Instruction for Use

respiraScreen 2
real time RT-PCR Kit

For the simultaneous in vitro detection and differentiation of RNA of SARS-CoV-2 (E gene, RdRP gene and S gene), Influenza Virus A (Flu A) and Influenza Virus B (Flu B), extracted from biological specimens.

REF	**G01131-96**	**G01131-384**	**G01131-768**
Σ	96	384	768

- 18°C

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1 Intended Use

The respiraScreen 2 real time RT-PCR Kit is a screening assay for the simultaneous detection of three groups of different respiratory viruses. The design allows the differentiation of Influenza Virus A (Flu A), Influenza Virus B (Flu B) from the pandemic coronavirus SARS-CoV-2 (E gene, RdRP gene and S gene), extracted from biological specimens.

2 Pathogen Information

Influenza Viruses belong to the family of Orthomyxoviridae. They are the causative agent of 'the flu'. Influenza A and B viruses have a single stranded RNA genome, consisting of 8 RNA segments. The genome of Influenza A Viruses is characterized by a high mutation frequency, the so-called 'antigenic drift'. Numerous subtypes of Influenza A Viruses are known. They can be categorized by their surface antigens H (haemagglutinin) and N (neuraminidase): Influenza A (H1N1) Virus, Influenza A (H5N1) Virus etc. Therefore, yearly in silico analysis of the sequences of newly emerged subtypes is done, to prevent false negative results caused by primer and/or probe mismatches. Influenza B viruses show a 2 – 3 times slower mutation rate then type A.

Coronaviruses (CoV) are a large family of viruses that cause illness ranging from the common cold to more severe diseases such as Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS). The novel Coronavirus (SARS-CoV-2) is a new strain within the Sarbecoviruses that has been previously identified in humans and causes the pulmonary disease COVID-19.

Coronaviruses are zoonotic, meaning they are transmitted between animals and people. Detailed investigations found that SARS-CoV was transmitted from civet cats to humans and MERS-CoV from dromedary camels to humans. Several known Coronaviruses are circulating in animals that have not yet infected humans.

Common signs of infection include respiratory symptoms, fever, cough, shortness of breath and breathing difficulties. In more severe cases, infection can cause pneumonia, severe acute respiratory syndrome, kidney failure and even death.

Standard recommendations to prevent infection spread include regular hand washing, covering mouth and nose when coughing and sneezing, thoroughly

cooking meat and eggs. Avoid close contact with anyone showing symptoms of respiratory illness such as coughing and sneezing.

3 Principle of the Test

The respiraScreen 2 real time RT-PCR Kit contains specific primers and dual-labelled probes for the amplification of RNA (cDNA) of Influenza A (M gene), Influenza B (NEP gene) and SARS-CoV-2 (E gene, RdRP gene and S gene) extracted from biological specimen.
Furthermore, respiraScreen 2 real time RT-PCR Kit contains a Control RNA (Internal Process Control, IPC), which is added during RNA extraction and detected in the same reaction by a HEX-labelled probe.
The Control RNA allows the detection of RT-PCR inhibition and acts as control that the nucleic acid was isolated from the biological specimen.

4 Package Contents

The reagents supplied are sufficient for 96, 384 or 768 reactions, respectively.

Table 1: Components of the respiraScreen 2 real time RT-PCR Kit

Label	Lid Colour	Content		
		96	384	768
Reaction Mix	yellow	1 x 1325 µl	4 x 1325 µl	8 x 1325 µl
Enzyme	blue	1 x 19.2 µl	1 x 76.8 µl	2 x 76.8 µl
Positive Control	red	1 x 150 µl	1 x 300 µl	1 x 300 µl
Negative Control	green	1 x 150 µl	1 x 300 µl	1 x 300 µl
Control RNA	colourless	1 x 480 µl	2 x 960 µl	4 x 960 µl

5 Equipment and Reagents to be Supplied by User

- RNA isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004, NukEx Mag RNA/DNA, gerbion Cat. No. G05012).
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid or optical PCR reaction plate with optical foil
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

The respiraScreen 2 real time RT-PCR Kit is shipped on dry ice or cool packs. All components must be stored at maximum -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Up to 20 freeze and thaw cycles are possible. For convenience, opened reagents can be stored at +2-8°C for up to 6 months. Protect kit components from direct sunlight during the complete test run.

7 Warnings and Precautions

Read the Instructions for Use carefully before using the product.
Before first use check the product and its components for:

- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipet tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner.

Always wear disposable gloves in each area and change them before entering a different area.

- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave the optical PCR reaction plate after the PCR since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.

8 Sample Material

Starting material for respiraScreen 2 real time RT-PCR Kit is RNA isolated from biological specimens (respiratory samples).

9 Sample Preparation

Commercial kits for RNA isolation such as the following are recommended:

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

Please follow the Instructions for Use of the respective extraction kit.

Important:
In addition to the samples always run a ‚water control' in your extraction. Treat this water control analogous to a sample.
Comparing the amplification of the Control RNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time RT-PCR. Furthermore, possible contaminations during RNA extraction will be detectable.

Please note the chapter ‚Control RNA'.

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the manufacturer.

10 Control RNA

A Control RNA is supplied as extraction control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

Add 5 µl Control RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer‘s instructions.

The Control RNA must be added to the Lysis Buffer of the extraction kit.

11 Real time RT-PCR

11.1 Important Points Before Starting:

- Please pay attention to the chapter 7 ‚Warnings and Precautions‘.
- Before setting up the real time RT-PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the RT-PCR set up.
- In every RT-PCR run one Positive Control and one Negative Control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed (except the Enzyme) and centrifuged very briefly.
- Due to the high viscosity of the Enzyme (blue lid), prewarming at room temperature for 15 min is recommended.

11.2 Procedure

The Master Mix contains all of the components needed for the real time RT-PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2:Preparation of the Master Mix

Volume per Reaction	Volume Master Mix
13.8 µl Reaction Mix	13.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

Real time RT-PCR set-up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument / take an optical PCR reaction plate.
- Pipet **14 µl** of the Master Mix into each optical PCR reaction tube / the optical PCR reaction plate.
- Add **6 µl** of the eluates from the RNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding optical PCR reaction tube / the optical PCR reaction plate (Table 3).
- Close the optical PCR reaction tubes / the optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 3: Preparation of the real time RT-PCR

Component	Volume
Master Mix	14.0 µl
Sample	6.0 µl
Total Volume	20.0 µl

11.3 Instrument Settings

For the real time RT-PCR use the thermal profile shown in Table 4.

Table 4: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of cDNA			45
Denaturation	10 sec	95°C	
Annealing and Extension	40 sec Acquisition at the end of this step	60°C	

Further instrument settings have to be adjusted according to the table below.

Table 5: Overview of the instrument settings required for the respiraScreen 2 real time RT-PCR

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes
Bio-Rad CFX96	SARS-CoV-2 (S gene)	FAM	Reference Dye: None
	Control RNA (IPC)	HEX	
	SARS-CoV-2 (E gene, RdRP gene)	ROX	
	Flu B	Cy5	
	Flu A	Cy5.5	
NEOS-96 qPCR	SARS-CoV-2 (S gene)	FAM	
	Control RNA (IPC)	HEX	
	SARS-CoV-2 (E gene, RdRP gene)	ROX	
	Flu B	Cy5	
	Flu A	Alexa Fluor 680	
Quant Studio 5	SARS-CoV-2 (S gene)	FAM	QuantStudio™ 5 Spectral Calibration Plate for the dye Cy5.5 needed Reference Dye: None
	Control RNA (IPC)	HEX	
	SARS-CoV-2 (E gene, RdRP gene)	ROX	
	Flu B	Cy5	
	Flu A	Cy5.5	

12 Data Analysis

Following results can occur:

Signal/C_T Values					**Interpretation**
FAM SARS-CoV-2	**ROX** SARS-CoV-2	**Cy5.5** Flu A	**Cy5** Flu B	**HEX** Control RNA (IPC)	
positive [1]	**positive** [1]	negative	negative	positive or negative [2]	**Positive result. The sample contains RNA of SARS-CoV-2.**
negative	negative	**positive**	negative	positive or negative [2]	**Positive result. The sample contains RNA of Flu A.**
negative	negative	negative	**positive**	positive or negative [2]	**Positive result. The sample contains RNA of Flu B.**
negative	negative	negative	negative	≤ 34	**Negative result. The sample contains no RNA of Flu A, Flu B, or SARS-CoV-2.**
negative	negative	negative	negative	negative or > 34 [3]	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.

1 If the amount of SARS-CoV-2 RNA is below the limit of detection, the results from the FAM and the ROX channel may differ. In this case, a single positive result in one of the two channels is sufficient for a positive result.

2 A strong positive signal in the FAM, ROX, Cy5.5 or Cy5 channel can inhibit the IPC. In such cases the result for the Control RNA can be neglected.

3 In case of high C_T values, the IPC should be compared to the water extraction control as described in the chapter 'Assay validation'.

Figure 1, Figure 2, Figure 3, Figure 4 and **Figure 5** show examples for positive and negative real time RT-PCR results.

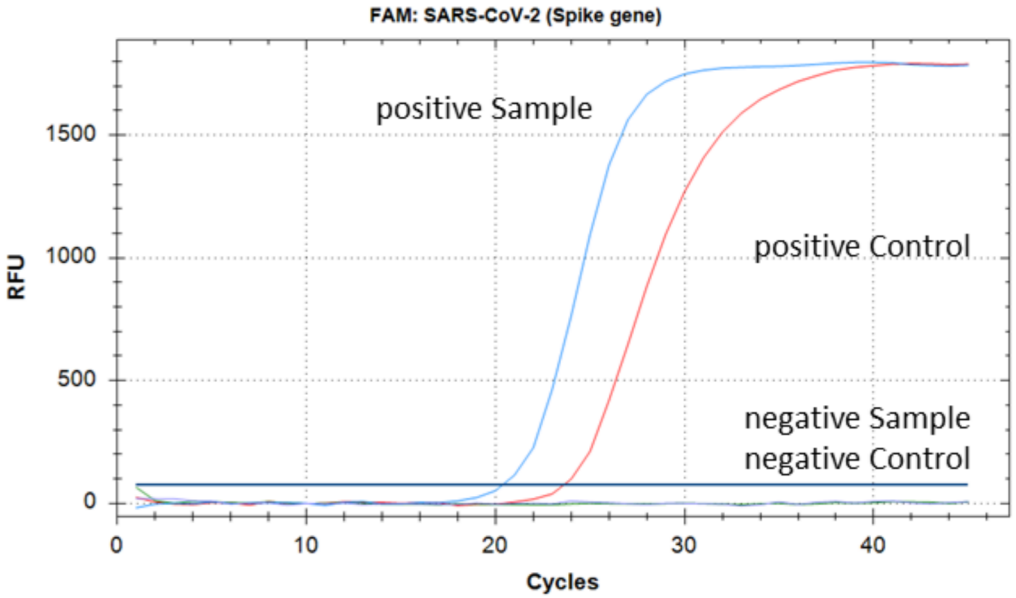

Figure 1: The positive sample shows pathogen specific amplification in the FAM channel (positive SARS-CoV-2 (S gene) sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (CFX96).

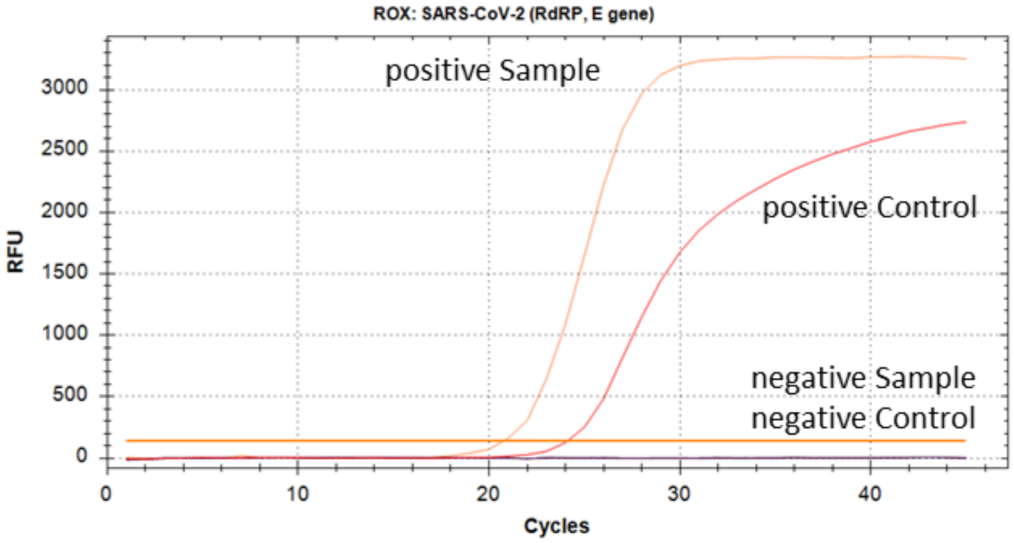

Figure 2: The positive sample shows pathogen specific amplification in the ROX channel (positive SARS-CoV-2 (E gene, RdRP gene) sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (CFX96).

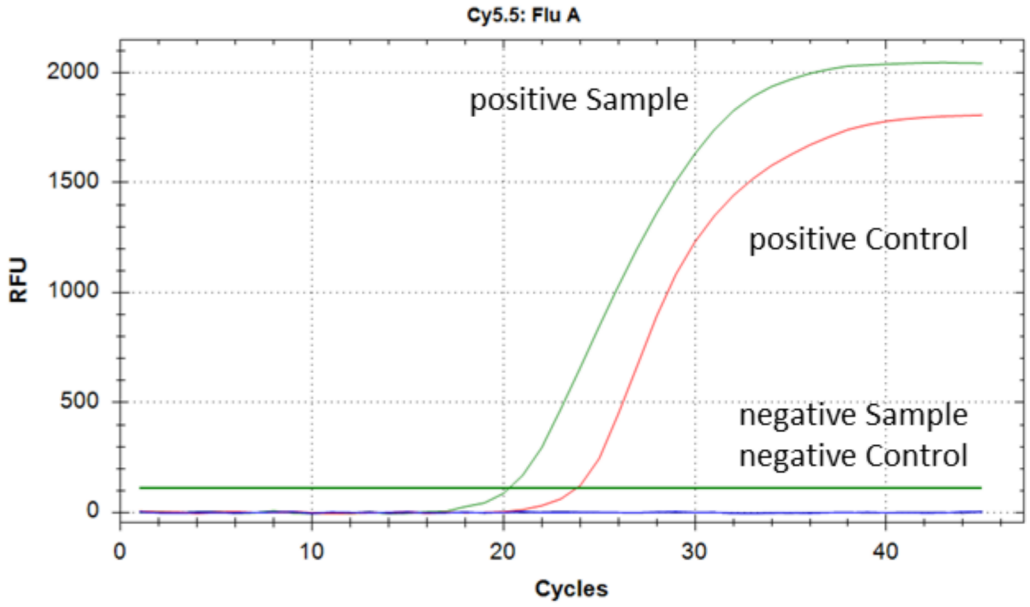

Figure 3: The positive sample shows pathogen specific amplification in the Cy5.5 channel (positive Flu A sample and Positive Control), whereas no fluorescence signal is detected in the negative sample or the Negative Control (CFX96).

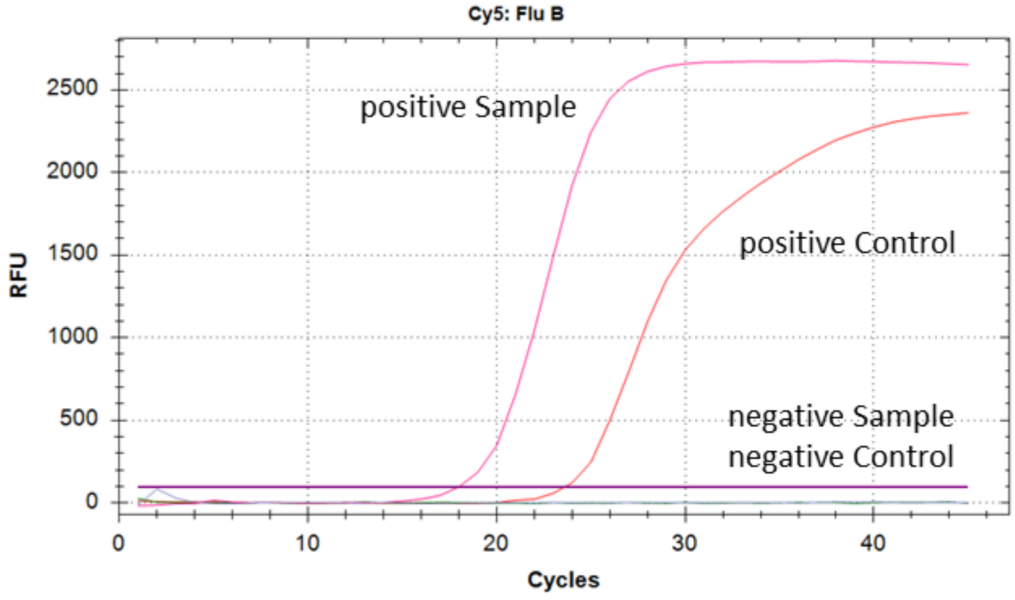

Figure 4: The positive sample shows pathogen specific amplification in the Cy5 channel (positive Flu B sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (CFX96).

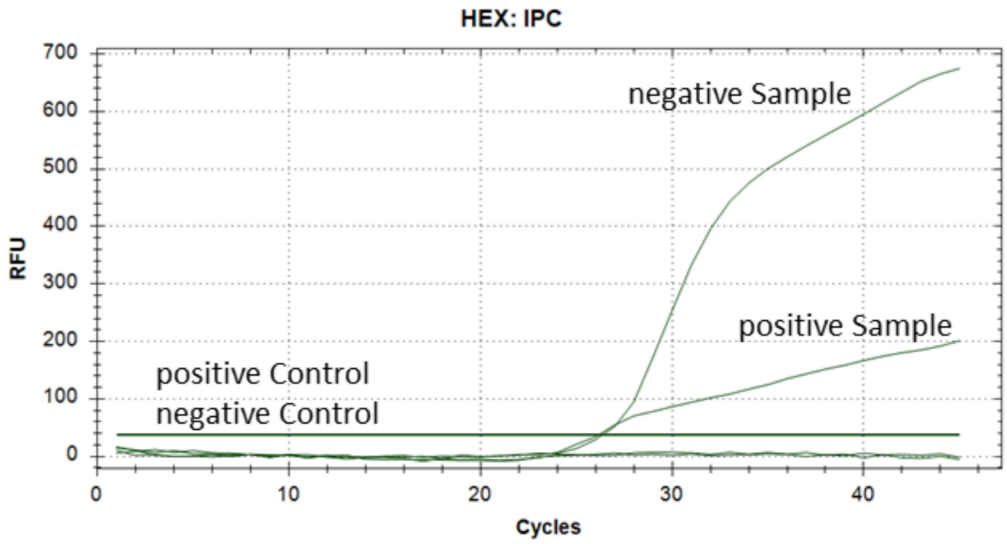

Figure 5:: The positive sample and the negative sample show an amplification curve, whereas the Positive Control and the Negative Control don't show a signal in the Control RNA specific HEX channel (CFX96). The amplification signal of the Control RNA in the negative eluate shows that the missing signal in the specific channels is not due to PCR inhibition or failure of RNA isolation, but that the eluate is a true negative.

13 Assay Validation

Negative Control

The Negative Control must show no C_T in the channels Cy5.5, FAM, HEX, ROX and Cy5.

Positive Control

All parameters in the Positive Control must show a positive (i.e. exponential) amplification curve in the different channels Cy5.5, FAM, ROX and Cy5. The Positive Controls must fall below a C_T of 30.

Internal Controls

The following values for the amplification of the internal controls are valid using gerbion nucleic acid extraction kits NukEx Mag RNA/DNA or NukEx Pure RNA/DNA. The Control RNA (IPC) must show a positive (i.e. exponential) amplification curve and fall below a C_T of 34. If the Control RNA is above C_T 34 this points to a purification problem or a strong positive sample that can inhibit the IPC. In the latter case, the assay is valid. It is recommended to

perform the extraction of a water control in each run. The IPC in the water control must fall below a C_T of 34.
If other nucleic acid extraction kits are used, the customer must define own cut-offs. In this case the C_T value of the Control RNA (IPC) in an eluate from a sample should not be delayed for more than 4 C_T in comparison to an eluate from an extracted water control.

14 Limitations of the Method

- Strict compliance with the Instruction for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR and in vitro diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of RT-PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the Flu A, Flu B and SARS-CoV-2 genomes covered by the primers and/or probes used in the kit may result in failure to detect the respective RNA.
- As with any diagnostic test, results of the respiraScreen 2 real time RT-PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

15 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time RT-PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal for the Positive Control in the channel Cy5.5 and/or FAM and/or ROX and/or Cy5.	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the SARS-CoV-2 (S gene) specific amplification, the ROX channel for analysis of the SARS-CoV-2 (RdRP gene and E gene) specific amplification, the Cy5 channel for analysis of the Flu B specific amplification, the Cy5.5 channel for analysis of the Flu A specific amplification and the HEX channel for the amplification of the Control RNA.
Incorrect preparation of the Master Mix	Make sure that the Enzyme is added to the Master Mix (chapter 11).
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with 'Procedure' on page 7.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol 'Instrument Settings' in Table 4 and 5.
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in 'Transport, Storage and Stability'.
Weak or no signal of the Control RNA (HEX) and simultaneous absence of a signal in the channel FAM and /or ROX and /or Cy5 and /or Cy5.5	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions in Table 4 and Table 5.
real time RT-PCR inhibited	Make sure that you use an appropriate isolation method (see chapter 'Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed.
sample material not sufficient	Make sure that enough sample material has been applied to the extraction. Use an appropriate isolation method (see chapter 'Sample Preparation') and follow the manufacturer's instructions.
RNA loss during isolation process	Lack of an amplification signal in the 533-580 channel can indicate that the RNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in 'Transport, Storage and Stability'.

Detection of a fluorescence for the Negative Control signal in the channel FAM and /or ROX and /or Cy5 and /or Cy5.5 and /or HEX	
Contamination during preparation of the real time RT-PCR	Repeat the real time RT-PCR in replicates. If the result is negative in the repetition, the contamination occurred when the samples were pipetted. Make sure to pipet the Positive Control last. If the same result occurs, one or more of the kit components might be contaminated. Make sure that workspace and instruments are decontaminated regularly. Use a new kit and repeat the real time RT-PCR.

16 Kit Performance

16.1 Analytical Sensitivity

The limit of detection (LoD) of respiraScreen 2 real time RT-PCR Kit was determined testing serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on a CFX96 real time PCR instrument. The estimated LoD of respiraScreen 2 real time RT-PCR Kit is ≤ 10 genome copies per reaction for each parameter (SARS-CoV-2, Flu A, Flu B).

16.2 Analytical Specificity

The specificity of the respiraScreen 2 real time RT-PCR Kit was evaluated with different other relevant viruses and bacteria found in clinical samples and basing on in silico analyses.

The results for the sample analysis are shown in table 6, the results for the in silico analysis are shown in Table 7.

Table 6: Eluted RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of respiraScreen 2 real time RT-PCR Kit.

Eluates with known status	Result SARS-CoV-2 (S gene)	Result SARS-CoV-2 (E gene, RdRP gene)	Result Flu A	Result Flu B
Parainfluenzavirus 1	negative	negative	negative	negative
Parainfluenzavirus 2	negative	negative	negative	negative
Parainfluenzavirus 3	negative	negative	negative	negative
Parainfluenzavirus 4	negative	negative	negative	negative
Metapneumovirus	negative	negative	negative	negative
Adenovirus	negative	negative	negative	negative
Enterovirus	negative	negative	negative	negative
Legionella pneumophila	negative	negative	negative	negative
Mycoplasma pneumophila	negative	negative	negative	negative
Mycobacterium tuberculosis complex	negative	negative	negative	negative
Bordetella pertussis	negative	negative	negative	negative
Bordetella parapertussis	negative	negative	negative	negative
Staphylococcus aureus	negative	negative	negative	negative
MRSA	negative	negative	negative	negative
Streptococcus ssp.	negative	negative	negative	negative
MERS-CoV	negative	negative	negative	negative
HCoV-229E	negative	negative	negative	negative
HCoV-OC43	negative	negative	negative	negative
SARS-CoV-2	positive	positive	negative	negative
Influenza A H1N1	negative	negative	positive	negative
Influenza A H3N2	negative	negative	positive	negative
Influenza A H5N1	negative	negative	positive	negative
Influenzavirus B	negative	negative	negative	positive
Respiratory Syncytial Virus A	negative	negative	negative	negative
Respiratory Syncytial Virus B	negative	negative	negative	negative

Table 7: Inclusivity of the respiraScreen 2 real time RT-PCR Kit Primers and Probes (in silico analysis).

1000 - 5000 whole genome sequences		Homology	Comment
Flu B	Forward Primer	1000 sequences: 100%	no mismatch
	Reverse Primer	1000 sequences: 100%	no mismatch
	Probe	998 sequences: 100%	2 sequences: 96% (1 mismatch)
Flu A	Forward Primer	5000 sequences: 100%	no mismatch
	Reverse Primer	5000 sequences: 100%	no mismatch
	Probe	5000 sequences: 100%	no mismatch
RdRP gene	Forward Primer	2313 sequences: 100%	7 sequences: 95% (1 mismatch)
	Reverse Primer	2320 sequences: 100%	no mismatch
	Probe	2318 sequences: 100%	2 sequences: 95% (1 mismatch)
S gene	Forward Primer	2315 sequences: 100%	5 sequences: 96% (1 mismatch)
	Reverse Primer	2312 sequences: 100%	8 sequences: 96% (1 mismatch)
	Probe	2309 sequences: 100%	11 sequences: 95% (1 mismatch)
E gene	Forward Primer	2319 sequences: 100%	1 sequence: 96% (1 mismatch)
	Reverse Primer	2318 sequences: 100%	2 sequences: 95% (1 mismatch)
	Probe	2317 sequences: 100%	3 sequences: 96% (1 mismatch)

16.3 Clinical Samples

Positive (106) and negative (171) confirmed samples (oral and nasal swabs) from the pandemic COVID-19 outbreak 2020 and 2021 in Europe were tested. The RNA was extracted by using the NukEx Mag RNA/DNA (gerbion Cat. No. G05012) extraction kit on a KingFisher™ Flex 96 Purification System.
The testing of the confirmed samples with respiraScreen 2 real time RT-PCR Kit showed a sensitivity of 100% and a specificity of 100%.

	SARS-CoV-2 positive samples	SARS-CoV-2 negative samples
respiraScreen 2 positive SARS-CoV-2	106	0
respiraScreen 2 negative SARS-CoV-2	0	171
	Sensitivity (%)	Specificity (%)
	100	100

16.4 Linear Range

The linear range of the respiraScreen 2 real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of in vitro transcripts of the target sequences.

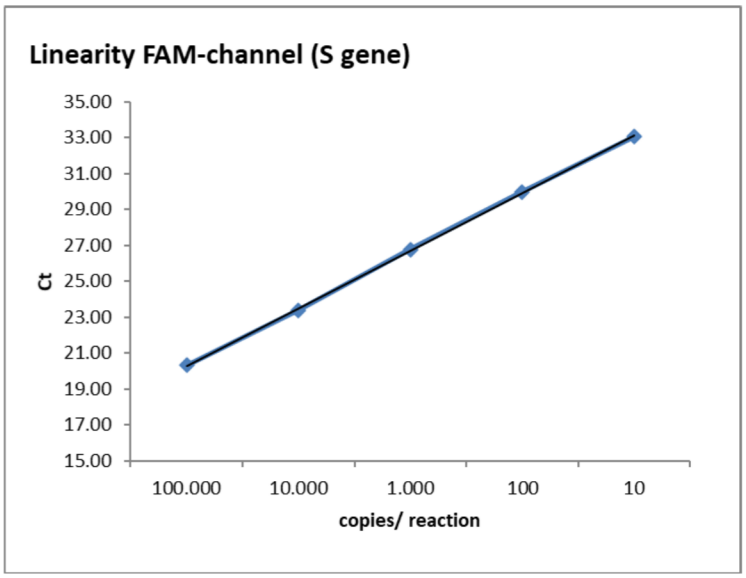

Figure 6: Determination of the linear range of respiraScreen 2 real time RT-PCR Kit for SARS-CoV-2 (S gene) in the FAM channel.

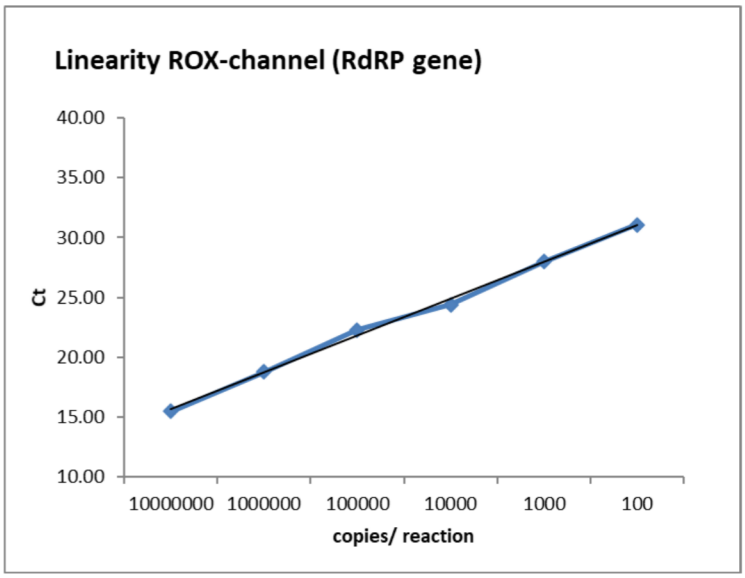

Figure 7: Determination of the linear range of respiraScreen 2 real time RT-PCR Kit for SARS-CoV-2 (RdRP gene) in the ROX channel.

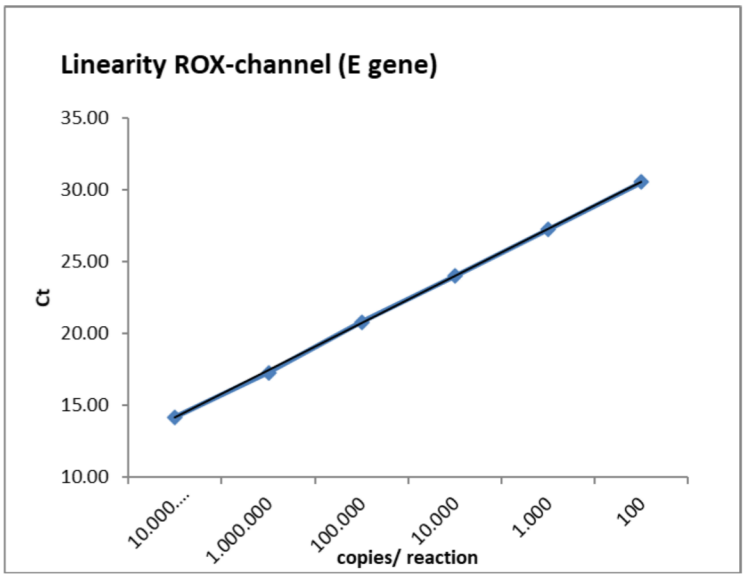

Figure 8: Determination of the linear range of respiraScreen 2 real time RT-PCR Kit for SARS-CoV-2 (E gene) in the ROX channel.

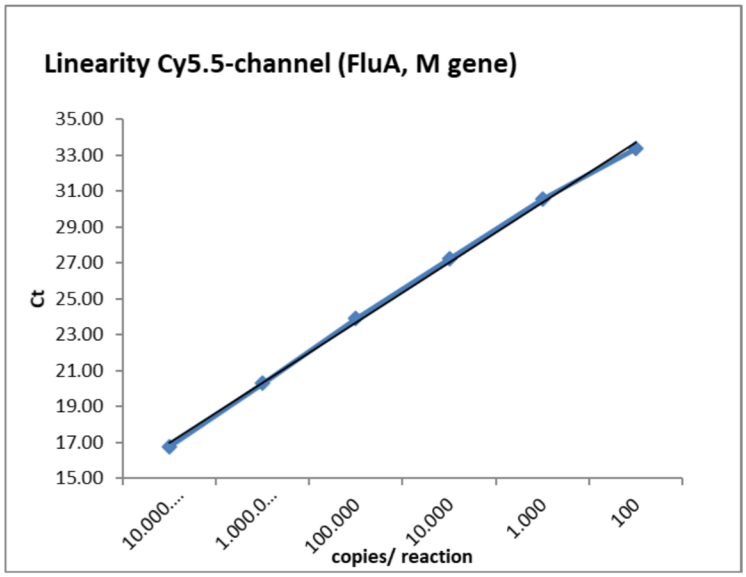

Figure 9: Determination of the linear range of respiraScreen 2 real time RT-PCR Kit for Flu A in the Cy5.5 channel.

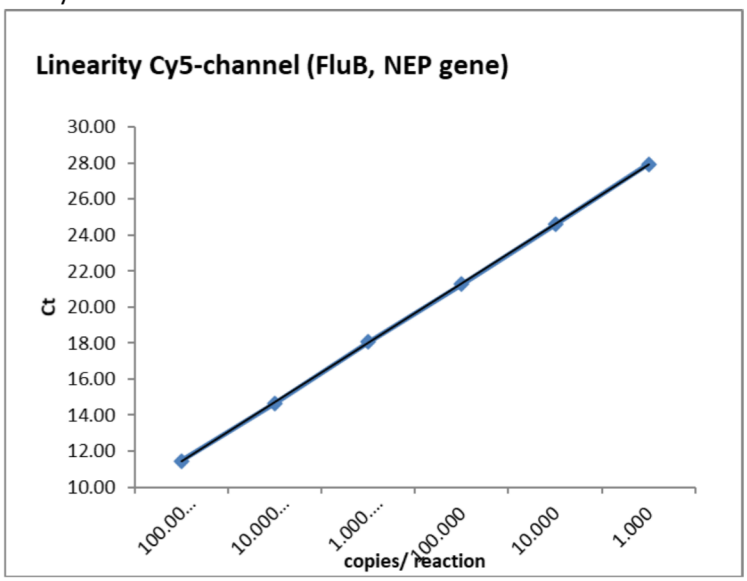

Figure 10: Determination of the linear range of respiraScreen 2 real time RT-PCR Kit for Flu B in the Cy5 channel.

16.5 Precision

The precision of the respiraScreen 2 real time RT-PCR Kit was determined as intra-assay variability, inter-assay variability and inter-lot variability.
Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of Flu A in vitro transcripts, Flu B in vitro transcripts, SARS-CoV-2 (E gene, RdRP gene and S gene) in vitro transcripts and on the threshold cycle of the Control RNA (IPC). The results are shown in Table 8.

Table 8: Precision of the respiraScreen 2 real time RT-PCR Kit.

SARS-CoV-2, S gene (FAM)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	1000	0.13	0.41
Inter-Assay-Variability	1000	0.20	0.66
Inter-Lot-Variability	1000	0.16	0.54
SASR-CoV-2, RdRP gene and E gene (ROX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	500	0.16	0.53
Inter-Assay-Variability	500	0.10	0.32
Inter-Lot-Variability	500	0.10	0,31
Flu A (Cy5.5)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	1000	0.07	0.24
Inter-Assay-Variability	1000	0.04	0.14
Inter-Lot-Variability	1000	0.08	0.26
Flu B (Cy5)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	500	0.17	0.56
Inter-Assay-Variability	500	0.17	0.55
Inter-Lot-Variability	500	0.04	0.11
IPC (HEX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	1000	0.10	0.37
Inter-Assay-Variability	1000	0.34	1.26
Inter-Lot-Variability	1000	0.16	0.60

16.6 Diagnostic Sensitivity

The diagnostic sensitivity of real time (RT-)PCR assays is mainly dependent on the DNA/RNA extraction method used to isolate DNA and RNA from various biological specimens. DNA/RNA extraction reagents are not part of the gerbion real time (RT-)PCR kits. gerbion real time (RT-)PCR kits include an

extraction control and guidelines for the validation criteria of the extraction control in each reaction. The extraction control indicates inhibition of the real time (RT-)PCR and/or inefficient nucleic acid extraction. It cannot be used as a calibrator.
Therefore, gerbion guarantees the analytical sensitivities and specificities of the real time (RT-)PCR kits, performed with eluted DNA and RNA from reference materials and ring trial samples and with synthetic nucleic acid fragments. gerbion does not guarantee diagnostic sensitivities. If diagnostic sensitivities are mentioned in manuals of gerbion real time (RT-)PCR kits, the data are strictly correlated to a specific nucleic acid extraction method that has been used during the validation of the respective kits and cannot be transferred to other extraction methods. It is the responsibility of the user to qualify the extraction methods used for DNA/RNA isolation from biological samples.

AccuPlex™ SARS-CoV-2, Flu A/B and RSV Verification Panel member 1 was spiked in 20 samples of pooled confirmed negative pharyngeal swab samples in UTM® with a final concentration of 1000 copies/ml and 500 copies/ml. Nucleic acids were extracted with the NukEx Mag RNA/DNA on the KingFisher™ Flex 96 Purification System as described above. The results of the experiment on the CFX96 can be found in Table 9 and Table 10.

Table 9: Confirmation of the LoD on the CFX96

Concentration 1000 virus/mL				
replicate	SARS-CoV-2		Flu A	Flu B
	S gene	RdRP gene / E gene		
1	33.42	31.71	40.38	33.47
2	32.54	31.51	-	32.48
3	33.28	31.32	35.75	32.66
4	34.18	31.82	38.08	33.48
5	34.08	31.37	36.53	33.19
6	33.44	31.57	35.98	32.64
7	33.02	30.87	34.75	33.23
8	33.07	31.49	37.69	33.26
9	34.54	31.52	35.57	33.41
10	33.77	31.65	35.62	33.22
11	32.70	31.39	35.31	33.62
12	33.69	31.43	37.78	32.56
13	32.95	32.37	37.27	33.07
14	33.70	31.63	35.64	32.85
15	33.58	31.09	37.62	32.91
16	32.73	31.18	35.50	32.28
17	33.26	31.35	35.84	33.08
18	33.22	31.41	37.50	32.96
19	33.27	31.55	35.25	33.25
20	34.31	32.32	36.03	33.08
Mean C_T	33.44	31.53	36.53	33.04
SD	0.55	0.35	1.38	0.37
CoV	1.64	1.12	3.78	1.11
Result	20/20	20/20	19/20	20/20

Table 9: Confirmation of the LoD on the CFX96

Concentration 500 virus/mL				
replicate	SARS-CoV-2		Flu A	Flu B
	S gene	RdRP gene / E gene		
1	33.64	32.01	-	32.74
2	34.63	32.34	37.93	34.18
3	33.48	32.75	-	34.20
4	34.69	32.39	37.35	33.97
5	35.07	32.05	-	34.14
6	34.88	33.20	-	34.22
7	34.25	32.28	-	34.18
8	33.93	32.07	-	33.45
9	34.21	32.04	37.03	33.41
10	34.55	33.24	35.79	34.52
11	33.47	32.96	36.00	34.37
12	35.48	32.14	-	33.31
13	34.50	31.84	36.46	33.85
14	33.80	32.03	35.29	34.72
15	33.97	33.25	37.59	33.37
16	33.98	32.52	37.62	33.88
17	34.89	32.19	37.46	33.55
18	34.47	32.55	-	33.79
19	36.79	33.46	-	33.27
20	34.90	32.31	39.17	33.86
Mean C_T	34.48	32.48	37.06	33.85
SD	0.77	0.49	1.11	0.49
CoV	2.24	1.52	2.99	1.45
Result	20/20	20/20	11/20	20/20

The respiraScreen 2 real time RT-PCR Kit in combination with the NukEx Mag RNA/DNA nucleic acid extraction kit on a KingFisher™ Flex 96 Purification System and the CFX96 system detected 20/20 replicates at a concentration of 500 copies/ml for SARS-CoV-2 and Flu B and 19/20 replicates at a concentration of 1000 copies/ml for Flu A.
Consequently, the confirmed LoDs are:

- 500 copies/ml for SARS-CoV-2
- 500 copies/ml for Flu B
- 1000 copies/ml for Flu A

17 Abbreviations and Symbols

RNA	Ribonucleic Acid		Upper limit of temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction		Manufacturer
REACTION MIX	Reaction Mix		Use by YYYY-MM-DD
ENZYME	Enzyme	LOT	Batch code
CONTROL +	Positive Control	CONT	Content
CONTROL −	Negative Control		Consult instruction for use
CONTROL RNA IPC	Control RNA (IPC)	IVD	*In vitro* diagnostic medical device
REF	Catalog number	CE	European Conformity
	Content sufficient for <n> tests		

18 Literature

[1] www.who.int/health-topics/coronavirus
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[3] www.nature.com/articles/s41564-020-0695-z, 02/March/2020
[4] https://www.ncbi.nlm.nih.gov/research/coronavirus/
[5] https://www.nhs.uk/conditions/sars/