# ViroReal® Kit SARS-CoV-2 Multiplex

## Kit Version 2.1

# Instructions for Use

CE

IVD **For *in vitro* diagnostic use**

REF **DHUV02413** Σ **100**

REF **DHUV02413x5** Σ **500**

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# Explanation of symbols

| | | | |
|---|---|---|---|
| LOT | Batch code | | Use by date |
| REF | Catalogue number | | Manufacturer |
| Σ | Contains sufficient for <n> tests | | Temperature limit (Store at) |
| CE | This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices | IVD | In vitro diagnostic medical device |
| | Consult instructions for use | UDI | Unique device identifier |
| | Keep away from sunlight | QTY | Contents |

# 1 Intended purpose

ViroReal® Kit SARS-CoV-2 Multiplex is a non-automated IVD test, based on one-step reverse transcription real-time PCR (RT-PCR), for the qualitative detection of RNA of the N gene of SARS-CoV-2, SARS-CoV and SARS-related coronavirus (universal detection of Sarbecovirus) and of the RdRp gene of SARS-CoV-2 (specific detection of SARS-CoV-2).

Proper specimens are RNA extracts isolated from samples of the human respiratory tract (throat rinsing fluid, nasopharyngeal and oropharyngeal swabs, anterior nasal swab and mid-turbinate nasal swab specimens, nasopharyngeal wash/aspirate and nasal aspirates).

This test is suitable for patients of all ages with or without a suspected SARS-CoV-2 or SARS coronavirus infection and is intended as an aid in the diagnosis of such infections in combination with patient history and additional clinical information.

The test is intended for professional use and is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostic procedures.

# 2 Product description

ViroReal® Kit SARS-CoV-2 Multiplex is a one-step reverse transcription real-time PCR test and detects RNA of the nucleocapsid protein gene (N gene) of SARS-CoV-2, SARS-CoV and SARS-related coronavirus (Sarbecovirus) as well as of the RdRp gene of SARS-CoV-2.

A probe-specific amplification-curve in the FAM channel indicates the amplification of the N gene specific RNA. A probe-specific amplification-curve in the VIC channel indicates the amplification of RdRp gene specific RNA. An internal RNA positive control (IPC) is detected in Cy5 channel and serves as a control for RNA extraction and possible RT-PCR inhibition. The target for the RNA IPC (artificial target RNA) is added during sample extraction.

The multiplex approach allows universal detection of all so far known SARS coronavirus strains (Sarbecovirus) including SARS-CoV-2, and the discrimination between SARS-CoV-2 and the other Sarbecovirus strains.
The chosen design for the N gene includes a highly conserved region in all SARS coronavirus clusters of the N gene, which allows the universal detection of previously known Sarbecoviruses including SARS-CoV-2. Due to its universal character, it takes into account possible future mutations in the SARS-CoV-2 virus sequence. Because of its design, the test has the potential to detect new SARS corona viruses in the future. For the RdRp gene, a sequence range highly specific for SARS-CoV-2 was chosen, which allows the specific detection of SARS-CoV-2. The primer and probe designs chosen are not identical with designs published by the WHO.

This test has been validated with the ABI® 7500 Fast instrument (fast cycle parameters are not supported, Thermo Fisher Scientific) and was also tested with QuantStudio™ 7 Pro (Thermo Fisher Scientific), MIC instrument (bio molecular systems), Mx3005P® (Agilent), qTOWER$^3$G (Analytik Jena) and LightCycler® 480 II (Roche Diagnostics). It is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC and Cy5 channel (e.g., QuantStudio™ 5 (Thermo Fisher Scientific), cobas z 480 Analyzer (Roche)).
When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR-platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

Ingenetix ViroReal®, BactoReal®, MycoReal®, PanReal and ParoReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA can be analysed in one run.

# 3 Pathogen information

Coronaviruses are positive single-stranded RNA viruses of the family *Coronaviridae*. Several different strains of coronaviruses are currently known to infect humans (HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, MERS-CoV, SARS-CoV, SARS-CoV-2, NCoV and HCoV-EMC).

Strains HCoV-229E, HCoV-NL63, HCoV-OC43, MERS-CoV and HCoV-HKU1 cause cold, upper respiratory infection, bronchiolitis and pneumonia in humans.

SARS-CoV, a beta coronavirus, causes the Severe Acute Respiratory Syndrome (SARS).

SARS-CoV-2, a beta corona virus, causes COVID-19 (corona virus disease 2019). Fever, cough, and breathing difficulties are described as the most common initial symptoms, and infection can subsequently lead to pneumonia. The coronavirus spreads mainly by droplet and contact transmission.

# 4 Principle of one-step reverse transcription real-time PCR

The test is based on multiplex one-step reverse transcription real-time PCR (RT-PCR) by 5´-nuclease-assay technology. In a first step, a specific RNA sequence is transcribed into cDNA and subsequently the DNA is amplified. The generated PCR-products are detected by an oligonucleotide-probe labelled with a fluorescent dye. This allows the sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence rises significantly above the background fluorescence.

# 5 Contents of the kit, stability, and storage

**Table 1**

| | | **DHUV02413** | **DHUV02413x5** | |
|---|---|---|---|---|
| **Labelling** | **Content** | **Amount 100 reactions** | **Amount 500 reactions** | **Storage** |
| SARS-CoV-2 Multiplex + IPC3 Assay Mix (green cap) | Primer + probes for detection of<br>- N gene (FAM)<br>- RdRp gene (VIC)<br>- RNA IPC (Cy5) | 1 x 100 µl | 1 x 500 µl | -25 to -15 °C |
| IPC-Target (RNA) (orange cap) | Target for RNA IPC (internal RNA positive control system) | 1 x 200 µl | 1 x 500 µl | -25 to -15 °C |
| SARS-CoV-2 Multiplex Positive Control (red cap) | RNA Positive Control for the N gene and RdRp gene (à 1000 target copies/µl) | 1 x 300 µl | 1 x 500 µl | -25 to -15 °C |
| RNA Reaction Mix (white cap) | PCR reaction mix for RNA amplification | 1 x 500 µl | 5 x 500 µl | -25 to -15 °C |
| Nuclease-free water (blue cap) | Nuclease-free water | 1 x 1000 µl | 3 x 1000 µl | -25 to -15 °C |

**RNA Reaction Mix:** The Master Mix provided with the kit has been designed for reliable, high-sensitivity one-step reverse transcription real-time PCR even in the presence of common reaction inhibitors. The Master Mix contains a thermostable MMLV Reverse Transcriptase, an RNase inhibitor, a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

**Delivery and Storage**

Shipment is with dry ice. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.

**Quality Control Release Testing**

In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality.
Quality control is performed with artificial RNA representing parts of the pathogen RNA. The RNA concentration was determined using a standard curve of a plasmid containing parts of the pathogen sequence.

# 6 Additionally required materials and devices

- Reagents and devices for RNA-extraction which are appropriate for the listed sample material (see 9. Preparation of the samples)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Vortex-Mixer
- Benchtop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument detecting and differentiating fluorescence in FAM, VIC and Cy5 channel
- Appropriate 96 well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: Laminar flow box
- Optional: PCR workstation

# 7 Precautions and safety information

## 7.1 General information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.
- Transportation of clinical specimens must comply with local regulations for the transport of biological materials.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Discard specimens, reagents, and waste according to your local safety regulations.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of RNA has a profound impact on the test performance. Ensure that the used RNA extraction system is compatible with real-time PCR technology.
- The real-time PCR instrument should be calibrated, serviced, and cleaned regularly.
- Protect kit components from light.
- Avoid mixing reagents of different kits and lots and check expiry date of the kit.

## 7.2 Specific information

A workflow must be followed to avoid false positive results due to detection of contaminating RNA.

<u>Recommended measures to avoid RNA contamination:</u>

- Use separated workspaces for specimen preparation, set-up of real-time PCR and amplification. Supplies and equipment must be dedicated to each of these separate workspaces to ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and devices must be cleaned regularly.
- Sample preparation should be performed in a laminar flow box. Clean laminar flow box regularly in all areas.

- If possible, leave consumables and pipettes in the laminar flow sterile bench and PCR workstation.
- Preparation of real-time PCR should be done in a PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only RNA-free consumables.
- Wear lab coat.
- Work only with powder-free disposable gloves, do not touch the outer surface of the palm and fingers of the gloves when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads of open vials.
- Caution is advised when handling specimens and positive control to avoid cross-contamination.
- Store positive and potentially positive material separately from all other reagents.
- For a valid interpretation of results, a negative control shall be included during RNA-extraction (e.g., extraction of water instead of sample material), to exclude false-positive results due to contamination with RNA of pathogen during extraction.
- Optional: include a negative control of PCR per PCR-run (nuclease-free water instead of sample, NTC).

# 8 Limitations

- Reliable results with this test are only achieved by appropriate specimen collection, transport and storage, as well as an appropriate RNA extraction procedure.
- RNA extraction and SARS-CoV-2 detection have been validated for swabs from the respiratory tract with this kit. Test performance with other specimen types has not yet been assessed.
- A negative test result does not exclude the possibility of a SARS-CoV or Sarbecovirus infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or viral quantities below the assay sensitivity. The presence of PCR inhibitors may lead to invalid results.
- In general, sequence variability in the target region of previously unknown clinical subtypes may lead to false negative or less sensitive results. The universal test character of ViroReal® Kit SARS-CoV-2 Multiplex takes into account possible future mutations of the SARS-CoV-2 virus sequence and thus reduces the probability of false negative or less sensitive results due to mutations in the target region.
- Results should be interpreted in context of other clinical and laboratory findings.

# 9 Preparation of samples

ViroReal® Kit SARS-CoV-2 Multiplex is suitable for analysis of RNA extracts of samples from the human respiratory tract (throat rinsing fluid, nasopharyngeal and oropharyngeal swabs, anterior nasal swab and mid-turbinate nasal swab specimens, nasopharyngeal wash/aspirate and nasal aspirates).

Sample preparation should be performed with the recommended measures to avoid RNA contamination (see precautions and safety information). Always include an extraction negative control during RNA-extraction (e.g., extraction of water instead of sample material).

Purified RNA should be stored at -25 to -15 °C or at -80 to -60 °C.

## 9.1 Sample collection and storage

Samples from the respiratory tract can be stored in microcentrifuge tubes. Swabs are stored either dry or in isotonic saline solution (NaCl 0.9%, not provided). Swabs can be collected with swab material proper for PCR (e.g., sterile polyester or rayon swabs with aluminium or plastic shaft).
It is recommended to process samples immediately after collection. Store samples at 2-8 °C for no longer than 48 hours. Do not freeze samples prior to extraction.

## 9.2 Recommended extraction methods

Ensure that the extraction system used is not contaminated with RNA of pathogens detected with ViroReal® Kit SARS-CoV-2 Multiplex. Extract samples with an RNA extraction system compatible with RT real-time PCR technology and appropriate for the sample material. Extract RNA from 140-200 µl sample (depending on the extraction method) and elute in approx. 50 µl.

<u>For manual extraction recommended</u>
- QIAamp Viral RNA Mini Kit (Qiagen)

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.

## 9.3 Quality control for RNA extraction and RT PCR inhibition with IPC

The RNA IPC system (internal RNA positive control) is used as a control for RNA extraction, identifies possible RT PCR inhibition and confirms the integrity of kit reagents.
For this, an artificial RNA (IPC-Target (RNA), approx. $5x10^5$ copies/µl) is added during extraction.

**Note:** The Cq values of the IPC depend on the extraction method and on the type of sample material. Negative samples should show Cq values of the IPC between 27-30. The extraction method used must be validated accordingly with sample material. Use the IPC-Target (RNA) freshly diluted (1:10 with nuclease-free water) in the extraction, if Cq values < 27 are determined during validation with samples.

### 9.3.1 Application of IPC as control of extraction and RT real-time PCR

The IPC-Target (RNA) must be added during extraction.

→ Per sample spike <u>1 µl IPC-Target (RNA)</u> (orange cap) directly into the appropriate volume of lysis buffer (or spike it to the sample <u>after</u> the lysis buffer has been added to the sample), then continue the extraction procedure.

**Caution:** The IPC-Target (RNA) shall not be added to sample material in the absence of lysis buffer, as degradation may occur. It must be added to the lysis buffer.

### 9.3.2 Application of IPC as quality control for RT PCR reaction

If the IPC-Target (RNA) has not been added during extraction, it can be added at a later stage to the PCR master mix.

→ Freshly dilute the IPC-Target (RNA) 1:100 with nuclease-free water and add 1 µl of the dilution/PCR reaction.

**Caution:** The IPC-Target (RNA) shall not be added undiluted to the master mix (inhibition of PCR).

# 10 Preparation of real-time PCR

- Include one positive control (red cap), one extraction negative control and optionally one PCR negative control (NTC, e.g., nuclease-free water) per PCR run.
- It is generally recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- Best use RNA immediately after extraction and always keep it on ice. Alternatively, use RNA stored at -80 °C to -15 °C. Thaw RNA on ice, avoid prolonged exposure to room temperature and immediately refreeze the RNA after use.
- Just before use, thaw the RNA Reaction Mix on ice, and invert 2 to 3 times to ensure a homogenous solution. The RNA Reaction Mix does not freeze at -20°C, but gelling may occur. The RNA reaction mix should not be warmed to room temperature. Thaw the other kit components completely at room temperature. When thawed, mix components carefully, centrifuge briefly with low speed and subsequently put on ice. Prepare Master Mix for RT-PCR on ice and use as soon as possible.
- **Positive Control**
  → As positive control, use 10 µl of the Positive Control (red cap). Always, pipette positive control at last. The Positive Control is a mixture of two *in vitro* synthesized RNA fragments with a concentration of $10^3$ copies/µl each. It has to be stored at -20 °C. Before use, gently mix Positive Control, do not vortex. To avoid frequent freeze/thaw cycles, it can also be temporarily stored at 4 °C if used several times on the same day.

## 10.1 Pipetting scheme

| | | Per sample |
|---|---|---|
| **Preparation of Master Mix** (mix well) | Nuclease-free Water* | 4.0 µl |
| | RNA Reaction Mix | 5.0 µl |
| | SARS-CoV-2 Multiplex + IPC3 Assay Mix | 1.0 µl |
| | **Total volume Master Mix** | **10.0 µl** |
| **Preparation of PCR** | Master Mix | 10.0 µl |
| | RNA-Sample* | 10.0 µl |
| | **Total volume** | **20.0 µl** |

*10 µl sample can be used. When using a volume other than 10 µl, the volume of nuclease free water must be adjusted accordingly.

→ **If IPC-Target (RNA) was not added during extraction:** Freshly dilute the IPC-Target (RNA) (orange cap) 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. In this case, the IPC-Target (RNA) is used as a quality control for the RT PCR reaction.

**Caution:** The use of undiluted IPC-Target (RNA) might cause inhibition of the real-time PCR reaction.

- Prepare the Master Mix according to the number of samples, calculating an additional volume of approx. 10% to account for pipetting loss.
- Pipette 10 µl of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 10 µl of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

## 10.2 Programming of temperature profile

Please find further information on programming of the real-time PCR instrument in the respective operator's manual. Keep in mind that some PCR-platforms must be calibrated with the corresponding dye before performing a multiplex-PCR.

**Sample Volume:** 20 µl
**Ramp speed:** "Standard" without "fast cycling" parameter for ABI® 7500 Fast Instrument, QuantStudio™ (Thermo Fisher Scientific)

| **Program 1**<br>Cycles: 1<br>Analysis: None | **Program 2**<br>Cycles: 1<br>Analysis: None | **Program 3**<br>Cycles: 45<br>Analysis: Quantification<br>Acquisition at 60 °C |
|---|---|---|
| 50 °C<br>15 min | 95 °C<br>2 min | 95 °C<br>5 sec<br>60 °C<br>1 min |

**Ad program 1:** This temperature profile can be used for all ingenetix ViroReal®, BactoReal®, MycoReal, PanReal and ParoReal® kits for the detection of DNA or RNA.
**Ad program 2:** the previous temperature profile with 20 sec in program 2 can still be used.

**<u>Select detection channels</u>**

**FAM channel:** Universal detection of SARS-CoV-2 or SARS-CoV (Sarbecovirus)
**VIC channel:** Specific detection of SARS-CoV-2
**Cy5 channel:** Detection of IPC

**For ABI® 7500 Instrument, QuantStudio™ 5/6/7 (Thermo Fisher Scientific), Mx3005P® (Agilent)**
FAM-TAMRA
VIC-NONE
Cy5-NONE
Passive refence dye: ROX

**For MIC Instrument (bio molecular systems)**
FAM Green
VIC Yellow
Cy5 Red
Passive reference dye: no ROX needed

**For cobas z 480 Analyzer / LightCycler® 480 II (Roche)**
Detection format: 3 Color Hydrolysis Probe
FAM: Excitation at 465 nm, Emission at 510 nm
VIC: Excitation at 540 / 533 nm, Emission at 580 nm
Cy5: Excitation at 610 / 618 nm, Emission at 670 / 660 nm
Passive reference dye: no ROX needed

The color compensation for FAM and VIC must be selected from the Roche database after the analysis of Cy5.

# 11 Interpretation of PCR-data

For the analysis of the PCR results, select the fluorescence display options FAM and VIC channel for the pathogen target and Cy5 channel for the IPC.
Please note that some PCR platforms require Color Compensation when using a multiplex PCR.

**IMPORTANT:** Samples should be checked in both logarithmic (Roche instrument: Abs Quant/Fit Points) and linear view and compared to the negative control. In addition to the Cq values, also check the amplification curves and adjust the threshold (noise band) if necessary.
After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid. If results of controls are not valid, no interpretation of results with clinical samples is possible.

**Negative samples:**
Samples without amplification curves (no Cq values, undetermined) are regarded as negative. No RNA was detected in these samples because there is no infection with the corresponding pathogen or the pathogen RNA concentration is below the detection limit of the test.

**Positive samples:**
Samples with Cq values < 45 in the fluorescence channel for the pathogen target are regarded as positive.

Table 2 shows the criteria for valid positive and negative controls. Table 3 shows interpretation of data with clinical samples.

## 11.1 Controls

**Table 2** Criteria for valid positive and negative controls, IPC-Target (RNA) was added during extraction

| | Cq FAM channel N gene | Cq VIC channel RdRp gene | Cq Cy5 channel IPC[1] | Interpretation | Action |
|---|---|---|---|---|---|
| **Positive control** | <28 | <28 | Negative | Valid | - |
| **Positive control** | Negative | Negative | Negative | Invalid | See 12.1 |
| **Positive control** | <28 | Negative | Negative | Invalid | See 12.1 |
| **Positive control** | Negative | <28 | Negative | Invalid | See 12.1 |
| **Extraction negative control** | Negative | Negative | 27-30[2] | Valid | - |
| **Extraction negative control** | Negative | Negative | Negative | Invalid | See 12.1 |
| **Extraction negative control** | Positive | Positive | 27-30[2] | Invalid | See 12.3 |
| **Extraction negative control** | Positive | Negative | 27-30[2] | Invalid | See 12.3 |
| **Extraction negative control** | Negative | Positive | 27-30[2] | Invalid | See 12.3 |
| **NTC PCR (optional)** | Negative | Negative | Negative | Valid | - |
| **NTC PCR (optional)** | Positive | Positive | Negative | Invalid | See 12.2 |
| **NTC PCR (optional)** | Negative | Positive | Negative | Invalid | See 12.2 |
| **NTC PCR (optional)** | Positive | Negative | Negative | Invalid | See 12.2 |

[1] If the IPC-Target (RNA) was added directly to the master mix, all samples in the Cy5 channel must be positive.
[2] Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).

### 11.2 Clinical samples

Samples with positive Cq-values are considered positive, see Table 3.

**Table 3** Interpretation of data with clinical samples

| | Cq FAM channel N gene | Cq VIC channel RdRp gene | Cq Cy5 channel IPC | Interpretation | Action |
|---|---|---|---|---|---|
| **Clinical sample** | Negative | Negative | 27-30[1] | Negative | - |
| **Clinical sample** | Positive | Positive | Positive/Negative[2] | Positive for SARS-CoV-2 | - |
| **Clinical sample** | Negative | Positive | Positive/Negative[2] | Positive for SARS-CoV-2 | - |
| **Clinical sample** | Positive[3] | Negative[3] | Positive/Negative[2] | Positive for Sarbecovirus[3] | - |
| **Clinical sample** | Negative | Negative | Negative | Invalid | See 12.5 |

[1] A positive signal of the IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR. Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).
[2] High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the IPC.
[3] If the sample is weak positive (approx. Cq >33) in FAM channel and negative in VIC channel, there is a low virus concentration. In this case it is not possible to distinguish between SARS-CoV-2 or any other sarbecovirus.

In case of invalid data, analysis has to be repeated with the remaining or newly extracted RNA sample (see 12. Troubleshooting).

## 12 Troubleshooting

### 12.1 No virus specific signal with positive control and with IPC

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.
  → Compare temperature profile and setting of detection channels with details specified in the protocol.
- Incorrect configuration of the PCR reaction.
  → Check your pipetting steps with the pipetting scheme and repeat PCR, if necessary.
- The RNA may have been degraded.
- The IPC-Target (RNA) was added undiluted directly to the mastermix and not freshly diluted 1:100. The PCR reaction is therefore inhibited.
  → Freshly dilute IPC-Target (RNA) 1:100 and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of the reverse transcription real-time PCR only, 1 µl of freshly 1:100 diluted IPC-Target (RNA) must be added to the mastermix. If the IPC-Target (RNA) has been forgotten to be added:
  → Freshly dilute IPC-Target (RNA) and repeat PCR.
- For control of RNA extraction and PCR inhibition, the undiluted IPC-Target (RNA) must be added during extraction to the lysis buffer. If the IPC-Target (RNA) has been forgotten to be added:
  → Repeat RNA extraction.

### 12.2 Virus specific signal with negative control

- A contamination occurred during preparation of the RT-PCR.
  → Repeat the RT-PCR with new reagents in replicates.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals.

### 12.3 Virus specific signal with negative control of extraction

- A contamination occurred during extraction.
  → Repeat RNA extraction and RT-PCR using new reagents.
  → Make sure that workspace and instruments are cleaned at regular intervals.

### 12.4 IPC specific signal with PCR negative control and positive control

- The IPC-Target (RNA) has been added to lysis buffer during extraction, but there is IPC specific signal with PCR negative control and positive control: Contamination with the IPC-Target (RNA).
  → Make sure that workspace and instruments are cleaned at regular intervals.

### 12.5 No signal with IPC and no virus specific signal with sample

- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- Incorrect RT-PCR conditions.
  → Check the RT-PCR conditions and repeat the RT-PCR, if necessary.
- The RNA might be degraded.
- If IPC-Target (RNA) was added during extraction:
  - Inhibition of RT-PCR may have occurred.
  - RNA extraction was unsuccessful.
  - The IPC-Target (RNA) was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the RT-PCR reaction.

  → No statement is possible. Verify you use a recommended method for RNA isolation and re-examine the single steps of the RNA extraction.
- → If no operating mistakes during RNA extractions can be retraced, it is recommended to repeat the RT-PCR with lower amounts of RNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of nuclease-free water).

# 13 Specifications and performance evaluation

## 13.1 Test performance on different real-time PCR instruments

Performance of ViroReal® Kit SARS-CoV-2 Multiplex with an ABI® 7500 Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 1.

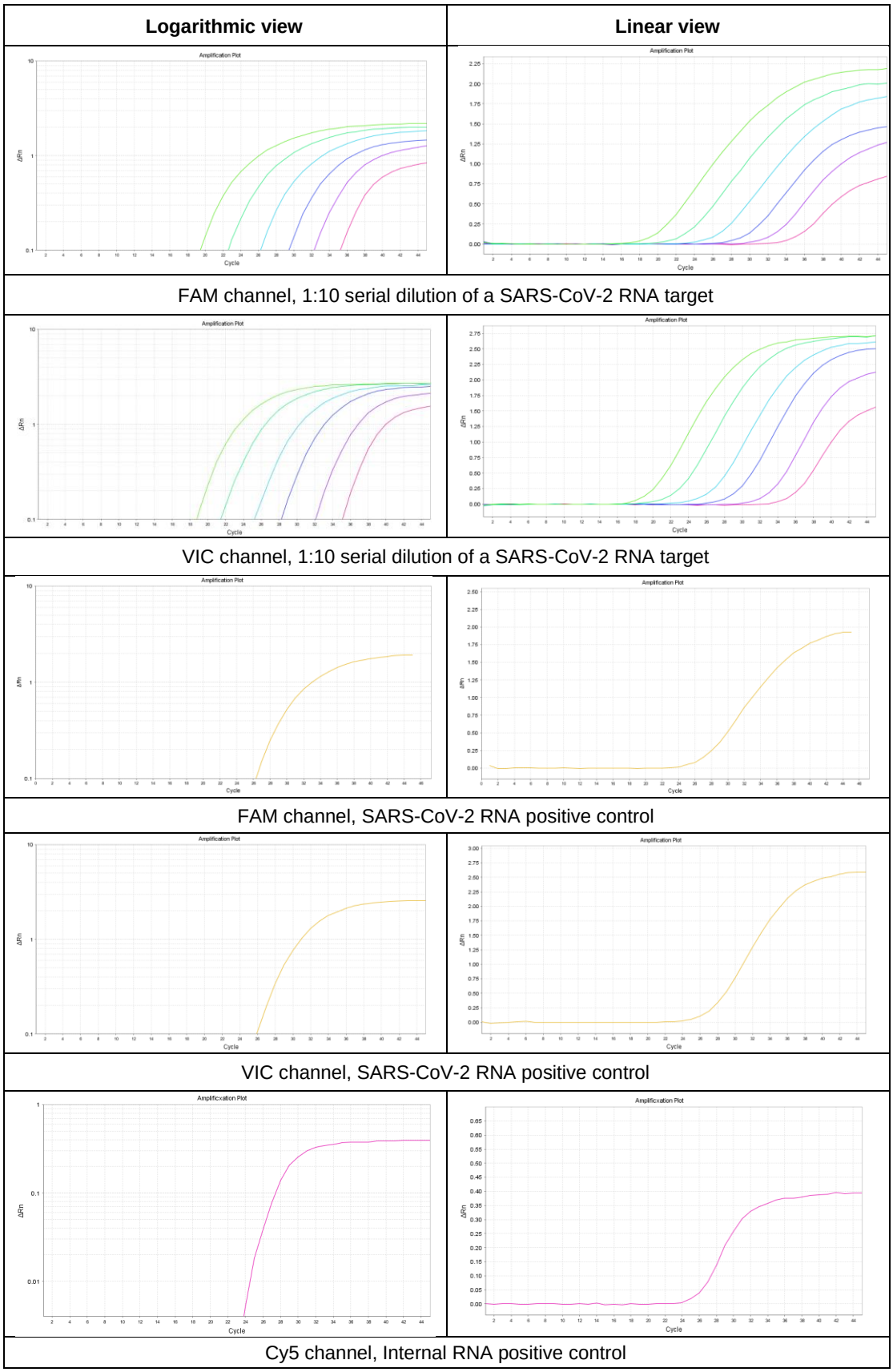


**Figure 1** Performance of ViroReal® Kit SARS-CoV-2 Multiplex with ABI® 7500

This test has been validated with the ABI® 7500 Real-time PCR System (Thermo Fisher Scientific) and tested with a LightCycler® 480 Instrument II (Roche), MIC instrument (bio molecular systems), QuantStudio™ 7 Pro (Thermo Fisher Scientific), Mx3005P® (Agilent) and qTOWER$^3$G (Analytik Jena), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC and Cy5 channel.

### 13.2 Limit of detection, LoD95%

**Method:** ViroReal® Kit SARS-CoV-2 Multiplex was tested with a 10-fold dilution series of a CE certified RNA reference material (AMPLIRUN® CORONAVIRUS SARS-CoV-2 RNA CONTR, Vircell, order no. MBC137-R). The limit of detection (LoD95% = smallest number of copies of target RNA which can be detected in 95% of cases) was determined with dilutions of the RNA around the analytical sensitivity using a non-linear curve fitting with the Graph Pad Prism Software.

**Result:** The LoD95% is 6 target copies/reaction for the N gene and 5 target copies/reaction for the RdRp gene.

### 13.3 Linearity and dynamic measuring range

**Method:** Linearity was determined using a 10-fold dilution series ($10^6$ – $10^1$ target copies/reaction) of two synthetic RNAs representing fragments of SARS-CoV-2 RNA. The number of determinations (n) per dilution was ten.

**Result:** For the N gene, the assay shows linearity over the range of 10 to 1,000,000 target copies/reaction with a slope of -3.382 ± 0.04229 and an $R^2$ of 0.9910 as shown in Figure 2.
For the RdRp gene, the assay shows linearity over the range of 10 to 1,000,000 target copies/reaction with a slope of -3.390 ± 0.04703 and an $R^2$ of 0.9890 as shown in Figure 3.

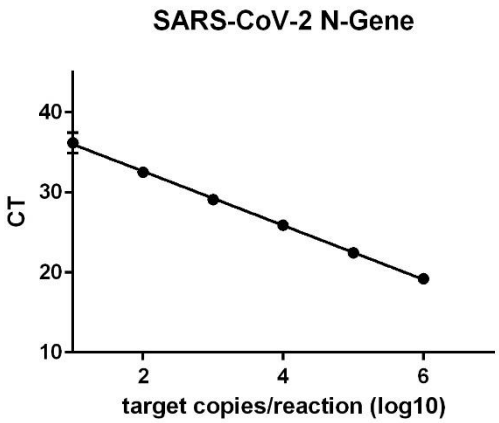


**Figure 2** Ten-fold dilution series of N-gene RNA plotted against CT

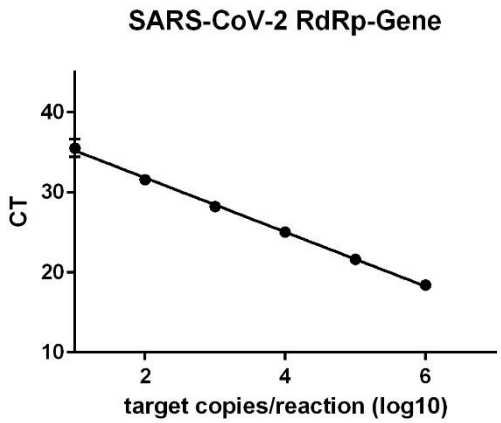


**Figure 3** Ten-fold dilution series of RdRp-gene RNA plotted against CT

### 13.4 Precision

**Method:** Precision within a run (intra-assay), between multiple runs (inter-assay) and between two lots (inter-lot) was determined with 10-fold dilution series ($10^6$ – $10^1$ target copies/reaction) of RNA.

**Result:** For the N gene, the mean values of the coefficients of variation (CV%) are 1.1% for intra-assay precision, 1.0% for inter-assay precision and 0.7% for inter-lot precision.

For the RdRp gene, the mean values of the coefficients of variation (CV%) are 1.4% for intra-assay precision, 1.6% for inter-assay precision and 1.3% for inter-lot precision.

### 13.5 Analytical specificity

**Method BLAST analysis:** The selection of highly specific primers and probes ensures analytical specificity. The specificity of primer and probes was validated *in silico* using the Basic Local Alignment Search Tool (BLAST) against the NCBI database. Furthermore, sequence analyses were performed in the GISAID database. Primers and probes have been checked for possible homologies to currently published sequences. This analysis validates the detection of all so far known SARS coronavirus strains including SARS-CoV-2.

**Result:** Based on BLAST analysis, no relevant sequence variabilities were observed in the primer and probe region of so far known SARS-CoV-2 variants. ViroReal® Kit SARS-CoV-2 Multiplex detects the nucleocapsid protein gene (N gene) of SARS-CoV-2, SARS-CoV and SARS-related coronavirus (Sarbecovirus) as well as the RdRp gene of SARS-CoV-2.
For the N gene, a highly conserved region in all SARS coronavirus clusters (SARS-CoV-2 as well as SARS-CoV and SARS-related bat coronavirus) was chosen as target region. The selected primers and probe are intended to cover possible future mutations in the virus sequence. All data in the NCBI and GISAID database showed homology of the sequence region of primers and probe in all SARS coronavirus clusters. This approach allows universal detection of all so far known SARS coronavirus strains including SARS-CoV-2.
For the RdRp gene, a highly specific region for SARS-CoV-2 was chosen, the RdRp gene of other coronaviruses are not detected, with exception of Bat coronavirus RaTG13, which is the coronavirus most closely related to SARS-CoV-2.

**Method testing inclusivity:** Specificity was experminetally validated by testing against a SARS-CoV-2 isolate.

**Result:** SARS-CoV-2 was detected.

**Method testing exclusivity:** Analytical specificity has been further evaluated by testing genomic DNA or RNA of viruses (adenovirus, enterovirus, metapneumovirus, respiratory syncytial virus, rhinovirus, influenza A, influenza B) and of bacteria (*Bordetella pertussis, Bordetella parapertussis).*

**Result:** No cross-reactions have been observed. All samples were correctly analysed negative with ViroReal® Kit SARS-CoV-2 Multiplex.

### 13.6 Diagnostic evaluation

**Method:** ViroReal® Kit SARS-CoV-2 Multiplex (kit version 2.0) was tested by an external service provider (Labor Mustafa, Vienna) in May 2022 with extracts from 275 swab samples from the upper respiratory tract or gargeling samples from routine COVID-19 testing, from suspected COVID-19 patients or patients with signs and symptoms of COVID-19. Extraction was performed using TANBead DNA/RNA extraction Kit for TANBead (Maelstrom 9600; Taiwan Advanced Nanotech Inc.) with 300 µl sample. RNA was eluted with 100 µl elution buffer.
From this, 10 µl of eluates were used for PCR analysis with ViroReal® Kit SARS-CoV-2 Multiplex (kit version 2.0). Runs were performed using QuantStudio™ 7 Pro (Thermo Fisher Scientific). Single determinations were performed for each sample.
The test panel included 99 samples tested positive (range from highly positive to weakly positive with Cq values from 15-35) and 176 samples tested negative for SARS-CoV-2. Original data were obtained using ViroReal® Kit SARS-CoV-2 & SARS allowing single detection of the N gene of SARS-CoV-2 in the FAM channel. Samples with Cq-values above 35 with the reference method were excluded from the analysis.

**Result:** ViroReal® Kit SARS-CoV-2 Multiplex which detects two separate genes of SARS-CoV-2, the N and RdRp gene, confirmed the presence of SARS-CoV-2 in all 99 positive samples. In all 176 samples negatively tested for SARS-CoV-2, ViroReal® Kit SARS-CoV-2 Multiplex (kit version 2.0) confirmed the absence of SARS-CoV-2 (Table 4, Table 5).
The Cq value (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)) corresponds to the number of PCR cycles required until a positive signal is obtained and is therefore a measure of the virus concentration in the sample material. However, the Cq values are also depending on the instrument software and on the quality of swab samples.
Guidelines for interpretation of the Cq values can be found on the website of the Robert Koch Institute.

**Table 4** Diagnostic evaluation of ViroReal® Kit SARS-CoV-2 Multiplex

| | Reference | | | |
|---|---|---|---|---|
| | | pos | neg | Total |
| ViroReal® Kit SARS-CoV-2 Multiplex | pos | 99 | 0 | 99 |
| | neg | 0 | 176 | 176 |
| | Total | 99 | 176 | 275 |

**Table 5** Results of clinical validation compared with to ViroReal® Kit SARS-CoV-2 & SARS

| | Value |
|---|---|
| **Sensitivity** | 100 % |
| **Specificity** | 100 % |
| **NPV** | 100 % |
| **PPV** | 100 % |

# 14 References

Huang, Chaolin & Wang, Yeming & Li, Xingwang & Ren, Lili & Zhao, Jianping & Hu, Yi & Zhang, Li & Fan, Guohui & Xu, Jiuyang & Gu, Xiaoying & Cheng, Zhenshun & Yu, Ting & Xia, Jiaan & Wei, Yuan & Wu, Wenjuan & Xie, Xuelei & Yin, Wen & Li, Hui & Liu, Min & Cao, Bin. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. The Lancet. 10.1016/S0140-6736(20)30183-5.
https://www.who.int/emergencies/diseases/novel-coronavirus-2019

# 15 Revision history

| Revision | Date | Description |
|---|---|---|
| 1.1 | 13 October 2020 | 8.3. Close the plate with appropriate optical closing material.<br>9. Samples with positive Cq-values are considered positive (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)). |
| 1.2en | 25 May 2022 | **General update for new kit version 2.0:**<br>The test design has been improved to increase the stability of the master mix and to improve performance. For this, the forward primers for the N gene and the RdRp gene were modified.<br><br>**Explanation of symbols**<br>Update<br>**1. Intended purpose**<br>Intended use specified in more detail<br>**5. Contents, stability and storage**<br>Changed fill volume of RNA IPC Target and Positive Control. Changed name for Assay Mix and Positive Control.<br>**6. Precautions and safety instructions**<br>Inclusion of additional information on specimen collection and storage, processing of specimens after collection, storage conditions of specimens, transport and storage conditions of purified RNA.<br>**9. Preparation of samples**<br>Update<br>**10. Preparation of real-time PCR**<br>The temperature profile has changed - the activation step in program 2 has been extended from 20 sec to 2 min.<br>Information added, that the Master Mix shall be used as soon as possible.<br>**11. Interpretation of PCR data**<br>Update of tables for results with positive and negative controls and clinical samples.<br>**13. Specifications and performance evaluation**<br>Update for new kit version 2.0. Test performance (LoD95) has been improved. |
| 1.3en | 01 May 2023 | **5. Contents, stability and storage**<br>Kit version 2.1:<br>Changed filling volume of RNA IPC-Target and Positive Control in DHUV02413x5<br>The RNA IPC-Target is not stored in RNA/DNA Stabilizer anymore, concentration was changed from approx. $6x10^5$ copies/µl to approx. $6x10^4$ copies/µl and was renamed to IPC-Target (RNA).<br>**7. Precautions and safety instructions**<br>Update of this section<br>Inclusion of additional information on specimen collection and storage, processing of specimens after collection, storage conditions of specimens, transport and storage conditions of purified RNA.<br>Recommended measures to avoid RNA contamination added.<br>**9. Preparation of samples**<br>Update of paragraph of Quality control for RNA extraction and PCR inhibition<br>Note that for Cq values <27, the RNA IPC-Target must be diluted prior to extraction.<br><br>**10. Preparation of real-time PCR**<br>Dilution of IPC-Target (RNA) was changed from 1:500 to 1:100<br>**11. Interpretation of PCR data**<br>Table 1 and Table 2: Update |

**Note:**
Any serious incident that has occurred in relation to the product shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

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