virellaSARS-CoV-2 seqc

real time RT-PCR Kit 2.0

Validation of this test has not been reviewed by FDA. Review under the Emergency Use Authorization (EUA) program is pending

For the in vitro detection of RNA of novel coronavirus (SARS-CoV-2) extracted from biological specimens.

For use with
Mx3000P/Mx3005P qPCR System (Agilent)

REF	**G01128-96**	**G01128-384**
Σ	96	384
- 18°C		

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IVD

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1 Intended Use

The virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 is an assay for the qualitative detection of RNA from the novel coronavirus (SARS-CoV-2) extracted from nasopharyngeal swabs, oropharyngeal swabs and nasal washes, from individuals who are suspected of COVID-19 by their healthcare provider.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests.

The results are for the identification of SARS-CoV-2 RNA. The viral RNA is usually detectable in stages of an acute infection with the virus. While positive results indicate the presence of SARS-CoV-2 RNA, the clinical correlation with the patient history and other diagnostic information are necessary to determine the infection status. Positive results for SARS-CoV-2 RNA do not exclude co-infections with other bacteria and viruses. Laboratories located in the United States and its territories need to report all positive results to their public health authorities.

Negative results do not completely exclude a SARS-CoV-2 infection and should not be used for treatment decisions. Negative results should be aligned with clinical observations.

The virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 is intended for use by trained clinical laboratory personnel, specifically instructed and trained in the techniques of real time PCR and in vitro diagnostic procedures. *Validation of this test has not been reviewed by FDA. Review under the EUA program is pendin*g.

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.
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 virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 contains specific primers and dual-labeled probes for the amplification of RNA (cDNA) of SARS-CoV-2 (both, RdRP gene and S gene, FAM channel) and the RNA (cDNA) of the Subgenus Sarbecoviruses (SARS-CoV-1 and SARS-CoV-2, E gene, Cy5 channel) extracted from biological specimens. Both, E gene and RdRP gene are target sequences of the viral genome recommended by the WHO. The simultaneous detection of 3 target sequences (RdRP gene, S gene and E gene) increases the diagnostic reliability, even in cases of target sequence mutations.
Furthermore, virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 contains a Control RNA (Internal Process Control, IPC), which is added during RNA extraction and detected in the same reaction by a HEX-labeled 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.
Additionally, virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 contains an Internal System Control (ISC). The ISC consists of primers and probes for the detection of a house keeping gene (Beta-actin, multi species) 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 Beta-actin target sequence is measured in the ROX channel.

4 Package Contents

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

Table 1: Components of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0

Label	Lid Color	Content	
		96	384
Reaction Mix	yellow	1 x 1325 µl	4 x 1325 µl
Enzyme	blue	1 x 19.2 µl	1 x 76.8 µl
Positive Control	red	1 x 150 µl	1 x 150 µl
Negative Control	green	1 x 150 µl	1 x 150 µl
Control RNA	colorless	1 x 480 µl	2 x 960 µl

5 Equipment and Reagents to be Supplied by User

- Real time PCR instrument: Mx3000P/Mx3005P qPCR system (Agilent, Santa Clara, California, USA)
- MxPro qPCR Software v4.01
- Appropriate optical PCR reaction tubes with lid or optical PCR reaction plate with optical foil
- Universal Transport Medium™, UTM® (Copan, Murrieta, California, USA, or similar product according to FDA recommended alternatives in cases of product shortages) for processing of swab specimens
- Nucleic acid extraction instrument: KingFisher™ Flex Purification System (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Appropriate plastic material for the KingFisher™ Flex Purification System
- RNA isolation kit: NukEx Mag RNA/DNA, gerbion Cat. No. G05012
- Proteinase K (gerbion, Cat. No. G07001)
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Powder-free gloves (disposable)

6 Transport, Storage and Stability

The virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 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.
Real time stability experiments on shelf life were performed for the product family of real time RT-Multiplex PCR due the identical chemistry in the critical components (Enzymes, Buffer, Salt Concentration).
Data is available on request at info@gerbion.com.

7 Warnings and Precautions

This assay is for in vitro diagnostic use under the FDA Emergency Use Authorization only.
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 pipette 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 virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 is RNA isolated from nasopharyngeal swabs, oropharyngeal swabs or nasal washes using the NukEx Mag RNA/DNA nucleic acid extraction kit on a KingFisher™ Flex Purification System.

9 Sample Preparation

Follow the instructions for use in the NukEx Mag RNA/DNA manual (gerbion, Cat. No. G05012).
For the extraction of viral RNA using the NukEx Mag RNA/DNA nucleic acid extraction kit with the KingFisher™ Flex Purification System 200 µl swab wash is used.
Prepare a working solution as described in the manual of the extraction kit and in Table 2 for each sample and preload the 96 Deepwell plates of the KingFisher™ Flex Purification System according to Table 3.
The program for the automated extraction is shown in Table 4. The corresponding *.bdz file for the KingFisher™ Flex Purification System is available at gerbion. Please contact our scientists via info@gerbion.com

Table 2: Preparation of the Working solution for the extraction of viral RNA with the KingFisher™ Flex Purification System in combination with NukEx Mag RNA/DNA

Volume needed per sample	Mastermix working solution
500 µl Binding Buffer (P1)	500 µl x (N+1)
4 µl PolyA/Carrier RNA (PA)	4 µl x (N+1)
50 µl Proteinase K [20 mg/mL]	50 µl x (N+1)
5 µl Control RNA (IPC)	5 µl x (N+1)

Table 3: Preparation of the 96 Deepwell plates for the KingFisher™ Flex Purification System in combination with NukEx Mag RNA/DNA

Tip plate	Microtiter DW 96 plate	
Lysis/Binding		
Name	*Well volume [µl]*	*Type*
Magnet Beads	20	Reagent
Working Solution	550	Reagent
Extraction Control	See Instruction Manual of the respective PCR Kit.	Reagent
Sample	200	Sample
Inhibitor Removal Buffer (P2)	Inhibitor Removal	
Name	*Well volume [µl]*	*Type*
Inhibitor Removal	500	Reagent
1st Wash Buffer (P3)	Inhibitor Removal	
Name	*Well volume [µl]*	*Type*
Wash Buffer	450	Reagent
2nd Wash Buffer (P3)	2nd Wash Buffer	
Name	*Well volume [µl]*	*Type*
Wash Buffer	450	Reagent
Elution Buffer (P4)	Elution	
Name	*Well volume [µl]*	*Type*
Elution Buffer	100	Reagent

Table 4: Program for the extraction of viral RNA with the KingFisher™ Flex Purification System in combination with NukEx Mag RNA/DNA

	Tip 1		96 DW tip comb	
		Pick-Up	Tip plate	
		Binding	Lysis	
			Pause	
		Beginning of step	Precollect	No
			Release beads	Yes
		Mixing / heating	Mixing time, speed	00:10:00, Bottom mix
			Heating during mixing	Yes
			Heating temperature [°C]	60
		End of step	Postmix	No
			Collect count	4
			Collect time [s]	3
		Inhibitor Removal Buffer	Inhibitor Removal	
		Beginning of step	Precollect	No
			Release time, speed	00:00:30, Medium
		Mixing / heating	Shake 1 time, speed	00:00:30, Bottom mix
			Shake 2 time, speed	00:00:30, Half mix
			Heating during mixing	No
		End of step	Postmix	No
			Collect count	4
			Collect time [s]	3
		1st Wash Buffer	1st Wash Buffer	
		Beginning of step	Precollect	No
			Release time, speed	00:00:30, Medium
		Mixing / heating	Shake 1 time, speed	00:00:30, Bottom mix
			Shake 2 time, speed	00:00:30, Half mix
			Heating during mixing	No
		End of step	Postmix	No
			Collect count	3
			Collect time [s]	2
		2nd Wash Buffer	2nd Wash Buffer	
		Beginning of step	Precollect	No
			Release time, speed	00:00:30, Medium
		Mixing / heating	Mixing time, speed	00:01:00, Bottom mix
			Heating during mixing	No
		End of step	Postmix	No
			Collect count	3
			Collect time [s]	2
		Bead Drying		
			Dry time	00:05:00
			Tip position	Outside well / tube
		Elution	Elution	
		Beginning of step	Precollect	No
			Release time, speed	00:00:30, Fast
		Mixing / heating	Mixing time, speed	00:10:00, Slow
			Heating temperature [°C]	56
			Preheat	Yes
		End of step	Postmix	No
			Collect count	5
			Collect time [s]	4
		Leave	Tip plate	

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 and is used as extraction/inhibition 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 familiarize 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 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 Control RNA was added during RNA extraction (chapter 10 ‚Control RNA'). Prepare the Master Mix according to Table 5.

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 5:Preparation of the Master Mix (Control RNA was added during RNA extraction)

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 6).
- Close the optical PCR reaction tubes / the optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 6: 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 7.

Table 7: 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	

Table 8: Overview of the instrument settings required for the virellaSARS-CoV-2 seqc real time RT-PCR 2.0.

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes	
Stratagene Mx3000P / Mx3005P	RdRP gene / S gene	FAM	Gain 8	Reference Dye: None
	Control RNA (IPC)	HEX	Gain 1	
	ISC	ROX	Gain 1	
	E gene	Cy5	Gain 4	

12 Data Analysis

Following results can occur:

Signal/Ct Values				**Interpretation**
FAM Channel RdRP gene S gene	**Cy5 Channel** E gene	**ROX Channel** ISC	**HEX Channel** Control RNA (IPC)	
positive	**positive**	positive or negative	positive or negative[1]	**Positive result, the sample contains SARS-CoV-2 RNA.** Report result to healthcare provider and appropriate public health authorities.
positive	negative	positive or negative	positive or negative[1]	**Positive result, the sample contains SARS-CoV-2 RNA[2].** Report result to healthcare provider and appropriate public health authorities.
negative	**positive**	positive or negative	positive or negative[1]	**Positive result, the sample contains SARS-CoV-2 RNA or SARS-CoV-1 -RNA[2,3].** Report result to healthcare provider and appropriate public health authorities.
negative	negative	positive	≤ 34	**Negative result, the sample does not contain detectable amounts of SARS-CoV-2 RNA or SARS-CoV-1 RNA[3].** Report result to healthcare provider and appropriate public health authorities.
negative	negative	negative	≤ 34	**No diagnostic statement can be made.** Amount or quality of sample material not sufficient. A new sample needs to be collected and tested.
negative	negative	positive	negative or > 34	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.[4]
negative	negative	negative	negative or > 34	**Caution!** The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction. Amount or quality of sample material not sufficient.[5]

1) The IPC detection can be reduced or fail due to a high SARS-CoV-2 RNA load in the sample.

2) 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]).

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

4) Repeat the PCR with a 1:3 dilution of the RNA Eluate.

5) Repeat the PCR with a 1:3 dilution of the RNA Eluate or collect and test a new sample.

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

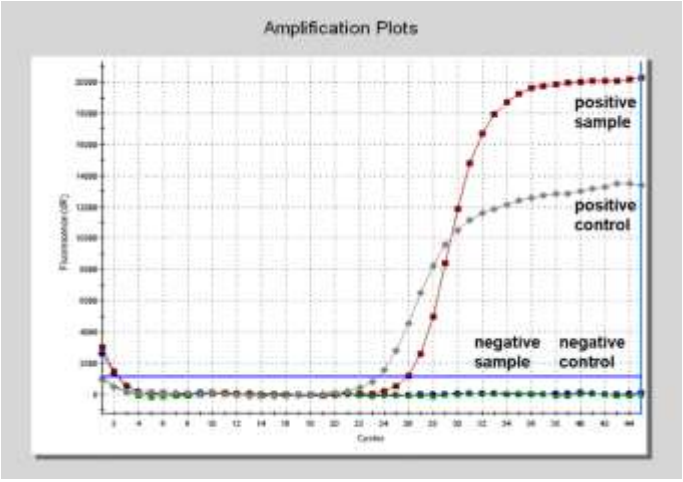

Figure 1: The positive sample shows pathogen specific amplification in the FAM channel (positive sample and Positive Control), whereas no fluorescence signal is detected in the negative sample or the Negative Control.

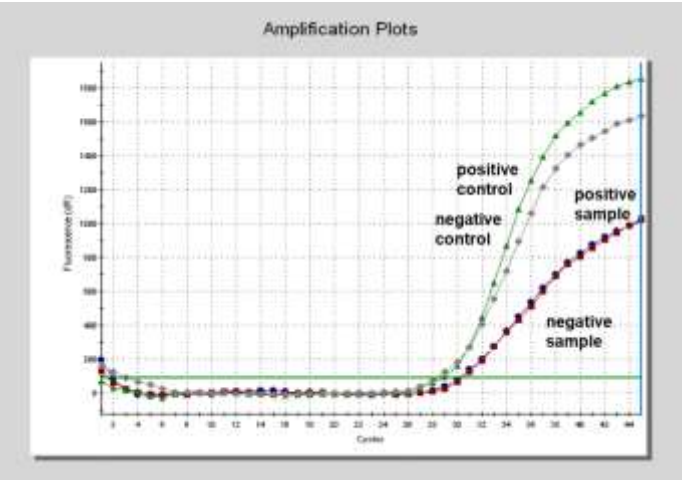

Figure 2: The positive sample, the Positive Control, the Negative Control as well as the negative sample show a signal in the Control RNA specific HEX channel (IPC). The amplification signal of the Control RNA in the negative sample shows that the missing signals in the pathogen specific channels FAM and Cy5 are not due to RT-PCR inhibition or failure of RNA isolation, but that the sample is a true negative sample.

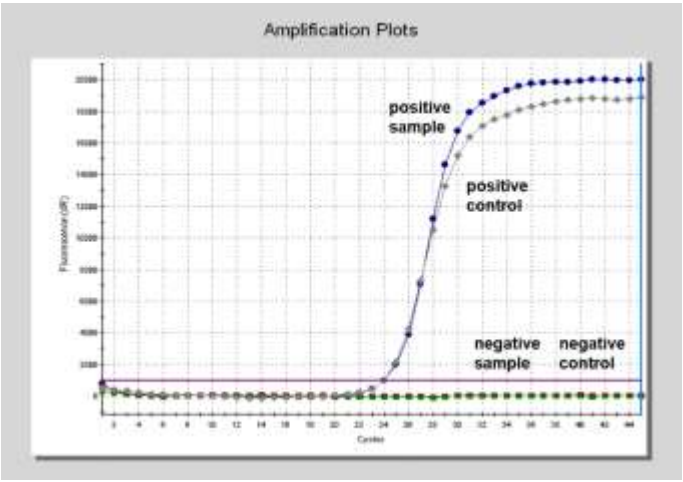

Figure 3: The positive sample shows pathogen specific amplification in the Cy5 channel (positive sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control.

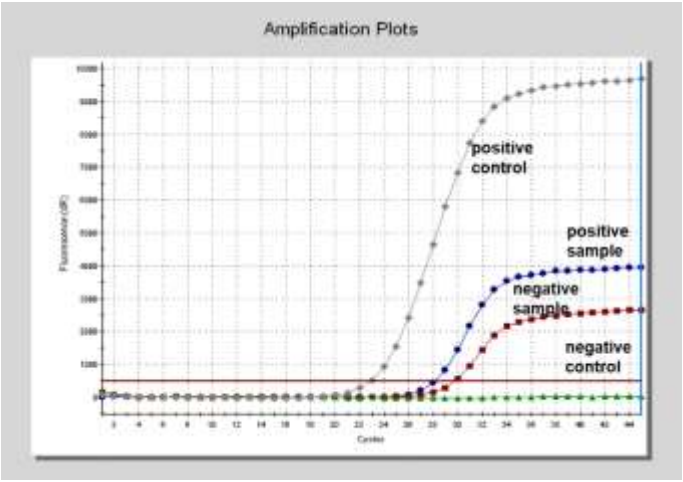

Figure 4: Signals of the amplification of the ISC in the ROX channel. The Figure shows the C_T values of eluates from respiratory swabs after nucleic acid extraction using NukEx Mag RNA/DNA nucleic acid extraction kit.

13 Assay Validation

To increase process safety IPC is included in the Negative Control and Positive Control.

Negative Control

The Negative Control must show no C_T in the FAM and Cy5 channel. The HEX channel (IPC) in the Negative Control must show a C_T of below 34. Due to the high sensitivity of the virellaSARS-CoV-2 real time RT-PCR Kit 2.0, a weak positive result in the ROX channel (ISC) caused by slight contaminations with human DNA during RT-PCR set up cannot completely be ruled out. This does not affect the validity of the respective run (see also Internal Controls).

Positive Control

All the Positive Controls must show a positive (i.e. exponential) amplification curve in the different channels FAM, Cy5, ROX and HEX. The Positive Controls must fall below a C_T of 30 except for the HEX channel, which must show a C_T of below 34. The Positive Control includes in vitro transcripts and synthetic DNA of approximately 10^4 copies per reaction for RdRP gene, E gene and ISC.

Internal Controls

The following values for the amplification of the Internal Controls are valid using the gerbion nucleic acid extraction kit NukEx Mag RNA/DNA on a KingFisher™ Flex Purification System (Thermo Fisher Scientific). 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 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 (ISC) and the HEX channel (IPC) 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 cutoffs. 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 and Betacoronavirus 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 virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 need to be interpreted in consideration of all clinical and laboratory findings.
- Laboratories should include a statement that the test has been validated but FDA's independent review of this validation is in test reports reports to healthcare providers.

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 Cy5 channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the SARS-CoV-2 specific amplification, the Cy5 channel for analysis of the Sarbecovirus specific amplification, the HEX channel for the amplification of the Control RNA and the ROX channel for the amplification of the ISC.
Incorrect preparation of the Master Mix	Make sure, 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" on page 9.
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 Cy5 channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 7).

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 Cy5 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 work space 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 (>35), the contamination is negligible. If the FAM and Cy5 channel are negative in the Negative Control, the PCR is still valid for the detection of SARS-CoV-2.

16 Kit Performance

16.1 Analytical Sensitivity

16.1.1 Estimation of the Limit of Detection (LoD)

Serial dilutions of heat inactivated SARS-CoV-2 cell culture supernatant (1.7E+07 copies/mL; Strain BetaCoV/Munich/ChVir984/2020 INSTAND e.V. Duesseldorf, Germany) were used.

For extraction, 900 µL of pooled negative confirmed pharyngeal swabs in UTM® were spiked with 100 µL SARS-CoV-2 cell culture supernatant. The extraction was performed on a KingFisher™ Flex Purification System (Thermo Fisher Scientific) using the NukEx Mag RNA/DNA kit (gerbion, Cat. No. G05012). The volume used by the instrument for the nucleic acid extraction was 200 µl. Each dilution was extracted in triplets and tested with the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 on the Mx3005P qPCR system. The lowest concentration at which all replicates were tested positive was treated as the estimated LoD. The results are shown in Table 9, Table 10 and Table 11.

Table 9: Determination of the suspected LoD using the KingFisher™ Flex Purification System in combination with the NukEx Mag RNA/DNA nucleic acid extraction kit.

Target	Concentration [copies/mL]	Call rate	Replicate 1 C_T (FAM)	Replicate 2 C_T (FAM)	Replicate 3 C_T (FAM)
RdRP / S gene	1.70E+05	3/3	30.03	30.32	29.71
	1.70E+04	3/3	32.47	32.59	32.31
	1.70E+03	3/3	36.04	35.51	37.17
	1.70E+02	0/3	-	-	-
	1.70E+01	0/3	-	-	-

Table 10: Determination of the suspected LoD using the KingFisher™ Flex Purification System in combination with the NukEx Mag RNA/DNA nucleic acid extraction kit.

Target	Concentration [copies/mL]	Call rate	Replicate 1 C_T (Cy5)	Replicate 2 C_T (Cy5)	Replicate 3 C_T (Cy5)
E gene	1.70E+05	3/3	26.62	26.59	26.41
	1.70E+04	3/3	29.28	29.53	29.38
	1.70E+03	3/3	32.18	31.53	32.25
	1.70E+02	2/3	-	35.26	32.25
	1.70E+01	0/3	-	-	-

Table 11: Determination of the suspected LoD using the KingFisher™ Flex Purification System in combination with the NukEx Mag RNA/DNA nucleic acid extraction kit.

Target	Concentration [copies/mL]	Call rate	Replicate 1 C_T (ROX)	Replicate 2 C_T (ROX)	Replicate 3 C_T (ROX)
beta actin	1.70E+05	3/3	26.27	26.42	26.15
	1.70E+04	3/3	25.92	26.01	26.23
	1.70E+03	3/3	25.15	25.06	25.26
	1.70E+02	3/3	25.76	25.75	25.76
	1.70E+01	3/3	25.99	25.92	25.91

The virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 in combination with the KingFisher™ Flex Purification System and NukEx Mag RNA/DNA detected 3/3 replicates with a concentration of 1.7E+03 copies/mL for all targets (RdRP / S gene and E gene) on a Mx3005P qPCR system.
As a result, this concentration was considered the suspected LoD.
The low variation of C_T values for beta actin confirmed the homogeneous pool of negative pharyngeal swab samples.

16.1.2 Confirmation of the Limit of Detection

Based on the suspected LoD, heat inactivated SARS-CoV-2 cell culture supernatant was spiked in 20 samples of pooled negative confirmed pharyngeal swab samples in UTM® with a final concentration of 1.7E+03 copies/mL. Nucleic acids were extracted with the NukEx Mag RNA/DNA on the KingFisher™ Flex Purification System (Thermo Fisher Scientific) as described above. The results of the experiment on the Mx3005P qPCR System (Agilent) can be found in Table 12.

Table 12: Confirmation of the LoD on the Mx3005P qPCR system

SARS-CoV-2 concentration 1.7E+03 copies/mL					
#	interpretation	C_T Cy5	C_T FAM	C_T ROX	C_T HEX
		E gene	RdRP / S gene	beta actin	IPC
1	positive	31.38	34.62	25.23	29.14
2	positive	30.68	34.29	25.21	28.87
3	positive	30.63	35.83	24.96	28.92
4	positive	30.46	35.36	24.98	28.56
5	positive	30.76	35.85	24.99	28.95
6	positive	30.93	35.96	25.07	29.82
7	positive	30.86	34.56	24.99	29.33
8	positive	30.77	34.39	24.90	28.69
9	positive	31.28	34.55	25.60	29.52
10	positive	31.37	34.31	25.64	29.37
11	positive	30.80	37.12	25.08	29.39
12	positive	30.63	34.58	24.92	28.90
13	positive	30.97	35.21	25.01	29.40
14	positive	31.22	34.84	25.06	29.42
15	positive	30.83	35.00	24.93	28.57
16	positive	31.18	36.24	25.00	28.89
17	positive	30.95	35.78	25.09	29.46
18	positive	30.91	35.38	25.06	29.35
19	positive	31.32	35.11	25.38	29.53
20	positive	30.94	35.67	25.00	30.57
	Mean C_T	30.94	35.23	25.11	29.23
	SD	0.27	0.75	0.21	0.47
	Coefficient of variation	0.87	2.14	0.84	1.61
	Result	20/20			

The virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 in combination with the NukEx Mag RNA/DNA nucleic acid extraction kit on a KingFisher™ Flex Purification System and the Mx3005P qPCR system detected 20/20 replicates at a concentration of 1.7E+03 copies/mL.
Consequently, the confirmed LoD is 1.7E+03 copies/mL.

16.2 Analytical Specificity

16.2.1 Inclusivity

Inclusivity of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 was evaluated for different isolates of SARS-CoV-2 by wet testing (Table 13) or *in silico* analyses (Table 14).

Table 13: Inclusivity of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 (wet testing)

SARS-CoV-2 Strain/Isolate	Source/Sample Type	Concentration
BetaCoV/Munich/ ChVir984/2020*	INSTAND e.V.; Duesseldorf; Germany/ Heat inactivated cell culture supernatant	1.7E+07 copies/mL

* The strain was part of the round robin test in Germany (April 2020) and used for the evaluation of the performance of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0

Table 14: Inclusivity of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 (in silico analysis): 2320 whole genome sequences of which were 2213 published via GISAID e.V and 107 published via the National Center for Biotechnology Information (NCBI).

2320 whole genome sequences		**Homology**	**Comment**
RdRP	Forward Primer	2313 sequences: 100%	7 sequences: 95% (1 mismatch)
	Reverse Primer	2320 sequences: 100%	-
	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)

None of the analyzed sequences showed mismatches in more than one oligonucleotide and none showed mismatches in more than one specific system (RdRP, S gene, E gene). Therefore, the binding of the specific oligonucleotides included in the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 is not expected to be affected.

16.2.2 Exclusivity

The specificity of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 was evaluated with different other relevant viruses and bacteria found in clinical samples and basing on in silico analyses.
For wet testing, purchased (EVA: European Virus Archive, ATCC) or extracted (INSTAND e.V., QCMD) RNA/DNA of the viruses or organisms was used. Most of the species were diluted to concentrations between 1E+04 and 9E+04 copies/mL for comparable results (Table 15).

Table 15: Eluted DNA and RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0.

Eluates with known status	**Source**	**concentration**	**virellaSARS-CoV-2 seqc** E gene **Cy5 channel**	**virellaSARS-CoV-2 seqc** RdRP gene / S gene **FAM channel**
SARS-CoV-1	EVA	ca. 50.000 copies/mL	***positive***	negative
MERS-CoV	EVA	6.13 log10 TCID50/mL	negative	negative
HCoV-229E	EVA	ca. 50.000 copies/mL	negative	negative
HCoV-OC43	EVA	no data available	negative	negative
Influenza A H3N2	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Influenza A H5N1	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Influenzavirus B	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Respiratory Syncytial Virus A	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Respiratory Syncytial Virus B	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Parainfluenzavirus 1	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Parainfluenzavirus 2	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Parainfluenzavirus 3	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Parainfluenzavirus 4	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative

Metapneumovirus	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Adenovirus	QCMD	ca. 50.000 copies/mL	negative	negative
Rhinoviruses	EVA	no data available	negative	negative
Enteroviruses B6	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Human Bocavirus	EVA	TCID50: 6.81E+03	negative	negative
Legionella pneumoniae	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Mycoplasma pneumoniae	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Mycobacterium tuberculosis complex	ATCC-25177	ca. 50.000 copies/mL	negative	negative
Bordetella pertussis	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Bordetella parapertussis	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
MRSA	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
MSSA	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative
Streptococcus agalactiae.	INSTAND e.V.	ca. 50.000 copies/mL	negative	negative

The virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 showed a positive result for the sample containing SARS-CoV-1 in the E gene. This is expectable, since the E gene detects for Sarbecoviruses in general, whereas samples containing other pathogens were reliably tested negative for all three targets. The results are shown in table 7.

The sequences of all species from the wet testing were used in *in silico* analysis, showing that a positive result due to cross-reactivity is unlikely to occur. The results are shown in Table 16.

Table 16: Exclusivity (*in silico* analysis) for the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0

Species tested for *in silico* exclusivity			
	RdRP	**S gene**	**E gene**
SARS-CoV-1	neg.	neg.	**pos.**
MERS-CoV	neg.	neg.	neg.
HCoV-229E	neg.	neg.	neg.
HCoV-OC43	neg.	neg.	neg.
Influenza A H3N2	neg.	neg.	neg.
Influenza A H5N1	neg.	neg.	neg.
Influenzavirus B	neg.	neg.	neg.
Respiratory Syncytial Virus A	neg.	neg.	neg.
Respiratory Syncytial Virus B	neg.	neg.	neg.
Parainfluenzavirus 1	neg.	neg.	neg.
Parainfluenzavirus 2	neg.	neg.	neg.
Parainfluenzavirus 3	neg.	neg.	neg.
Parainfluenzavirus 4	neg.	neg.	neg.
Metapneumovirus	neg.	neg.	neg.
Adenovirus	neg.	neg.	neg.
Rhinoviruses	neg.	neg.	neg.
Enteroviruses	neg.	neg.	neg.
Human Bocavirus	neg.	neg.	neg.
Legionella pneumoniae	neg.	neg.	neg.
Mycoplasma pneumoniae	neg.	neg.	neg.
Mycobacterium tuberculosis complex	neg.	neg.	neg.
Bordetella pertussis	neg.	neg.	neg.
Bordetella parapertussis	neg.	neg.	neg.
MRSA	neg.	neg.	neg.
MSSA	neg.	neg.	neg.
Streptococcus agalactiae.	neg.	neg.	neg.

16.3 Clinical Samples

Positive (30) and negative (32) from oral swabs in UTM® and from nasopharyngeal swabs (positive 12, negative 17) in UTM® confirmed samples from the pandemic outbreak 2020 in Europe were tested. The samples were extracted with the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and the PCR performed using the Allplex™ 2019-nCoV Assay (Seegene, Seoul, South Korea) on a Rotorgene Instrument.
At gerbion, the RNA was extracted by using the NukEx Mag RNA/DNA (gerbion Cat. No. G05012) extraction kit on a KingFisher™ Flex Purification System. The PCR experiments were performed on a Mx3005P Stratagene Cycler. The testing of the confirmed samples with virellaSARS-CoV-2 seqc 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 virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 the eluates of all samples were retested and showed the same results.

Table 17: Results from testing clinical samples (oral swabs in UTM®)

	positive samples	negative samples
virellaSARS-CoV-2 seqc positive	30	0
virellaSARS-CoV-2 seqc negative	0	32
	Sensitivity (%)	Specificity (%)
	100	100

Table 18: Results from testing clinical samples (nasopharyngeal swabs in UTM®)

	positive samples	negative samples
virellaSARS-CoV-2 seqc positive	12	0
virellaSARS-CoV-2 seqc negative	0	17
	Sensitivity (%)	Specificity (%)
	100	100

16.4 Precision

The precision of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0 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 in vitro transcripts and E gene in vitro transcripts, ISC specific DNA and on the threshold cycle of the Control RNA (IPC).

Table 19: Precision of the virellaSARS-CoV-2 seqc real time RT-PCR Kit 2.0.

RdRP gene and S gene (FAM)	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.23	0.77
Inter-Assay-Variability	25	0.51	1.71
Inter-Lot-Variability	25	0.76	2.56
E gene (Cy5)	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.27	0.84
Inter-Assay-Variability	25	0.51	1.50
Inter-Lot-Variability	25	0.52	1.61
ISC (ROX)	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.28	0.90
Inter-Assay-Variability	25	0.40	1.27
Inter-Lot-Variability	25	0.25	0.77
IPC (HEX)	copies/µl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.69	2.31
Inter-Assay-Variability	25	0.58	1.91
Inter-Lot-Variability	25	037	1.22

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
	Contains 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.who.int/publications/i/item/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117

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