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# Instruction for Use

# respiraScreen 1
## real time RT-PCR Kit

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

| REF | G01129-96 | G01129-384 |
|---|---|---|
| Σ (triangle) | 96 | 384 |

- 18°C

gerbion GmbH & Co. KG
Remsstr. 1
70806 Kornwestheim
Germany
phone: +49 7154 806 20 0
fax: +49 7154 806 20 29
e-mail: info@gerbion.com
www.gerbion.com

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# Index

# 1    Intended Use

The respiraScreen 1 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 (Flu A, Flu B) and Respiratory Syncytial Virus (RSV A, RSV B) from the pandemic coronavirus SARS-CoV-2 (E gene and RdRP gene), extracted from biological specimens.

# 2    Pathogen Information

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. Influenza B viruses show a 2 – 3 times slower mutation rate then type A.

Respiratory Syncytial Viruses are enveloped negative-sense, single stranded RNA Viruses of the Pneumoviridae family, genus *Orthopneumovirus.* RSV are divided into subgroups A and B. RSV is a virus that causes infections of the lungs and respiratory tract. It is so common that most children have been infected with the virus at the age of 2. RSV can also infect adults.
In adults and older, healthy children, the symptoms of RSV infections are mild and typically mimic the common cold. Self-care measures are usually all that is needed to relieve any discomfort. Infection with RSV can be severe in some cases, especially in premature babies and infants with underlying health conditions. RSV can also become serious in older adults, adults with heart and lung diseases, or anyone with a very weak immune system (immunocompromised).

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 1 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), RSV A (G gene), RSV B (G gene) and SARS-CoV-2 (E gene and RdRP gene) extracted from biological specimen.

Furthermore, respiraScreen 1 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 or 384 reactions, respectively.

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

| Label | Lid Colour | 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 | colourless | 1 x 480 µl | 2 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 1 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 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 respiraScreen 1 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 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

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* | | | |
| Denaturation | 10 sec | 95°C | 45 |
| Annealing and Extension | 40 sec | 60°C Acquisition at the end of this step | |

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 respiraScreen 1 real time RT-PCR.

| Real time PCR Instrument | Parameter Reaction Mix | Detection Channel | Notes | | |
|---|---|---|---|---|---|
| | | | Colour Compensation Kit (G070MP1-CC) required | | |
| | | | Melt Factor | Quant Factor | Max Integration Time (sec) |
| LightCycler 480II | Flu A / Flu B | 465-510 | 1 | 10 | 1 |
| | Control RNA (IPC) | 533-580 | 1 | 10 | 2 |
| | SARS-CoV-2 | 533-610 | 1 | 10 | 2 |
| | RSV A / RSV B | 618-660 | 1 | 10 | 3 |
| Stratagene Mx3000P / Mx3005P | Flu A / Flu B | FAM | Gain 8 | | |
| | Control RNA (IPC) | HEX | Gain 1 | Reference Dye: None | |
| | SARS-CoV-2 | ROX | Gain 1 | | |
| | RSV A / RSV B | Cy5 | Gain 4 | | |
| AriaMx Bio-Rad CFX96 | Flu A / Flu B | FAM | | | |
| | Control RNA (IPC) | HEX | Reference Dye: None | | |
| | SARS-CoV-2 | ROX | | | |
| | RSV A / RSV B | Cy5 | | | |
| ABI 7500 Quant Studio 5 | Flu A / Flu B | FAM | | | |
| | Control RNA (IPC) | JOE | Reference Dye: None | | |
| | SARS-CoV-2 | ROX | | | |
| | RSV A / RSV B | Cy5 | | | |

| Real time PCR Instrument | Parameter Reaction Mix | Detection Channel | Notes |
|---|---|---|---|
| Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000 | Flu A / Flu B | Green | Gain 5 |
| | Control RNA (IPC) | Yellow | Gain 5 |
| | SARS-CoV-2 | Orange | Gain 5 |
| | RSV A / RSV B | Red | Gain 5 |
| NEOS qPCR 48 | Flu A / Flu B | FAM | |
| | Control RNA (IPC) | HEX | |
| | SARS-CoV-2 | ROX | |
| | RSV A / RSV B | Cy5 | |
| NEOS qPCR 96 | Flu A / Flu B | FAM | |
| | Control RNA (IPC) | HEX | |
| | SARS-CoV-2 | ROX | |
| | RSV A / RSV B | Cy5 | |
| Mic qPCR Cycler | Flu A / Flu B | Green | Gain 8 |
| | Control RNA (IPC) | Yellow | Gain 10 |
| | SARS-CoV-2 | Orange | Gain 10 |
| | RSV A / RSV B | Red | Gain 10 |

# 12 Data Analysis

**Following results can occur:**

| Signal/$C_T$ Values | | | | |
|---|---|---|---|---|
| **FAM Channel** Flu A / Flu B | **ROX Channel** SARS-CoV-2 | **Cy5 Channel** RSV A / RSV B | **HEX Channel** Control RNA (IPC) | **Interpretation** |
| **positive** [3] | **positive** | **positive** | positive or negative [1] | **Positive result. The sample contains RNA of Flu A and/or Flu B, RSV A and/or RSV B and SARS-CoV-2.** |
| **positive** [3] | **positive** | negative | positive or negative [1] | **Positive result. The sample contains RNA of Flu A and/or Flu B and SARS-CoV-2.** |
| **positive** [3] | negative | negative | positive or negative [1] | **Positive result. The sample contains RNA of Flu A and/or Flu B.** |
| negative | **positive** | **positive** | positive or negative [1] | **Positive result. The sample contains RNA of RSV A and/or RSV B and SARS-CoV-2.** |
| negative | **positive** | negative | positive or negative [1] | **Positive result. The sample contains RNA of SARS-CoV-2.** |
| negative | negative | **positive** | positive or negative [1] | **Positive result. The sample contains RNA of RSV A and/or RSV B.** |
| negative | negative | negative | ≤ 34 | **Negative result. The sample contains no RNA of Flu A and/or Flu B, RSV A and/or RSV B or SARS-CoV-2.** |
| 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** A differentiation between Flu A and Flu B is possible with a second PCR (e.g. respiraRNA 2.0, gerbion Cat. No. G01084).

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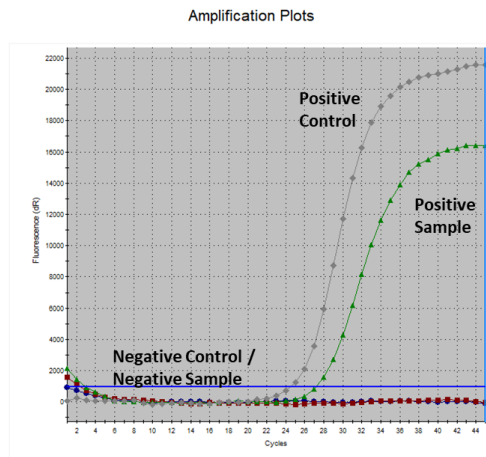


Figure 1: The positive sample shows pathogen specific amplification in the FAM channel (positive Flu sample and Positive Control), whereas no fluorescence signal is detected in the negative sample or the Negative Control (Mx3005P qPCR System).
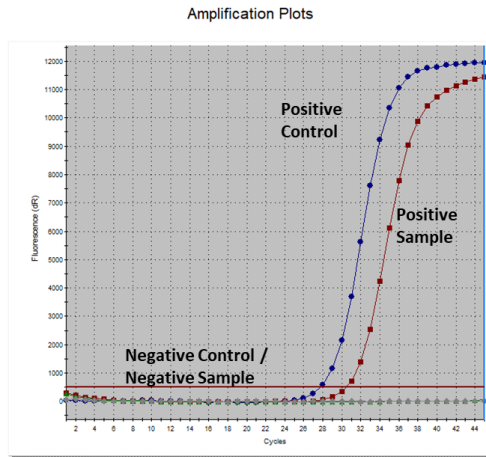
Figure 2: The positive sample shows pathogen specific amplification in the ROX channel (positive SARS-CoV-2 sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (Mx3005P qPCR System).
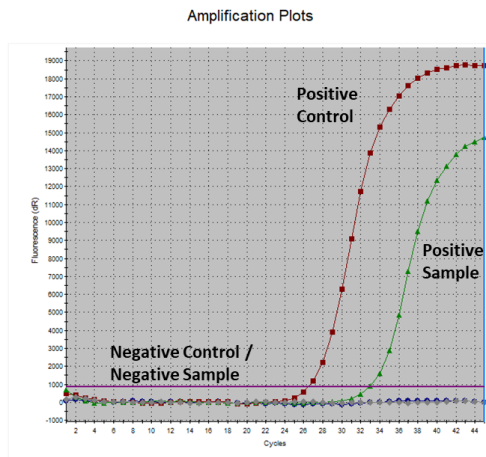


Figure 3: The positive sample shows pathogen specific amplification in the Cy5 channel (positive RSV sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (Mx3005P qPCR System).
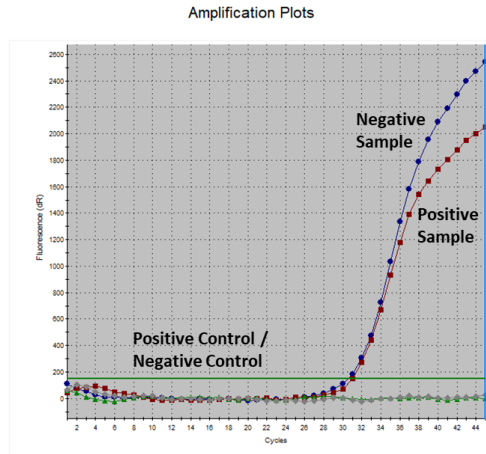
Figure 4: The positive sample and the negative sample show an amplification curve, whereas the Positive Control and the Negative Control don't show a signal in the Control RNA specific HEX channel (Mx3005P qPCR System).

## 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.

**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, RSV A, RSV 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 1 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 | Select the FAM channel for analysis of the Flu specific amplification, the ROX channel for analysis of the SARS-CoV-2 specific amplification, the HEX channel for the amplification of the Control RNA and the Cy5 channel for the amplification of the RSV. |
| 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 tables 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 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 tables 4 and 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 HEX 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 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. |

## 16   Kit Performance

### 16.1   Analytical Sensitivity

The limit of detection (LoD) of respiraScreen 1 real time RT-PCR Kit was determined testing serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on a Stratagene Mx3005P real time PCR instrument. The estimated LoD of respiraScreen 1 real time RT-PCR Kit is ≤ 10 genome copies per reaction for each parameter (Flu A, Flu B, SARS-CoV-2, RSV A, RSV B).

### 16.2   Analytical Specificity

The specificity of the respiraScreen 1 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 6, the results for the insilico analysis are shown in Table 7.

The Primers and Probes for SARS-CoV-2 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 [5].

Table 6: Eluted RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of respiraScreen 1 real time RT-PCR Kit.

| Eluates with known status | Result Flu A/ Flu B FAM channel | Result SARS-CoV-2 ROX channel | Result RSV A/ RSV B Cy5 channel | Result IPC HEX channel |
|---|---|---|---|---|
| Parainfluenzavirus 1 | negative | negative | negative | *positive* |
| Parainfluenzavirus 2 | negative | negative | negative | *positive* |
| Parainfluenzavirus 3 | negative | negative | negative | *positive* |
| Parainfluenzavirus 4 | negative | negative | negative | *positive* |
| Metapneumovirus | negative | negative | negative | *positive* |
| Adenovirus | negative | negative | negative | *positive* |
| Rhinovirus | negative | negative | negative | *positive* |
| Enterovirus | negative | negative | negative | *positive* |
| Human Bocavirus | negative | negative | negative | *positive* |
| Legionella pneumophila | negative | negative | negative | *positive* |
| Mycoplasma pneumophila | negative | negative | negative | *positive* |
| Mycobacterium tuberculosis complex | negative | negative | negative | *positive* |
| Bordetella pertussis | negative | negative | negative | *positive* |
| Bordetella parapertussis | negative | negative | negative | *positive* |
| Staphylococcus aureus | negative | negative | negative | *positive* |
| MRSA | negative | negative | negative | *positive* |
| MSSA | negative | negative | negative | *positive* |
| Streptococcus spp. | negative | negative | negative | *positive* |
| SARS-CoV-1 | negative | *positive* | negative | *positive* |
| MERS-CoV | negative | negative | negative | *positive* |
| SARS-CoV-2 | negative | *positive* | negative | *positive* |
| Influenzavirus A H1N1 | *positive* | negative | negative | *positive* |
| Influenzavirus A H3N3 | *positive* | negative | negative | *positive* |
| Influenzavirus A H5N1 | *positive* | negative | negative | *positive* |
| Influenzavirus B | *positive* | negative | negative | *positive* |
| Respiratory Syncytial Virus A | negative | negative | *positive* | *positive* |
| Respiratory Syncytial Virus B | negative | negative | *positive* | *positive* |

Table 7: Inclusivity of the respiraScreen 1 real time RT-PCR Kit Primers and Probes (in silico analysis).

| 12 - 5000 whole genome sequences | | | Homology | Comment |
|---|---|---|---|---|
| NCBI | 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) |
| NCBI | Flu A | Forward Primer | 5000 sequences: 100% | no mismatch |
| | | Reverse Primer | 5000 sequences: 100% | no mismatch |
| | | Probe | 5000 sequences: 100% | no mismatch |
| NCBI / GISAID | SARS-CoV-2 (RdRP) | 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) |
| NCBI / GISAID | 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) |
| NCBI | RSV A / RSV B | Forward Primer | 12 sequences: 100% | no mismatch |
| | | Reverse Primer | 2 sequences: 100% | 10 sequences: 96% (1 mismatch) |
| | | Probe | 10 sequences: 100% | 2 sequences: 96% (1 mismatch) |

### 16.3    Clinical Samples

Positive (35) and negative (172) 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 Mx3005P Stratagene Cycler. The testing of the confirmed samples with respiraScreen 1 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 respiraScreen 1 real time RT-PCR Kit the eluates of all samples were retested and showed the same results.

| | SARS-CoV-2 positive samples | SARS-CoV-2 negative samples |
|---|---|---|
| respiraScreen 1 positive SARS-CoV-2 | 35 | 0 |
| respiraScreen 1 negative SARS-CoV-2 | 0 | 172 |
| | Sensitivity (%) | Specificity (%) |
| | 100 | 100 |

### 16.4    Linear Range

The linear range of the respiraScreen 1 real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of in vitro transcripts and synthetic DNA fragments.
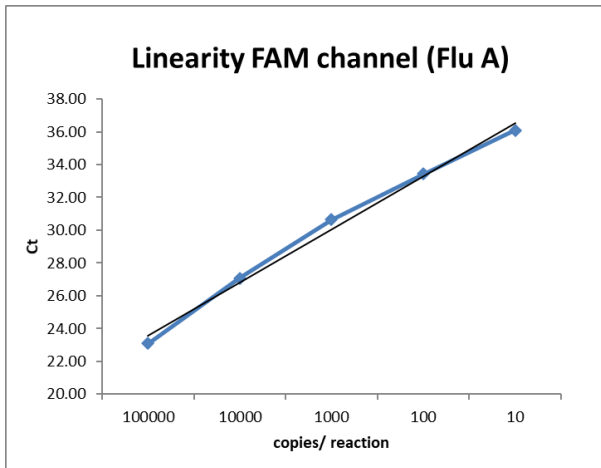
Figure 5: Determination of the linear range of respiraScreen 1 real time RT-PCR Kit for Flu A in the FAM channel.
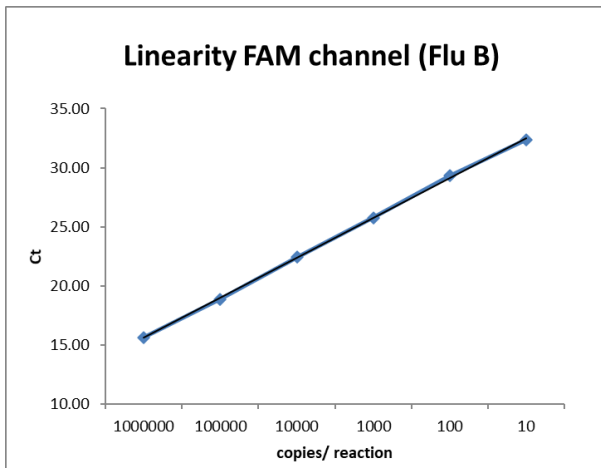


Figure 6: Determination of the linear range of respiraScreen 1 real time RT-PCR Kit for Flu B in the FAM channel.
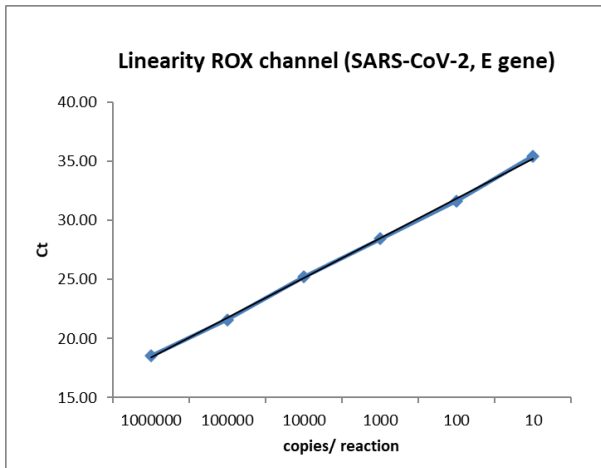
Figure 7: Determination of the linear range of respiraScreen 1 real time RT-PCR Kit for SARS-CoV-2 (E gene) in the ROX channel.
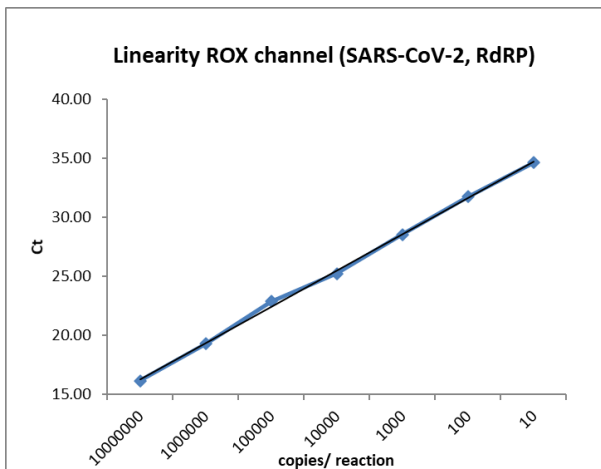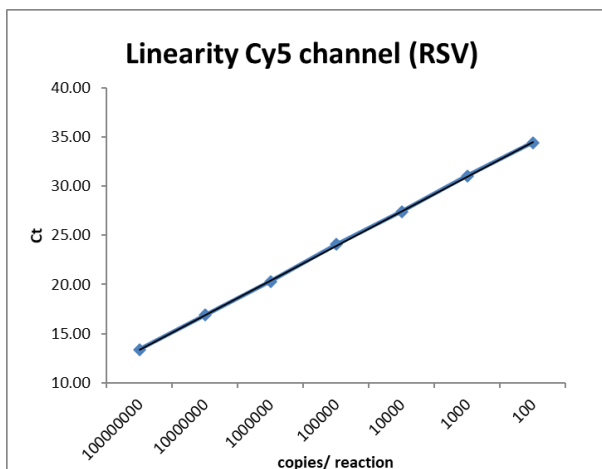


Figure 8: Determination of the linear range of respiraScreen 1 real time RT-PCR Kit for SARS-CoV-2 (RdRP gene) in the ROX channel.

Figure 9: Determination of the linear range of respiraScreen 1 real time RT-PCR Kit for RSV A and RSV B in the Cy5 channel.

## 16.5 Precision

The precision of the respiraScreen 1 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 M gene (Flu A) in vitro transcripts, NEP gene (Flu B) in vitro transcripts, SARS-CoV-2 (E gene and RdRP gene) in vitro transcripts, G gene in vitro transcripts (RSV) and on the threshold cycle of the Control RNA (IPC). The results are shown in Table 8.

Table 8: Precision of the respiraScreen 1 real time RT-PCR Kit.

| Flu A (FAM) | copies/ reaction | Standard Deviation | Coefficient of Variation [%] |
|---|---|---|---|
| Intra-Assay Variability | 50 | 0.30 | 0.88 |
| Inter-Assay-Variability | 50 | 0.22 | 0.65 |
| Inter-Lot-Variability | 50 | 0.22 | 0.65 |
| Flu B (FAM) | copies/ reaction | Standard Deviation | Coefficient of Variation [%] |
| Intra-Assay Variability | 10 | 0.28 | 0.89 |
| Inter-Assay-Variability | 10 | 0.26 | 0.80 |
| Inter-Lot-Variability | 10 | 0.25 | 0.79 |

| SASR-CoV-2 (ROX) | copies/ reaction | Standard Deviation | Coefficient of Variation [%] |
|---|---|---|---|
| Intra-Assay Variability | 10 | 0.33 | 0.98 |
| Inter-Assay-Variability | 10 | 0.11 | 0.34 |
| Inter-Lot-Variability | 10 | 0.04 | 0.11 |
| RSV A / RSV B (Cy5) | copies/ reaction | Standard Deviation | Coefficient of Variation [%] |
| Intra-Assay Variability | 50 | 0.31 | 0.91 |
| Inter-Assay-Variability | 50 | 0.39 | 1.15 |
| Inter-Lot-Variability | 50 | 0.24 | 0.71 |
| IPC (HEX) | copies/ reaction | Standard Deviation | Coefficient of Variation [%] |
| Intra-Assay Variability | 1000 | 0.47 | 1.60 |
| Inter-Assay-Variability | 1000 | 0.48 | 1.61 |
| Inter-Lot-Variability | 1000 | 0.25 | 0.86 |

## 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 | | 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
[2] Corman et al. Detection of 2019 novel coronavirus (2019-nCoV) by real time RT-PCR. Eurosurveillance, Volume 25, Issue 3, 23/Jan/2020.
[3] www.nature.com/articles/s41564-020-0695-z, 02/March/2020
[4] https://www.who.int/publications/i/item/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117
[5] https://www.nhs.uk/conditions/sars/