Instruction for Use

respiraSC2-FluA/B seqc

real time RT-PCR Kit

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

REF	**G01130-96**	**G01130-384**
Σ	96	384
- 18°C		

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1 Intended Use

The respiraSC2-FluA/B seqc real time RT-PCR Kit is an assay for the detection of RNA of the pandemic coronavirus SARS-CoV-2 and Influenza Virus (Flu A and Flu B), extracted from biological specimens.

2 Pathogen Information

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 [1, 2, 3].

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.

Influenza Viruses belong to the family of Orthomyxoviridae and 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. The high mutation rate in Influenza A viruses leads to a lot of different generations, adding to some genetic variants a pandemic and zoonotic potential [4]. These generations like G4 H1N1 swine influenza virus will be detected, too. Influenza B viruses show a 2 – 3 times slower mutation rate then type A.

3 Principle of the Test

The respiraSC2-FluA/B seqc real time RT-PCR Kit contains one vial (Reaction Mix 1) with specific primers and dual-labelled probes for the amplification of RNA (cDNA) of SARS-CoV-2 (RdRP gene (FAM channel), E gene (ROX channel), S gene (Cy5 channel)).

The respiraSC2-FluA/B seqc real time RT-PCR Kit contains a second vial (Reaction Mix 2) with specific primers and dual-labelled probes for the amplification of RNA (cDNA) of Influenzavirus A (Flu A M gene, FAM channel) and Influenzavirus B (Flu B NEP gene, Cy5 channel).
Additionally, Reaction Mix 2 contains an Internal System Control (ISC). The ISC consists of primers and probes for the detection of a house keeping gene (human Succinate-Dehydrogenase) in the eluate from a biological specimen. The ISC helps preventing false negative results due to insufficient sample drawing or transport. The amplification of the Succinate-Dehydrogenase target sequence is measured in the ROX channel.

Furthermore, respiraSC2-FluA/B seqc 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. The amplification of the Control RNA (cDNA) is measured in both, Reaction Mix 1 and Reaction Mix 2.

4 Package Contents

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

Table 1: Components of the respiraSC2-FluA/B seqc real time RT-PCR Kit

Label	Lid Colour	Content	
		96	384
Reaction Mix 1	yellow	1 x 1325 µl	4 x 1325 µl
Reaction Mix 2	orange	1 x 1325 µl	4 x 1325 µl
Enzyme	blue	1 x 38.4 µl	1 x 153.6 µl
Positive Control 1 (RdRP gene, E gene, S gene)	red	1 x 150 µl	1 x 150 µl
Positive Control 2 (Flu A, Flu B, ISC)	violet	1 x 150 µl	1 x 150 µl
Negative Control	green	1 x 300 µl	1 x 300 µl
Control RNA	colourless	1 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 respiraSC2-FluA/B seqc 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 Instruction 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 reaction tubes 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 respiraSC2-FluA/B seqc real time RT-PCR Kit is RNA isolated from biological specimens (e.g. oral swabs, nasal swabs, nasal washes, sputum).

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 Instruction 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 and can be used as extraction control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

Control RNA used as extraction control:
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

If the Control RNA is used to control both, the real time RT-PCR and the RNA isolation procedure, please work as described in the following protocol.

Protocol

The Control RNA was added during RNA extraction (chapter 10 ‚Control RNA'). In this case, prepare the Master Mix according to Table 2.

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 (Control RNA was added during RNA extraction)

Volume per Reaction	Volume Master Mix
13.8 µl Reaction Mix 1 or 2	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 respective 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			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Acquisition at the end of this step	60°C	

Dependent on the real time PCR instrument used, further instrument settings have to be adjusted according to Table 5.

Table 5: Overview of the instrument settings required for the respiraSC2-FluA/B seqc real time RT-PCR.

Real time PCR Instrument	**Parameter Reaction Mix 1**	**Parameter Reaction Mix 2**	**Detection Channel**	**Notes**		
LightCycler 480II				Colour Compensation Kit (G070MP3-CC or G070MP1-CC) required		
				Melt Factor	Quant Factor	Max Integration Time (sec)
	RdRP gene	Flu A	465-510	1	10	1
	Control RNA (IPC)	Control RNA (IPC)	533-580	1	10	2
	E gene	ISC	533-610	1	10	2
	S gene	Flu B	618-660	1	10	3
Mx3000P / Mx3005P	RdRP gene	Flu A	FAM	Gain 8	Reference Dye: None	
	Control RNA (IPC)	Control RNA (IPC)	HEX	Gain 1		
	E gene	ISC	ROX	Gain 1		
	S gene	Flu B	Cy5	Gain 4		
AriaMx CFX96 NEOS-48 qPCR NEOS-96 qPCR	RdRP gene	Flu A	FAM		Reference Dye: None	
	Control RNA (IPC)	Control RNA (IPC)	HEX			
	E gene	ISC	ROX			
	S gene	Flu B	Cy5			
ABI 7500 QuantStudio 5	RdRP gene	Flu A	FAM		Reference Dye: None	
	Control RNA (IPC)	Control RNA (IPC)	JOE			
	E gene	ISC	ROX			
	S gene	Flu B	Cy5			

Real time PCR Instrument	Parameter Reaction Mix 1	Parameter Reaction Mix 2	Detection Channel	Notes	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	RdRP gene	Flu A	Green	Gain 5	Outlier Removal NTC Threshold 15%
	Control RNA (IPC)	Control RNA (IPC)	Yellow	Gain 5	
	E gene	ISC	Orange	Gain 5	
	S gene	Flu B	Red	Gain 5	
Mic qPCR Cycler	RdRP gene	Flu A	Green	Gain 8	
	Control RNA (IPC)	Control RNA (IPC)	Yellow	Gain 10	
	E gene	ISC	Orange	Gain 10	
	S gene	Flu B	Red	Gain 10	

12 Data Analysis

Table 6: Interpretation Reaction Mix 1

Signal/C_T Values				
FAM Channel RdRP gene	**ROX Channel** E gene	**Cy5 Channel** S gene	**HEX Channel** Control RNA (IPC)	**Interpretation**
positive	**positive**	**positive**	positive or negative [1]	**Positive result. The sample contains RNA of SARS-CoV-2.**
positive	**positive**	negative	positive or negative [1]	**Positive result. The sample contains RNA of SARS-CoV-2.**
positive [3]	negative	negative	positive or negative [1]	**Positive result. The sample contains RNA of SARS-CoV-2.**
negative	**positive**	**positive**	positive or negative [1]	**Positive result. The sample contains RNA of SARS-CoV-2.**
negative	**positive** [3]	negative	positive or negative [1]	**Positive result. The sample contains RNA of SARS-CoV-2 or SARS-CoV-1.**[4]
negative	negative	**positive** [3]	positive or negative [1]	**Positive result. The sample contains RNA of SARS-CoV-2.**
negative	negative	negative	≤ 34	**Negative result. The sample contains no RNA of SARS-CoV-2 and SARS-CoV-1*.**
negative	negative	negative	negative or > 34 [2]	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.

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

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

3 The WHO Guidelines for the detection of SARS-CoV-2 (March 19, 2020) recommend the detection of two different targets in areas with no known SARS-CoV-2 circulation (Lit. [5]).

4 SARS-CoV-1 infections have not been reported since 2004 (Lit. [6]).

Table 7: Interpretation Reaction Mix 2

Signal/C_T Values				**Interpretation**
FAM Channel Flu A	**Cy5 Channel** Flu B	**ROX Channel** ISC	**HEX Channel** Control RNA (IPC)	
positive	**positive**	positive or negative	positive or negative [1]	**Positive result. The sample contains RNA of Flu A and Flu B.**
positive	negative	positive or negative	positive or negative [1]	**Positive result. The sample contains RNA of Flu A.**
negative	**positive**	positive or negative	positive or negative [1]	**Positive result. The sample contains RNA of Flu B.**
negative	negative	positive	≤ 34	**Negative result. The sample contains no RNA of Flu A and no RNA of Flu B.**
negative	negative	negative	≤ 34	**No diagnostic statement can be made.** Amount or quality of sample material not sufficient.
negative	negative	positive	negative or > 34 [2]	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.

negative	negative	negative	negative or > 34 [2]	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction. Amount or quality of sample material not sufficient.

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

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

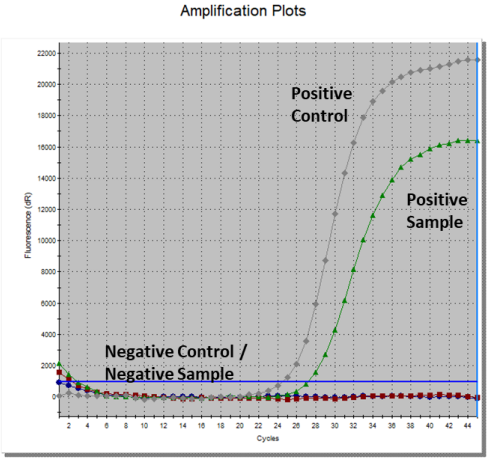

Figure 1: The positive sample shows pathogen specific amplification in the FAM channel (positive sample and Positive Control), whereas no fluorescence signal is detected in the negative sample or the Negative Control (Mx3005P qPCR System).

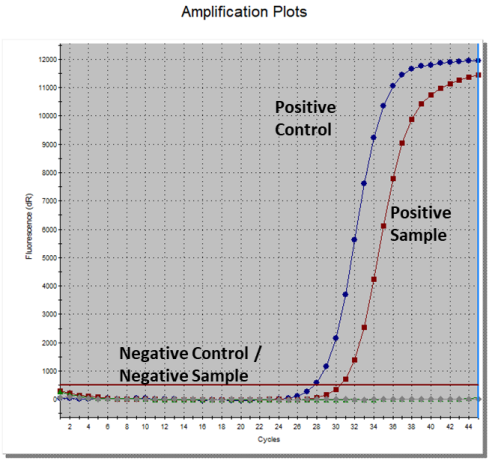

Figure 2: The positive sample shows pathogen specific amplification in the ROX channel of Reaction Mix 1 (positive sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (Mx3005P qPCR System).

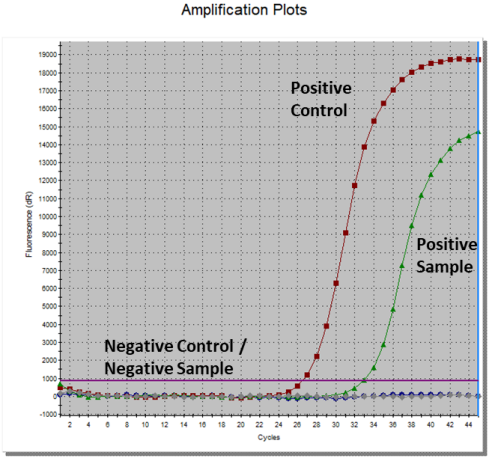

Figure 3: The positive sample shows pathogen specific amplification in the Cy5 channel (positive sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (Mx3005P qPCR System).

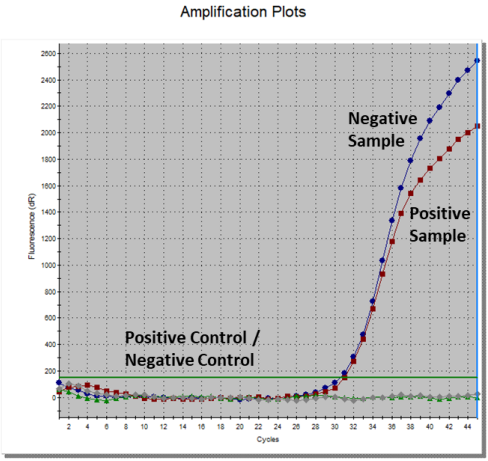

Figure 4: The positive sample and the negative sample show an amplification curve, whereas the Positive Control and the Negative Control don't show a signal in the Control RNA specific HEX channel (Mx3005P qPCR System).

13 Assay Validation

Negative Control

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

Positive Control

All parameters in the Positive Control must show a positive (i.e. exponential) amplification curve in the different channels FAM, Cy5 and ROX. The Positive Controls must fall below a C_T of 30. Positive Control 1 includes in vitro transcripts of SARS-CoV-2 (RdRP gene, E gene and S gene). Positive Control 2 includes in vitro transcripts of Flu A (M gene) and Flu B (NEP gene) and synthetic DNA (ISC, human Succinate-Dehydrogenase).

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. All internal controls (ISC and IPC, seqc – sample and extraction quality control) must show a positive (i.e. exponential) amplification curve. The Control RNA (IPC) must 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. For accurately drawn respiratory swab samples, the ISC (Mix 2, ROX channel) shows C_T values from app. 15 to app. 28. A heavily delayed signal of higher than a C_T of 34 indicates a low sample amount. Therefore, false negative results cannot be ruled out. In case of no amplifications neither in the FAM nor in the Cy5 channel, there must be an amplification curve in the ROX channel of Mix 2 (ISC) and the HEX (IPC) channel when using eluates of primary samples from multiple species such as mammals and birds.

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 SARS-CoV-2, Flu A and Flu B 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 respiraSC2-FluA/B seqc 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 in the FAM and/or ROX and/or Cy5 channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Reaction Mix 1: Select the FAM channel for analysis of the RdRP gene specific amplification, the ROX channel for analysis of the E gene specific amplification, the HEX channel for the amplification of the Control RNA and the Cy5 channel for the amplification of the S gene; Reaction Mix 2: Select the FAM channel for analysis of the Flu A specific amplification, the ROX channel for analysis of the ISC specific amplification, the HEX channel for the amplification of the Control RNA and the Cy5 channel for the amplification of Flu B.
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 8.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol 'Instrument Settings' in Table 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 and ISC and simultaneous absence of a signal in the FAM and/or ROX and/or Cy5 channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions in 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	In case the Control RNA was added before extraction, the lack of an amplification signal 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 signal in the FAM and/or ROX and/or Cy5 and/or HEX channel of the Negative Control	
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 into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. 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.
Detection of a fluorescence signal in the ROX channel of the Negative Control	
Contamination with human DNA during preparation of the real time RT-PCR	As long as the ROX channel shows very high Ct values, the contamination is negligible. If the FAM, Cy5 and HEX channel are negative in the Negative Control, the PCR is still valid for the detection of Flu A and Flu B.

16 Kit Performance

16.1 Analytical Sensitivity

The limit of detection (LoD) of respiraSC2-FluA/B seqc real time RT-PCR Kit was determined testing serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on a QuantStudio 5 real time PCR instrument. The estimated LoD of respiraSC2-FluA/B seqc real time RT-PCR Kit is ≤ 10 genome copies per reaction for the SARS-CoV-2 RdRP gene, Flu A and Flu B and 1 genome copy per reaction for the SARS-CoV-2 E gene and the S gene.

16.2 Analytical Specificity

The specificity of the respiraSC2-FluA/B seqc 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 wet analysis are shown in Table 8 and Table 9, the result for the in silico analysis of the Primer and Probe binding sites is shown in Table 10.

For in silico exclusivity testing, all Primers where used in BLAST analysis with exclusion of the specific PCR targets. Primers and Probes for SARS-CoV-2 E gene may detect SARS-CoV-1 as well, but since there is no report on SARS-CoV-1 cases since 2004, it is very unlikely to happen [6]. This is the only nontarget sequence detected in silico for potential amplification.

Table 8: AccuPlex™ SARS-CoV-2 Verification Panel

	Result SARS-CoV-2 **RdRP**	Result SARS-CoV-2 **E gene**	Result SARS-CoV-2 **S gene**	Result Influenza Virus A **M gene**	Result Influenza Virus B **NEP gene**
Member 1 100.000 copies/ml	positive	positive	positive	negative	negative
Member 2 10.000 copies/ml	positive	positive	positive	negative	negative
Member 3 1.000 copies/ml	positive	positive	negative	negative	negative
Member 4 5000 copies/ml RNase P	negative	negative	negative	negative	negative

Table 9: Eluted nucleic acid from bacterial and viral pathogens tested for the determination of the analytical specificity of respiraSC2-FluA/B seqc real time RT-PCR Kit.

Eluates with known status	Result SARS-CoV-2 **RdRP**	Result SARS-CoV-2 **E gene**	Result SARS-CoV-2 **S gene**	Result Influenza Virus A **M gene**	Result Influenza Virus B **NEP gene**
Parainfluenzavirus 1	negative	negative	negative	negative	negative
Parainfluenzavirus 2	negative	negative	negative	negative	negative
Parainfluenzavirus 3	negative	negative	negative	negative	negative
Parainfluenzavirus 4	negative	negative	negative	negative	negative
Metapneumovirus	negative	negative	negative	negative	negative
Adenovirus	negative	negative	negative	negative	negative
Rhinovirus	negative	negative	negative	negative	negative
Enterovirus	negative	negative	negative	negative	negative
Human Bocavirus	negative	negative	negative	negative	negative
Legionella pneumophila	negative	negative	negative	negative	negative
Mycoplasma pneumophila	negative	negative	negative	negative	negative
Mycobacterium tuberculosis complex	negative	negative	negative	negative	negative
Bordetella pertussis	negative	negative	negative	negative	negative
Bordetella parapertussis	negative	negative	negative	negative	negative
Staphylococcus aureus	negative	negative	negative	negative	negative
MRSA	negative	negative	negative	negative	negative
MSSA	negative	negative	negative	negative	negative
Streptococcus spp.	negative	negative	negative	negative	negative
SARS-CoV-2	***positive***	***positive***	***positive***	negative	negative
HCoV-OC43	negative	negative	negative	negative	negative
HCoV-229E	negative	negative	negative	negative	negative
MERS-CoV	negative	negative	negative	negative	negative
Influenzavirus A H1N1	negative	negative	negative	***positive***	negative
Influenzavirus A H3N2	negative	negative	negative	***positive***	negative
Influenzavirus A H5N1	negative	negative	negative	***positive***	negative
Influenzavirus B	negative	negative	negative	negative	***positive***
Respiratory Syncytial Virus A	negative	negative	negative	negative	negative
Respiratory Syncytial Virus B	negative	negative	negative	negative	negative

Table 10: Inclusivity of the respiraSC2-FluA/B seqc real time RT-PCR Kit Primers and Probes (in silico analysis).

1000 - 5000 whole genome sequences		Homology	Comment
Influenzavirus B	Forward Primer	1000 sequences: 100%	no mismatch
	Reverse Primer	1000 sequences: 100%	no mismatch
	Probe	998 sequences: 100%	2 sequences: 96% (1 mismatch)
Influenzavirus A	Forward Primer	5000 sequences: 100%	no mismatch
	Reverse Primer	5000 sequences: 100%	no mismatch
	Probe	5000 sequences: 100%	no mismatch
SARS-CoV-2 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)
SARS-CoV-2 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)
SARS-CoV-2 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 (36) and negative (171) confirmed samples (oral and nasal swabs) from the pandemic COVID-19 outbreak 2020 in Europe were tested.
The RNA was extracted by using the NukEx Mag RNA/DNA (gerbion Cat. No. G05012) extraction kit on a KingFisher Prime Duo Instrument.
The PCR experiments were performed on a QuantStudio 5 Cycler. The testing of the confirmed samples with respiraSC2-FluA/B seqc real time RT-PCR Kit showed a sensitivity of 100% and a specificity of 100%. None of the samples were inhibited in the real time RT-PCR. For the validation of the respiraSC2-FluA/B seqc real time RT-PCR Kit the eluates of all samples were retested and showed the same results.

field samples (2020)	SARS-CoV-2 positive samples	SARS-CoV-2 negative samples
respiraSC2-FluA/B seqc SARS-CoV-2 positive	36	0
respiraSC2-FluA/B seqc SARS-CoV-2 negative	0	171
	Sensitivity (%)	Specificity (%)
	100	100

Additionally, samples from different ring trials were tested with the respiraSC2-FluA/B seqc real time RT-PCR. For the detection of SARS-CoV-2, only ring trials from 2020 were available, while Influenza A and Influenza B were tested with ring trials from 2013 – 2020.

ring trials (2020)	SARS-CoV-2 positive samples	SARS-CoV-2 negative samples
respiraSC2-FluA/B seqc SARS-CoV-2 positive	11	0
respiraSC2-FluA/B seqc SARS-CoV-2 negative	0	19
	Sensitivity (%)	Specificity (%)
	100	100

ring trials (2013 - 2020)	Influenzavirus A positive samples	Influenzavirus A negative samples
respiraSC2-FluA/B seqc Influenzavirus A positive	32	0
respiraSC2-FluA/B seqc Influenzavirus A negative	0	30
	Sensitivity (%)	Specificity (%)
	100	100

ring trials (2013 - 2020)	Influenzavirus B positive samples	Influenzavirus B negative samples
respiraSC2-FluA/B seqc Influenzavirus B positive	21	0
respiraSC2-FluA/B seqc Influenzavirus B negative	0	41
	Sensitivity (%)	Specificity (%)
	100	100

Detailed information is available at gerbion GmbH & Co.KG.

16.4 Linear Range

The linear range of the respiraSC2-FluA/B seqc real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of in vitro transcripts (SARS-CoV-2 RdRP gene, S gene and E gene, Influenzavirus A M gene and Influenzavirus B NEP gene) and synthetic DNA fragments (human Succinate Dehydrogenase).

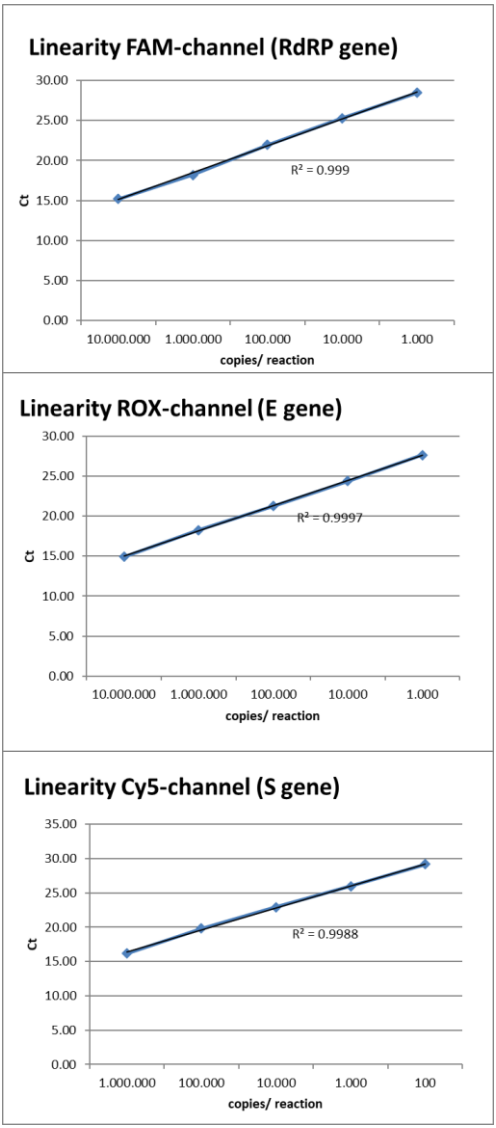

Figure 5: Determination of the linear range of respiraSC2-FluA/B seqc real time RT-PCR Kit for Mix 1.

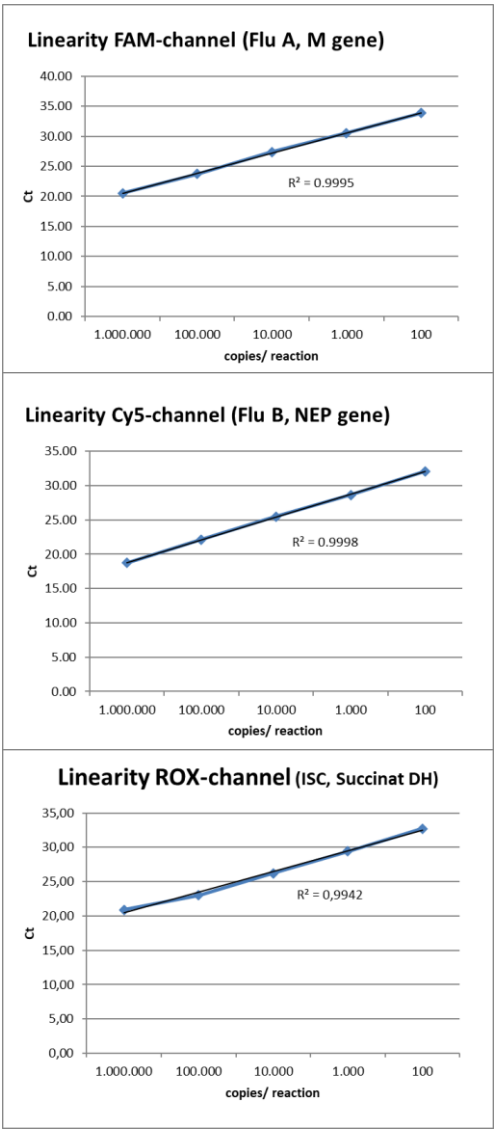

Figure 6: Determination of the linear range of respiraSC2-FluA/B seqc real time RT-PCR Kit for Mix 2.

16.5 Precision

The precision of the respiraSC2-FluA/B seqc 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 RdRP gene (SARS-CoV-2) in vitro transcripts, E gene (SARS-CoV-2) in vitro transcripts, S gene (SARS-CoV-2) in vitro transcripts, M gene (Influenzavirus A) in vitro transcripts, NEP gene (Influenzavirus B) in vitro transcripts, ISC specific DNA and on the threshold cycle of the Control RNA (IPC). The results are shown in Table 11.

Table 11: Precision of the respiraSC2-FluA/B seqc real time RT-PCR Kit.

Reaction Mix 1			
SARS-CoV-2, RdRP gene (FAM)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	100	0.18	0.58
Inter-Assay-Variability	100	0.70	2.25
Inter-Lot-Variability	100	0.17	0.54
SARS-CoV-2, E gene (ROX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	10	0.33	0.98
Inter-Assay-Variability	10	0.90	2.76
Inter-Lot-Variability	10	0.11	0.34
SARS-CoV-2, S gene (Cy5)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	10	0.15	0.47
Inter-Assay-Variability	10	0.21	0.65
Inter-Lot-Variability	10	0.18	0.56
IPC (HEX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	1000	0.78	2.45
Inter-Assay-Variability	1000	1.13	3.57
Inter-Lot-Variability	1000	0.42	1.33
Reaction Mix 2			
Influenzavirus A, M gene (FAM)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	100	0.23	0.69
Inter-Assay-Variability	100	0.20	0.59
Inter-Lot-Variability	100	0.03	0.08

Influenzavirus B, NEP gene (Cy5)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	100	0.17	0.53
Inter-Assay-Variability	100	0.10	0.30
Inter-Lot-Variability	100	0.22	0.70
ISC (ROX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	100	0.15	0.45
Inter-Assay-Variability	100	0.88	2.67
Inter-Lot-Variability	100	0.32	0.99
IPC (HEX)	copies/ reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	1000	0.42	1.29
Inter-Assay-Variability	1000	1.37	4.20
Inter-Lot-Variability	1000	0.19	0.58

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.

17 Abbreviations and Symbols

RNA	Ribonucleic Acid		Content sufficient for <n> tests
RT-PCR	Reverse Transcription Polymerase Chain Reaction		Upper limit of temperature
	Reaction Mix 1		Manufacturer
	Reaction Mix 2		Use by YYYY-MM-DD
	Enzyme		Batch code
	Positive Control 1		Content
	Positive Control 2		Consult instruction for use
	Negative Control		*In vitro* diagnostic medical device
	Control RNA (IPC)		European Conformity
	Catalog number		

18 Literature

[1] www.who.int/health-topics/coronavirus

[2] www.nature.com/articles/s41564-020-0695-z

[3] Corman et al. Detection of 2019 novel coronavirus (2019-nCoV) by real time RT-PCR. Eurosurveillance, Volume 25, Issue 3, 23/Jan/2020.

[4] Sun et al. Prevalent Eurasian avian-like H1N1 swine influenza virus with 2009 pandemic viral genes facilitating human infection. PNAS July 21, 2020 117 (29) 17204-17210

[5] https://www.who.int/publications/i/item/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117

[6] https://www.nhs.uk/conditions/sars/