample (negative control, NC). If the NC is omitted or classified as “invalid”, all results obtained with this test will be evaluated as “invalid” by the EURORealTime Analysis software. If the evaluation is performed using the software of the real-time PCR cycler, all test results should then also be classified as “invalid”. Since amplified RNA/DNA could be carried over into the samples via RNA purification components, EUROIMMUN recommends including the negative control already during RNA preparation.
It is mandatory to add the IC to the negative control. Negative controls without an amplification signal for the IC are classified as “invalid” by the EURORealTime Analysis software. If the evaluation is performed using the software of the real-time PCR cycler, negative controls without an amplification signal for the IC should then also be classified as “invalid”. If the IC is used as extraction control, EUROIMMUN recommends using the IC also for the negative control already during RNA preparation.

Positive control:
In order to verify whether the reaction mix has been prepared correctly and whether the RNA detection will be valid under the given conditions, the SARS-CoV-2/Influenza A/B positive control (PC) provided with the kit must be tested in each run. The positive control must be added directly to the PCR reaction and must not be used for extraction. If the PC is omitted or classified as “invalid”, all results obtained with this test will be classified as “invalid” by the EURORealTime Analysis software. If the evaluation is performed using the software of the real-time PCR cycler, all test results should then also be classified as “invalid”.

Note: If the EURORealTime Analysis software is not used, the addition of the IC in each sample is nevertheless mandatory and the external controls PC and NC must be included for the validation of results.

Preparation of run

Real-time PCR cycler programming

EUROIMMUN recommends importing the temperature profile via the file generated by the EURORealTime Analysis software. Further information about using the file can be found in the user manual of the EURORealTime Analysis software (EUROIMMUN document no. YG 0661_A_UK_CXX).

If the EURORealTime Analysis software is not used, the instrument settings and the temperature profile must be entered manually into the software of the real-time PCR cycler as follows:

Instrument settings

7500 Fast Real-Time PCR Instrument (Applied Biosystems):

Reaction Volume Per Well:	20 µl
Passive reference:	none
Cycler type:	7500 Fast (96 wells)
Experiment type:	Quantitation-Standard Curve
Reagent:	TaqMan® Reagents
Run-time:	Standard (~ 2 hours to complete run)

CFX 96 Touch (Bio-Rad):

Sample volume:	20 µl
Plate Type:	BR Clear
Plate Size:	96 Wells
Scan Mode:	All Channels

qTOWER³ (Analytik Jena):

Heating/Cooling Rate (for all steps):	3.3
Scan / Measurement:	Blue (FAM), Yellow (HEX_3) , Orange (ROX), Red (Cy5)
Meas. repeats:	3
Gain:	Blue (FAM): 2, Yellow (HEX_3): 5, Orange (ROX): 5, Red (Cy5): 5

Reporter:

Parameter	7500 Fast Real-Time PCR Instrument (Applied Biosystems)	CFX 96 Touch (Bio-Rad)	qTOWER³ (Analytik Jena)
SARS-CoV-2	FAM (Quencher None)	FAM	Blue (FAM)
Influenza A	ROX (Quencher None)	ROX	Orange (ROX)
Influenza B	Cy5 (Quencher None)	Cy5	Red (Cy5)
IC	VIC (Quencher None)	HEX	Yellow (HEX_3)

Temperature profile:

	Number of cycles	Acquisition	Temperature [°C]	Time [min:sec]	↗(°C/s) (only qTOWER³)
Reverse transcription	1	None	45	10:00	3.3
Denaturation	1	None	95	02:00	3.3
Amplification	45	None	95	00:15	3.3
		Acquisition*	58	00:45	3.3
Cooling	1	None	37	00:20	3.3

*The definition of acquisition depends on the real-time PCR cycler used:

7500 Fast Real-Time PCR Instrument (Applied Biosystems)	Data Collection On
CFX 96 Touch (Bio-Rad)	Plate Read
qTOWER³ (Analytik Jena)	Scan

Note: The PCR program given and the settings were optimised for the real-time PCR cyclers 7500 Fast Real-Time PCR Instrument (Applied Biosystems), CFX 96 Touch (Bio-Rad) and qTOWER³ (Analytik Jena) using the PCR reaction vessels recommended above. The use of other real-time PCR cyclers and/or other PCR reaction vessels may influence the amplification result and thus alter the performance data of the test, including sensitivity and specificity. The use of other instruments or vessels should be validated and may require adaptation of the temperature program. Due to the possibility of instrument deviations, for identical instruments we recommend validating the instrument with precharacterised samples and performing regular checks.

Creation of a run in EURORealTime Analysis software

EUROIMMUN recommends using the EURORealTime Analysis software to support, evaluate and document each run. Please familiarise yourself with the contents of the manual for the EURORealTime Analysis software before first use. To summarise, the following steps are performed:

Creating a run:

Create a protocol in the EURORealTime Analysis software under "New protocol":

- Define the real-time PCR instrument, the respective ID (in case of a previous definition of instrument IDs), the PCR vessels used and the sample arrangement.
- Enter the samples (via scanning or manually, from LIMS or csv import) and select “SARS-CoV-2/Influenza A/B” as the test.

- Enter the kit lot.

The master mix calculation and pipetting scheme are generated automatically and can be printed. The software automatically integrates the controls required for automatic evaluation of the test (positive and negative control).

Further information about the definition of a new protocol can be found in the user manual of the EURORealTime Analysis software.

Exporting the work protocol:

- After successful creation of a run, select “Save” and select “Export Working Protocol”.
- Select the file name and storage location (network directory or local data carrier) in the “Save under” window.
- The export of cycler- and run-specific template and sample layout file is carried out automatically.

If the EURORealTime Analysis software is not used, create the run as follows and according to the manufacturer’s instructions of the real-time PCR cycler used:

- Enter the name and the position of each sample
- Define the sample type for each sample (positive/negative control or patient sample)
- Apply the detection filters for each sample
- Choose the cycler-specific temperature profile

Real-time PCR performance

Note: RNA sample material, controls and PCR mixes are fragile. It is highly recommended to use appropriate cooling blocks or ice during the entire procedure and keep samples and reagents at +2°C to +8°C throughout the pipetting procedure.

Thawing of reagents and controls

Remove PCR Mixes A and B and controls from the freezer approx. 15 minutes before pipetting the PCRs and thaw in dedicated area/room at +2°C to +8°C. Protect from light, do not exceed 60 minutes at +2°C to +8°C!

Preparation of master mix

The following steps must be performed in the **pre-PCR area** (room 1):

- Prepare the PCR plate.
- After thawing at +2°C to +8°C, mix the PCR Mixes A and B by multiple inversion (**do not vortex!**) and centrifuge to collect the solutions at the bottom.
- Prepare the PCR master mix according to the pipetting scheme calculated by EURORealTime Analysis software. Pipette the indicated amount of PCR Mixes A and B as well as IC or RNA/DNA/nuclease-free water into a 1.5 ml reaction vessel using a new pipette tip each time. Mix thoroughly by pipetting up and down several times.

  The basis for master mix calculation in the EURORealTime Analysis software is[2]:

Component	Volume [µl]
PCR Mix A	5
PCR Mix B	5
IC[3]	1
∑	11

If the EURORealTime Analysis software is not used, calculate the volume of master mix for the required number of reactions manually according to the table above. EUROIMMUN recommends always taking into account a surplus of 10% to counterbalance pipetting inaccuracies.

- Pipette 10 µl of master mix into each well (see plate layout, one single pipette tip may be used).

[2] The EURORealTime Analysis software automatically calculates a surplus of 10% to counterbalance for pipetting inaccuracies.

[3] Only add the IC to the master mix if it has not yet been used during RNA isolation (see **“Preparation of RNA from patient samples”**). If the IC has already been used as sample extraction control, it MUST NOT be added at this stage. Instead, add the corresponding amount of RNA/DNA/nuclease-free water to the master mix.

Note: Avoid air bubbles during pipetting! Store all kit components again at -18°C to -25°C immediately after use.

Addition of samples

The following steps must be performed in the **sample preparation area** (room 2):

- If required, thaw the RNA samples and controls, mix by vortexing briefly and centrifuge for a short period to collect the solutions at the bottom.
- Pipette 10 µl of each RNA sample or control (positive or negative control) into the corresponding well containing the master mix. **Change the pipette tip with every step!**
- Seal the wells with care; when using 96-well plates ensure that the film is placed correctly!
- Centrifuge the PCR plate or strips briefly to collect the solutions at the bottom.

Amplification reaction

The following steps must be performed in the **amplification area** (room 3):

- Place the prepared PCR plate into the real-time PCR cycler.
- If the EURORealTime Analysis software is used[4], import the run data to the real-time PCR cycler according to the following description:

  7500 Fast Real-Time PCR Instrument (Applied Biosystems):
  - Start the software according to the manufacturer's instructions
  - Open the template file for the selected cycler (experiment properties, fluorophore selection and temperature profile are already included)
  - In the set-up "Plate Setup", go to "Import…" under "File"
  - Select the txt file in the "Import Plate Setup" window and confirm with "Start Import"
  - The fluorophores and sample descriptions are contained in the protocol

  Note: In the software version 2.3, the preset selection of the passive reference is not recognised by the software and the PCR run cannot start. First choose under "Assign Targets and Samples" in the column "Select the dye to use as the passive reference" any option that differs from "None" and then set it back to "None".

  CFX 96 Touch (Bio-Rad):
  - Start the software according to the manufacturer's instructions
  - Open cycler template file for the selected cycler (fluorophore selection, temperature profile and sample designation are already included)

  qTOWER³ (Analytik Jena):
  - Start software according to the manufacturer's instructions.
  - Choose "File" and "Import LIMS" in the menu bar to open the cycler template file for the selected cycler (fluorophore selection, temperature profile and sample designations are already contained).

  Note: After import of the cycler template file the setting for "Gain" in the tab "Settings" under "Scan" for the channel "Blue/Dye "FAM" must be manually set to 2!

  - Start the real-time PCR cycler.

Warning: Heated lid and incubation block of the real-time PCR cycler may reach temperatures of up to +110°C. There is a risk of skin burns. Please observe the operating instructions with respect to the instrument.

[4] If not using the EURORealTime Analysis software, please verify manually that correct settings, temperature profile and sample assignment have been chosen.

Preparations for evaluation using EURORealTime Analysis software

Export of raw data from real-time PCR cycler:

7500 Fast Real-Time PCR Instrument (Applied Biosystems):

- Save the eds file choosing “File” and “Save as” in the desired location.

CFX 96 Touch (Bio-Rad):

- Tick “No Baseline Subtraction” under “Settings” and “Baseline Settings”.
- Select tab “Quantification Data”.
- Select “Results” in the dropdown box.
- Right-click on the table and select “Export to Excel”.
- Save file in the desired location.
- Select “RFU” in the dropdown box.
- Right-click on the table and select “Export to Excel”.
- Save file in the desired location.

qTOWER³ (Analytik Jena):

- Select the colour compensation “Standard1” in the tab “Settings” and “Scan” and save.
- In the tab “Settings” under “Samples” right-click on the table and select “Export table to Excel-file”
- Save the file at the desired location.
- In the tab “Monitoring” under “View” in the drop-down menu select “Raw data”.
  Note: Under the diagram in the channel selection “all colours” must be set to export the data for the four channels in one file!
- Right-click on the diagram and select “Save chart as CSV-file”.
- Save file at the desired location.

Import of raw data into the EURORealTime Analysis software

- Open the EURORealTime Analysis software
- Select “Evaluate Run” and select a run from the menu

7500 Fast Real-Time PCR Instrument (Applied Biosystems):

- In the dialog box, select the eds file belonging to the run. If the storage locations of the eds files are already contained in the EURORealTime Analysis software, there will be an automatic preselection.
- When the eds file is loaded or preselected, select “Load Protocol”.

CFX 96 Touch (Bio-Rad):

- In the dialog box, select the xls files belonging to the run:
  - With RFU Export (amplification results), open the xls file having “Quantification Amplification Results” in its name
  - With Results Export (Cq Results), open the xls file having “Quantification Cq Results” in its name
- When all xls files are loaded, select “Load Protocol”.

qTOWER³ (Analytik Jena):

- In the dialog box, select the xls and csv files belonging to the run.
  - For Plate Layout export, open the xls file.
  - For Raw Data export, open the csv file.
- When the xml and csv files have been loaded, select “Load Protocol”.

Test evaluation

The detection of SARS-CoV-2 RNA is performed using two target regions, which are both detected in the same fluorescence channel (FAM). The detection of the RNA of influenza virus type A is performed by using a target region which is detected in the fluorescence channel ROX. The detection of the RNA of influenza virus type B is performed by using a target region which is detected in the fluorescence channel Cy5.

Evaluation using EURORealTime Analysis software

All test results and partial results are automatically determined and issued by the EURORealTime Analysis software.

- The results for the parameters and all controls are shown in table format in the “Details” tab and the corresponding amplification curves are displayed in the “Qualitative Analysis” tab.
- The results are evaluated automatically by the software, **however, they must always be checked and validated by the user!** Results can only be saved after final approval/validation by the user.
- After the results have been approved by the user in the “Details” tab, the summarising result list for SARS-CoV-2, Influenza A and Influenza B is given on the tab “Result Summary”, including all the controls.
- Printing the results:
  Results with the parameters SARS-CoV-2, Influenza A, Influenza B and IC in the “Details” tab can be printed for documentation either completely (“Print parameters” button) or in parts (“Print selected parameters” button). By activating the “Show Amplification Curves” box, the diagrams can be integrated into the result report. Furthermore, the result list summary in the tab “Result Summary” can be printed either completely (“Print results” button) or as a selection (“Print selected results”). In this view, only the result for the parameters SARS-CoV-2, Influenza A and Influenza B is displayed.

Note: Only approved results can be printed.

EUROIMMUN recommends the following evaluation of results:

Patient samples

SARS-CoV-2:

detected:	The sample shows an amplification signal for SARS-CoV-2 RNA at “SARS-CoV-2”.
not detected:	The sample shows no amplification signal for SARS-CoV-2 RNA at “SARS-CoV-2”.

Influenza A

detected:	The sample shows an amplification signal for RNA of influenza virus type A at “Influenza A”.
not detected:	The sample shows no amplification signal for RNA of influenza virus type A at “Influenza A”.

Influenza B

detected:	The sample shows an amplification signal for RNA of influenza virus type B at “Influenza B”.
not detected:	The sample shows no amplification signal for RNA of influenza virus type B at “Influenza B”.
invalid:	The following reasons are possible: • The internal control (IC) was not detected. • The external negative control is invalid. • The external positive control is invalid.

Positive control (PC)

valid:	The positive control shows an amplification signal for SARS-CoV-2, influenza A and influenza B RNA at “SARS-CoV-2”, “Influenza A” and “Influenza B”.
invalid:	The positive control shows no amplification signal for SARS-CoV-2, influenza A and/or influenza B RNA at “SARS-CoV-2”, “Influenza A” and/or “Influenza B”.

Negative control (NC)

valid: The negative control shows no amplification signal for SARS-CoV-2, influenza A and influenza B RNA at “SARS-CoV-2”, “Influenza A” and “Influenza B”; the IC shows the expected amplification signal.

invalid: The negative control shows an amplification signal for SARS-CoV-2, influenza A and/or influenza B RNA at “SARS-CoV-2”, “Influenza A” and/or “Influenza B”; the negative control shows no or an inhibited amplification signal for the IC.

Internal control (IC)

detected: The sample shows the expected amplification signal for the internal control.

not detected: The sample does not show the expected amplification signal for the internal control.

Note: If the IC signal does not show the expected amplification signal but a clearly increased Cp value and/or a reduction in fluorescence intensity this can indicate errors during extraction and/or PCR inhibition. In such cases the IC signal is evaluated as “not detected” by the EURORealTime Analysis software.

Note: A sample that shows an ambiguous result is highlighted in orange in the overview tab. The result is in a borderline area and cannot be reliably interpreted by the classifier for methodological reasons. The corresponding curve is labelled with orange diamonds in the “Qualitative Analysis” tab. EUROIMMUN recommends repeating the analysis for this sample.

Additional explanations for test results:

In special cases, test results are appended with the following footnotes:

[1] Control data out of specification. Possible reasons: the IC of this sample is not detected, the PC and/or NC of the test run is invalid.

[2] Result modified by user.

- Transfer of results to LIMS:
  Results can be transferred to a connected LIMS. Only the results of samples whose analysis was assigned by the connected LIMS are transferred.
  - Click the “Transfer All to LIMS” or “Transfer Selection to LIMS” button.
  - The analysis results of all of the patient samples stored in the LIMS are transferred to the LIMS.
  - A successful transfer can be identified on the LIMS status symbol.

Evaluation with the software of the used real-time PCR cycler

EUROIMMUN recommends the following settings:

- 7500 Fast Real-Time PCR Instrument (Applied Biosystems):
  Check the default setting “automatic threshold” and correct it, if required, by adjusting the threshold value.
- CFX 96 Touch (Bio-Rad):
  Select the settings “Regression” (Cq Determination Mode), „Apply Fluorescence Drift Correction“ and „Baseline Subtracted Curve Fit“ (Baseline Setting), check and, if required, adjust.
- qTOWER³ (Analytik Jena):
  Check and, if required, adjust the presettings “Auto Threshold” and “Baseline correction”.
  Note: For correct evaluation use the software colour compensation “Standard1” (qPCRsoft 4.1).

EUROIMMUN recommends the following evaluation of results:

	SARS-CoV-2 FAM	Influenza A ROX	Influenza B Cy5	IC VIC/HEX/Yellow	Result
Sample	-	-	-	+	SARS-CoV-2, influenza A and influenza B RNA not detected
	-	-	-	-/inhibited[5]	invalid result since there is no or an inhibited amplification signal for internal control
	+	-	-	-/+	SARS-CoV-2 RNA detected, influenza A and influenza B virus RNA not detected
	+	+	-	-/+	SARS-CoV-2 and influenza A virus RNA detected, influenza B virus RNA not detected
	+	+	+	-/+	SARS-CoV-2, influenza A and influenza B virus RNA detected
	-	+	-	-/+	Influenza A virus RNA detected, SARS-CoV-2 and influenza B virus RNA not detected
	-	+	+	-/+	Influenza A and Influenza B RNA detected, SARS-CoV-2 RNA not detected
	+	-	+	-/+	SARS-CoV-2 and influenza B virus RNA detected, influenza A virus RNA not detected
	-	-	+	-/+	Influenza B virus RNA detected, SARS-CoV-2 and influenza A virus RNA not detected
Positive control	+	+	+	-/+	valid
	-	-	-	-/+	invalid[6]
	+	-	-	-/+	invalid[6]
	+	+	-	-/+	invalid[6]
	-	+	-	-/+	invalid[6]
	-	+	+	-/+	invalid[6]
	+	-	+	-/+	invalid[6]
	-	-	+	-/+	invalid[6]
Negative control	-	-	-	+	valid
	-	-	-	-/inhibited[7]	invalid result since there is no or an inhibited amplification signal for internal control[6]
	+	-	-	-/+	invalid, contamination[6]
	+	+	-	-/+	invalid, contamination[6]
	+	+	+	-/+	invalid, contamination[6]
	-	+	-	-/+	invalid, contamination[6]
	-	+	+	-/+	invalid, contamination[6]
	+	-	+	-/+	invalid, contamination[6]
	-	-	+	-/+	invalid, contamination[6]

Note: If the IC signal shows a clearly increased Cp value and/or a reduction in fluorescence intensity this can indicate errors during extraction and/or PCR inhibition.

General note for evaluation: EUROIMMUN recommends repeating the analysis for all samples showing an ambiguous or atypical curve that does not allow a clear interpretation. A negative result does not exclude an infection. The sample may contain an insufficient amount of pathogenic material despite an

[5] If no amplification signal is detected for the parameter and at the same time there is no or an inhibited amplification signal for the IC the test result is to be evaluated as “invalid”.

[6] If the negative control and/or the positive control show an invalid result all test results are to be evaluated as “invalid”.

[7] If the negative control shows no or an inhibited amplification signal for the IC all test results are to be evaluated as “invalid”.

ongoing infection. For diagnosis, the clinical symptoms of the patient should always be taken into account along with the obtained results.

Analytical performance

Analytical specificity / cross-reactivity: The specificity of the test system is ensured by the primer and probe design and the PCR conditions given in the instructions for use.
All primers and probes used in the test system were checked for potential homologies by means of sequence comparison analyses in order to exclude potential cross-reactivity. All available sequences in the "nr" database of the National Center for Biotechnology Information (NCBI) (SARS-CoV-2: 13th February 2020, influenza virus: 17th September 2020) were taken into account (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
Additionally, the following pathogens which may occur in the respiratory tract or are closely related with SARS-CoV-2 or influenza virus were tested for potential cross-reactions by using nucleic acids (1 ng/reaction) in the PCR system: respiratory syncytial virus A, parainfluenza virus 1 and 2, rhinovirus, coronavirus NL63, coronavirus MERS, coronavirus OC43, coronavirus SARS HKU39849, coronavirus 229E, enterovirus 71, HHV-5 (CMV), HHV-4 (EBV), measles virus as well as *Escherichia coli, Legionella pneumophila, Mycoplasma pneumoniae, Chlamydia pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae and Streptococcus pyogenes*.
Deviating amounts of nucleic acids <1 ng/reaction were tested for the following pathogens: coronavirus HKU1, parainfluenza virus 3 and 4, adenovirus 1, adenovirus 3, adenovirus 5, adenovirus 31, metapneumovirus, *Bordetella pertussis, Bordetella parapertussis, Mycobacterium tuberculosis* and *Pneumocystis jirovecii*. No cross-reactions were detected.
To exclude cross-reactions with human genomic DNA or RNA, 100 ng of each per reaction was used. No cross-reactions were detected.

The analytical specificity was analysed further during the evaluation study (see below).

Analytical sensitivity / Detection limit*:* The primers and probes used in the test system were developed on the basis of the following sequences:

Pathogen	Database entry
SARS-CoV-2	NC_045512.2
Influenza virus A subtype H3N2	NC_007367.1
Influenza virus A subtype H1N1	NC_026431.1
Influenza virus type B	NC_002211.1

The reference numbers refer to the entry at the National Center for Biotechnology Information (NCBI).

The limit of detection (LoD) was determined based on quantified SARS-CoV-2- and influenza virus A/B-specific RNA. The detection limit was confirmed in three independent investigations using three independent lots with 21 replicates in the presence of 200 ng of human nucleic acid in ≥ 95% of the reactions. The lower detection limit is the minimum detection limit. Usually, fewer copies (cp) of the RNA are detected with the test system.

Analyte	Reference material	Detection limit
SARS-CoV-2	Quantitative Synthetic SARS-CoV-2 RNA: ORF, E, N (ATCC)	1.5 cp/µl eluate
Influenza virus A subtype H3N2	Twist Synthetic Influenza H3N2 RNA control (Twist Bioscience)	1.5 cp/µl eluate
Influenza virus A subtype H1N1	Twist Synthetic Influenza H1N1 (2009) RNA control (Twist Bioscience)	1.5 cp/µl eluate
Influenza virus type B	Twist Synthetic Influenza B RNA control (Twist Bioscience)	3 cp/µl eluate

Furthermore, the lower detection limit, which is achieved with a probability of 95%, was calculated using a dilution series of quantified SARS-CoV-2- and influenza virus A/B-specific RNA by means of a Probit analysis. The results can be found in the following table:

Concentration in RNA sample [cp/µl eluate]	Number of replicates	Number of positive tests			
		SARS-CoV-2	Influenza virus A subtype H3N2	Influenza virus A subtype H1N1	Influenza virus type B
5	21	21	21	21	21
2.5	21	21	21	21	21
1	21	21	21	21	20
0.5	21	20	20	18	14
0.25	21	14	14	17	8
0.1	21	12	6	7	4
0.05	21	3	5	7	0
0.025	21	1	4	1	0
0.01	21	0	2	0	0
Calculated lower detection limit as per Probit analysis [cp/µl eluate]		0.55	0.92	0.67	1.21
95% confidence interval [cp/µl eluate]		0.37 – 1.03	0.56 – 1.96	0.43 – 1.29	0.83 – 2.21

***Reproducibility**:* The reproducibility was investigated using the following samples: Solutions containing the target sequences for the detection of SARS-CoV-2 and influenza virus types A/B in the copy number that corresponds to the 3x lower detection limit or a concentration of 1000 cp/µl were used as positive samples. Moreover, a negative sample was applied which did not contain any RNA from SARS-CoV-2 or the influenza virus types A and B.

***Intra-assay reproducibility**:* The samples mentioned above were analysed 10 times each in one test run by 2 different persons. The results were consistent with the pre-characterisation in all cases. The coefficient of variation of the Cp value measured for all analyses of samples with a concentration of 1000 cp/µl was 0.18% for SARS-CoV-2, 0.46% for influenza virus A subtype H3N2, 0.49% for influenza virus A subtype H1N1 und 0.27% for influenza virus type B.

Inter-assay reproducibility: The samples mentioned above were investigated three times each in 5 independent test runs, performed twice, by two different users on 5 different days. The results were consistent with the pre-characterisation in all cases. The coefficient of variation of the Cp value measured for all analyses of samples with a concentration of 1000 cp/µl was 0.63% for SARS-CoV-2, 1.33% for influenza virus A subtype H3N2, 1.04% for influenza virus A subtype H1N1 and 0.48% for influenza virus type B.

Inter-lot reproducibility: The samples mentioned above were investigated three times each, in 3 independent test runs, performed twice, by two different users using 3 different lots. The results were consistent with the pre-characterisation in all cases. The coefficient of variation of the Cp value measured for all analyses of samples with a concentration of 1000 cp/µl was 0.96% for SARS-CoV-2, 1.36% for influenza virus A subtype H3N2, 1.30% for influenza virus A subtype H1N1 and 0.44% for influenza virus type B.

***Interferences**:* The influence of the following substances was investigated by adding them to the sample before RNA extraction using the QIAamp® Viral RNA Mini Kit (the concentration given in brackets refers to the sample to be extracted): 2% lidocaine solution (5%), 20% benzocaine solution (5%), 0.2% chlorhexidine gluconate solution (10%), 100,000 I.U./ml nystatin solution (10%), guaiphenesin (5 mg/ml), nasal spray (active ingredient: xylometazoline, 10%), saline (0.9%, 3.5%), EDTA blood (5%), 20 mg/g miconazole gel (10%), acyclovir cream (10%). After proper purification using the QIAamp® Viral RNA Mini Kit, none of these substances produced an inhibitory effect on the SARS-CoV-2 or influenza viruses A/B detection in samples at the 3x limit of detection. It cannot be excluded that a false application of the preparation method may affect the analysis result. EUROIMMUN recommends performing the additional

centrifugation step before elution of the RNA according to the manufacturer's specifications to remove any ethanol residue from the purification. Ethanol has a strongly inhibitory effect on the PCR reaction and may lead to false negative or invalid test results.
It was further shown that microorganisms and viruses that can live in the respiratory tract or that are closely related to the pathogen to be detected, as well as human nucleic acids do not interfere with the detection of SARS-CoV-2 and influenza virus A/B. For this purpose, detection of SARS-CoV-2 and influenza virus A/B was confirmed at the 3x lower detection limit using the current test system in the presence of genomic nucleic acids of the following organisms and viruses: respiratory syncytial virus A, parainfluenza virus 1 and 2, rhinovirus, coronavirus NL63, coronavirus MERS, coronavirus OC43, coronavirus SARS HKU39849, coronavirus 229E, enterovirus 71, HHV-5 (CMV), HHV-4 (EBV), measles virus as well as *Escherichia coli, Legionella pneumophila, Mycoplasma pneumoniae, Chlamydia pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae and Streptococcus pyogenes*. Deviating amounts of nucleic acids <1 ng/reaction were tested for the following pathogens: coronavirus HKU1, parainfluenza virus 3 and 4, adenovirus 1, adenovirus 3, adenovirus 5, adenovirus 31, metapneumovirus, *Bordetella pertussis, Bordetella parapertussis, Mycobacterium tuberculosis* and *Pneumocystis jirovecii.* To exclude interferences with human genomic DNA or RNA, 100 ng of each per reaction were tested.

Rare cases of co-infection with SARS-CoV-2, influenza virus type A and/or influenza virus type B have been reported in the literature. In the evaluation it could be shown that the lower detection limit for SARS-CoV-2 is achieved in the presence of up to 10^5 cp/µl eluate of influenza virus type A and 10^5 cp/µl eluate of influenza virus type B. With an influenza viral load >10^5 cp/µl, the detection limit of SARS-CoV-2 cannot be guaranteed due to potential competition within the multiplex PCR. The lower detection limit for influenza virus types A and B cannot be guaranteed in the presence of any of the two other pathogens.

Clinical performance

Diagnostic sensitivity and specificity: It was evaluated whether the results obtained for a clinical sample panel using the EURORealTime SARS-CoV-2/Influenza A/B agreed with those obtained with other SARS-CoV-2 and influenza virus A and B reference tests. In addition to the listed investigations for the determination of performance data, the test system was also exposed to different influencing factors of preanalytics (sample withdrawal, sample quality, RNA purification).

The following samples panels were investigated:
Panel 1:
50 samples precharacterised as negative for SARS-CoV-2 and influenza virus (precharacterisation SARS-CoV-2: EURORealTime SARS-CoV-2 (EUROIMMUN), precharacterisation influenza virus: RealStar® Influenza Screen & Type RT-PCR Kit 4.0 (Altona Diagnostics))
Panel 2:
46 samples precharacterised consistently as positive for SARS-CoV-2 in two independent determinations (determinations 1 and 2: EURORealTime SARS-CoV-2 (EUROIMMUN))
Panel 3:
48 samples (43/5 influenza A virus positive/negative, 6/42 influenza B virus positive/negative) precharacterised consistently in two independent determinations (determination 1: 46 samples with Modular Dx Kit (TIB MOLBIOL, REF 53-0101-96, 58-0102-96, 50-0100-96), 2 samples with Xpert Flu/RSV (Cepheid), determination 2: 48 samples with RealStar® Influenza Screen & Type RT-PCR Kit 4.0 Kit (Altona Diagnostics))

SARS-CoV-2:

96 samples (throat swabs, panels 1 and 2)		Precharacterisation with SARS-CoV-2 real-time PCR reference test	
		positive	negative
Results EURORealTime SARS-CoV-2/Influenza A/B	**positive**	45	0
	negative	1[8]	50

Positive agreement: 97.8%
Negative agreement: 100%

Influenza virus type A:

98 samples (throat swabs, panels 1 and 3)		Precharacterisation with influenza virus real-time PCR reference test	
		positive	negative
Results EURORealTime SARS-CoV-2/ Influenza A/B	**positive**	40	0
	negative	3[9]	55

Positive agreement: 93%
Negative agreement: 100%

Influenza virus type B:

98 samples (throat swabs, panels 1 and 3)		Precharacterisation with influenza virus real-time PCR reference test	
		positive	negative
Results EURORealTime SARS-CoV-2/ Influenza A/B	**positive**	6	1[10]
	negative	0	91

Positive agreement: 100%
Negative agreement: 98.9%

The analysis was performed using RNA samples derived from throat swabs. For the extraction, the QIAamp® Viral RNA Mini Kit (Qiagen; 73 samples) and the CMG-2017 Prepito Viral DNA/RNA300 Kit (Chemagen; 71 samples) were used according to the manufacturers' instructions.

If not indicated otherwise, all above mentioned studies were carried out in the CFX 96 (Bio-Rad), using the Multiplate™ 96-Well PCR Plate, low profile, unskirted, clear (Bio-Rad) order no. #MLL9601 and evaluated with the EURORealTime Analysis software.

The transferability of validation results was shown for the real-time PCR instruments 7500 Fast Real-Time PCR Instrument (Applied Biosystems) and qTOWER³ (Analytik Jena) as well as the PCR reaction vessels described above ("Additional materials and equipment (not supplied in the test kit)").

[8] The sample showed a Cp value of >36 during precharacterisation. According to the precharacterisation the sample is therefore very weak positive.

[9] According to the precharacterisation, the samples are weak or very weak positive. Two of them were consistently tested as positive in two subsequent analyses. For one sample, a positive and a negative result were obtained in two subsequent analyses.

[10] The sample showed a Cp value of 37.86 with this test system. The result is therefore very weak positive. Two subsequent analyses each yielded a negative result which agreed with the precharacterisation.

Limitations of the procedure

- For diagnosis, the clinical symptoms of the patient should always be taken into account along with the molecular diagnostic results. The result of the PCR analysis must, if necessary, be assessed together with results from other diagnostic methods.
- The performance data were generated based on the reference sequences (see section “Analytical sensitivity/Detection limit”). With molecular biological tests, the performance data for sequence variants that deviate at the primer and probe binding sites from the reference sequences cannot be guaranteed.
- A negative result does not exclude an infection. The sample may contain an insufficient amount of pathogenic material for detection despite an ongoing infection.
- Real-time PCR is used to detect the genetic material of a pathogen. This detection method is not necessarily synonymous with the presence of intact pathogens in the sample that are able to reproduce. Under certain circumstances, a pathogen’s genetic material can still be detected after successful treatment.
- A device performance out of specifications and deviations from the described test procedure, specified storage conditions, materials, devices and/or recommended sample material may lead to deviations from the results that are obtained when all instructions are followed. The internal and external controls can help to detect errors. They may, however, not detect every possible error. Each laboratory should validate their modifications and ensure that the device specifications are met.
- Principally, substances in the sample may interfere with the detection of SARS-CoV-2 and influenza viruses, thus reducing the detection limit and leading to invalid or even false negative results. For the extraction kit QIAamp® Viral RNA Mini Kit it could be shown that the tested substances up to the given concentrations had no significant influence on the detection limit.
- A high quality of the nucleic acid samples used in the analysis is an essential prerequisite for the diagnostic relevance of the test results. An unsuitable sample, sample collection, sample storage, nucleic acid preparation or storage can lead to invalid or even false results. It is the responsibility of the laboratory to ensure and, if required, validate the suitability of the sample and preanalytical steps.

Literature

1. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. **The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2.** Nat Microbiol. 2020; 5(4): 536-44
2. Borges do Nascimento IJ, von Groote TC, O'Mathúna DP, Abdulazeem HM, Henderson C, et al. International Task Force Network of Coronavirus Disease 2019 (InterNetCOVID-19). **Clinical, labora-tory and radiological characteristics and outcomes of novel coronavirus (SARS-CoV-2) infection in humans: A systematic review and series of meta-analyses.** PLoS One. 2020; 15(9): e0239235
3. Gralinski LE, Menachery VD. **Return of the Coronavirus: 2019-nCoV.** Viruses 2020, 12(2), 135
4. Udugama B, Kadhiresan P, Kozlowski HN, Malekjahani A, Osborne M, Li VYC, et al. **Diagnosing COVID-19: The Disease and Tools for Detection.** ACS Nano. 2020 Apr 9.
5. Cheng MP, Papenburg J, Desjardins M, Kanjilal S, Quach C, Libman M, et al. **Diagnostic Testing for Severe Acute Respiratory Syndrome-Related Coronavirus-2: A Narrative Review.** Ann Intern Med. 2020 Apr 13
6. Krammer F, Smith GJD, Fouchier RAM, Peiris M, Kedzierska K, Doherty PC, et al. **Influenza.** Nat Rev Dis Primers. 2018; 4(1): 3
7. Paules C, Subbarao K. **Influenza.** Lancet. 2017; 390(10095): 697-708

8. RKI-Ratgeber: **Influenza (Teil 1): Erkrankungen durch saisonale Influenzaviren.** www.rki.de; abgerufen am 1. 9. 2020
9. Allen JD, Ross TM. **H3N2 influenza viruses in humans: Viral mechanisms, evolution, and evaluation.** Hum Vaccin Immunother. 2018; 14(8): 1840-7
10. Tafalla M, Buijssen M, Geets R, Vonk Noordegraaf-Schouten M. **A comprehensive review of the epidemiology and disease burden of Influenza B in 9 European countries.** Hum Vaccin Immunother. 2016; 12(4): 993-1002
11. Solomon DA, Sherman AC, Kanjilal S. **Influenza in the COVID-19 Era.** JAMA. 2020 Aug 14
12. Faury H, Courboulès C, Payen M, Jary A, Hausfater P, Luyt CE, et al. **Medical features of COVID-19 and influenza infection: A comparative study in Paris, France.** J Infect. 2020; S0163-4453(20)30551-X
13. Pérez-García F, Vásquez V, de Egea V, Catalán P, Rodríguez-Sánchez B, Bouza E. **Influenza A and B co-infection: a case-control study and review of the literature.** Eur J Clin Microbiol Infect Dis. 2016; 35(6): 941-6
14. **Good Laboratory Practice when Performing Molecular Amplification Assays**. Quality Guidance, Q 4, Issue no: 4.4
15. Dieffenbach CW, Dveksler GS. **Setting up a PCR laboratory.** PCR Methods Appl. 3 (1993) S2-S7.
16. Prince AM, Andrus L. **PCR: how to kill unwanted DNA.** Biotechniques 12 (1992) 358-360.

Technical support

In case of technical problems you can obtain assistance via the EUROIMMUN website (https://www.euroimmun.de/en/contact/).

Meaning of the symbols

Symbol	Meaning	Symbol	Meaning
PCR MIX A	PCR Mix A		Storage temperature
PCR MIX B	PCR Mix B		Unopened usable until (YYYY-MM-DD)
POS CONTROL	Positive control		Manufacturing date (YYYY-MM-DD)
INT CONTROL	Internal control		Manufacturer
IVD	In vitro diagnostic medical device		Observe instructions for use
LOT	Lot description	CE	CE marking
	Protect from sunlight	REF	Order number
Σ	Contents suffice for <n> analyses		