ViroReal® Kit
SARS Coronavirus & Influenza A/B

Kit Version 1.1

Instructions for Use

CE

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

REF **DHUV02513** Σ **100**

REF **DHUV02513x5** Σ **500**

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Index

1 Intended purpose 3
2 Product description 3
3 Pathogen information 3
4 Principle of one-step reverse transcription real-time PCR 4
5 Contents of the kit, stability, and storage 4
6 Additionally required materials and devices 5
7 Precautions and safety information 5
7.1 General information 5
7.2 Specific information 6
8 Limitations 6
9 Preparation of samples 7
9.1 Sample collection and storage 7
9.2 Recommended extraction methods 7
9.3 Quality control for RNA extraction and RT PCR inhibition with IPC 7
9.3.1 Application of IPC as control of extraction and RT real-time PCR 7
9.3.2 Application of IPC as quality control for RT PCR reaction 7
10 Preparation of real-time PCR 8
10.1 Pipetting scheme 8
10.2 Programming of the temperature profile 9
11 Interpretation of PCR-data 10
11.1 Controls 10
11.2 Clinical samples 11
12 Troubleshooting 11
12.1 No virus specific signal with positive control and with IPC 11
12.2 Virus specific signal with negative control 11
12.3 Virus specific signal with negative control of extraction 12
12.4 IPC specific signal with PCR negative control and positive control 12
12.5 No signal with IPC and no virus specific signal with sample 12
13 Specifications and performance evaluation 13
13.1 Kit performance 13
13.2 Limit of detection, LoD95% 14
13.3 Linearity and dynamic measuring range 14
13.4 Precision 15
13.5 Analytical specificity 15
13.6 Diagnostic evaluation 16
14 References 17
15 Revision history 17

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 Coronavirus & Influenza A/B 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 (Sarbecovirus), of the matrix protein gene of influenza A virus and of the hemagglutinin gene of influenza B virus.

Proper specimens are 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).
This test is suitable for patients of all ages with or without a suspected SARS-CoV-2, SARS coronavirus or influenza virus infection and is intended as an aid in the diagnosis of these 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 Coronavirus & Influenza A/B 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 matrix protein gene of influenza A virus and of the hemagglutinin gene of influenza B virus. Other beta coronaviruses or influenza viruses are not detected with this kit.

An internal RNA positive control (IPC) is detected in Cy5 channel and is used as RNA extraction as well as RT-PCR inhibition control. The target for the RNA IPC (artificial target RNA) is added during extraction of the sample.

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, without discriminating between strains. The primer and probe design chosen is not identical with the WHO design. Due to its universal character, it takes into account possible future mutations in the SARS-CoV-2 virus sequence. Due to this design, the test has the potential to detect new SARS corona viruses in the future. The N gene is detected in the fluorescence channel for FAM.

The chosen designs for the matrix protein gene and the hemagglutinin gene include highly conserved regions in influenza A and B virus, respectively. These genes are detected in the fluorescence channel for VIC. A distinction between influenza A virus and influenza B virus is not possible.

This test has been validated with the ABI® 7500 Fast Real-time PCR System (fast cycle parameters are not supported, Thermo Fisher Scientific) and was also tested with LightCycler® 480 II (Roche Diagnostics), MIC instrument (bio molecular systems) and Mx3005P® (Agilent). It is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC and Cy5 channel (eg., cobas z 480 Analyzer (Roche), QuantStudio™ 5 and QuantStudio™ 7 Pro (Thermo Fisher Scientific) and qTOWER^{3}G (Analytik Jena).
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 have to 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.
Influenza is an acute infectious disease caused by influenza virus A, B or, to a much lesser extent, influenza virus C. Influenza viruses are enveloped viruses with single-stranded, segmented RNA with negative polarity as genome. These viruses can be found worldwide. Epidemics and pandemics are mainly caused by influenza virus A, due to antigenic drift of the hemagglutinin and neuraminidase molecules. Type B and C influenza viruses are isolated almost exclusively from humans, while influenza A viruses infect a wide variety of warm-blooded animals.

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

		DHUV02513	**DHUV02513x5**	
Component	**Content**	**Quantity 100 reactions**	**Quantity 500 reactions**	**Storage**
SARS-CoV-2 & SARS + IPC3 Assay Mix (green cap)	Primers and probes for detection of - SARS-CoV-2 and SARS coronavirus (FAM) - RNA IPC (Cy5)	1 x 100 µl	1 x 500 µl	-25 to -15 °C
Influenza A/B Assay Mix (purple cap)	Primers and probes for detection of - influenza A virus and influenza B virus (VIC)	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 + Influenza A Positive Control (red cap)	RNA Positive Control for SARS-CoV-2 + influenza A virus (10^3 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 an 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.

Recommended measures to avoid RNA contamination:

- 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.
- With this kit virus detection has been validated for swabs and sputum from the respiratory tract. Test performance with other specimen types has not yet been assessed.
- A negative test result does not exclude the possibility of a SARS-CoV, Sarbecovirus, influenza A virus or influenza B virus 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 Coronavirus & Influenza A/B 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 clinical and laboratory findings.

9 Preparation of samples

ViroReal® Kit SARS Coronavirus & Influenza A/B 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 native samples prior to extraction. Samples in lysis buffer can be frozen.

9.2 Recommended extraction methods

Ensure that the extraction system used is not contaminated with RNA of pathogens detected with ViroReal® Kit SARS Coronavirus & Influenza A/B. 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.

For manual extraction recommended
- 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 1 µl IPC-Target (RNA) (orange cap) directly into the appropriate volume of lysis buffer (or spike it to the sample after 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*	3.0 µl
	RNA Reaction Mix	5.0 µl
	SARS-CoV-2 & SARS + IPC-3 Assay Mix	1.0 µl
	Influenza A/B Assay Mix	1.0 µl
	Total volume Master Mix	**10.0 µl**
Preparation of RT-PCR	Master Mix RNA-Sample*	10.0 µl 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 the 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 Cycles: 1 Analysis: None	**Program 2** Cycles: 1 Analysis: None	**Program 3** Cycles: 45 Analysis: Quantification Acquisition at 60 °C
50 °C 15 min	95 °C 2 min	95 °C 5 sec 60 °C 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: Detection of influenza A virus and influenza B virus
Cy5-NONE: 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 SARS	Cq VIC channel Influenza A / B	Cq Cy5 channel IPC[1]	Interpretation	Action
Positive control	< 29	< 29	Negative	Valid	-
Positive control	Negative	Negative	Negative	Invalid	See 12.1
Positive control	< 29	Negative	Negative	Invalid	See 12.1
Positive control	Negative	< 29	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 SARS Coronavirus	Cq VIC channel Influenza A / B	Cq Cy5 channel RNA IPC	Interpretation	Action
Clinical sample	Negative	Negative	25-28[1]	Negative	-
Clinical sample	Positive	Positive	Positive/Negative[2]	Positive for SARS Coronavirus[3] and influenza A or B virus	-
Clinical sample	Negative	Positive	Positive/Negative[2]	Positive for influenza A or B virus	-
Clinical sample	Positive	Negative	Positive/Negative[2]	Positive for SARS Coronavirus[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] Positive for SARS-CoV-2, SARS-CoV or SARS-related coronavirus (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

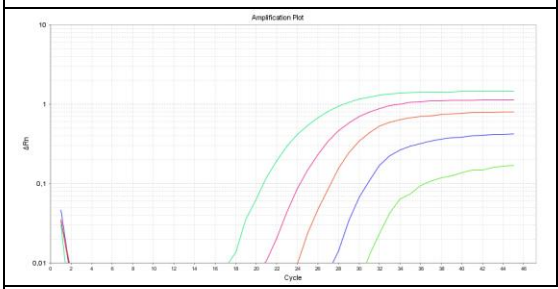
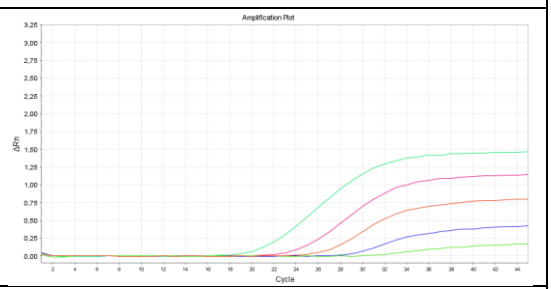
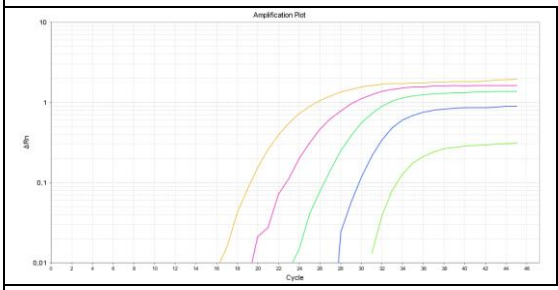
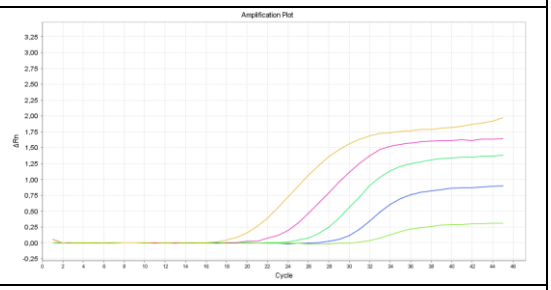
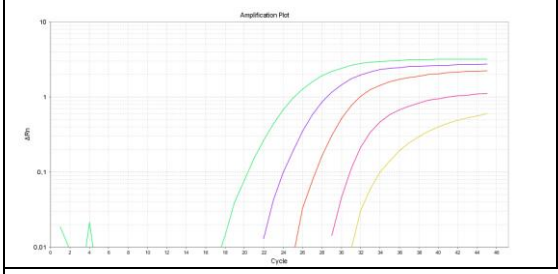
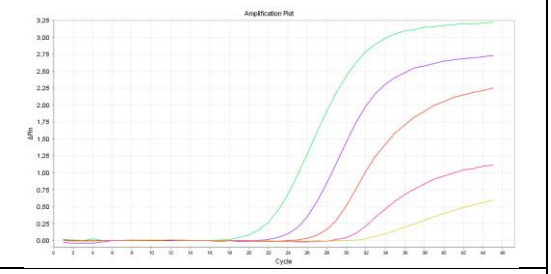
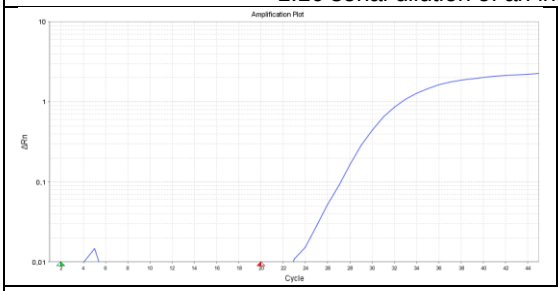
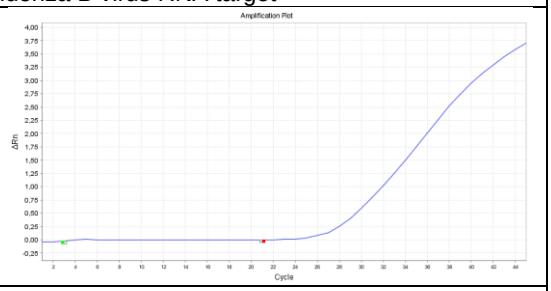
- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- Incorrect RT-PCR conditions.
  → Check the RT-PCR conditions and repeat the RT-PCR, if necessary.
- The RNA might be degraded.
- If IPC-Target (RNA) was added during extraction:
  - 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 Kit performance

Performance of ViroReal® Kit SARS Coronavirus & Influenza A/B with an ABI® 7500 Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 1.

Logarithmic view	Linear view
	
FAM channel 1:10 serial dilution of a SARS-CoV-2 RNA target	
	
VIC channel 1:10 serial dilution of an influenza A virus RNA target	
	
VIC channel 1:10 serial dilution of an influenza B virus RNA target	
	
FAM channel SARS-CoV-2 RNA positive control	

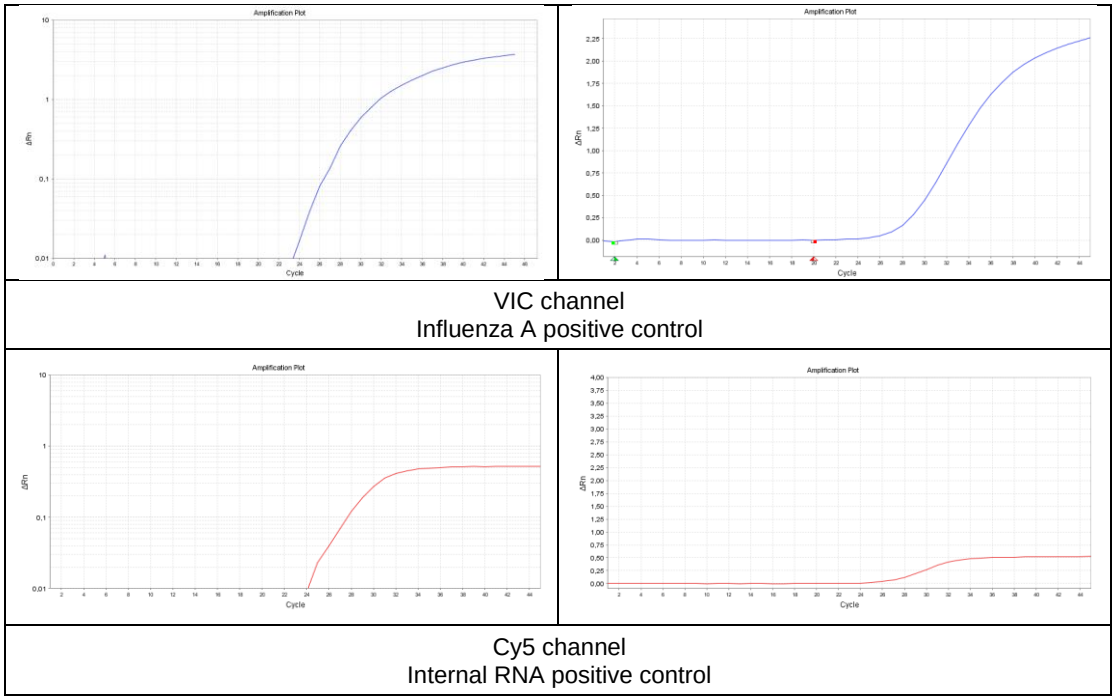


Figure 1 Performance of ViroReal® Kit SARS Coronavirus & Influenza A/B

ViroReal® Kit SARS Coronavirus & Influenza A/B was validated with the ABI® 7500 Fast Real-time PCR System (Thermo Fisher Scientific), Mx3005P® (Agilent), LightCycler® 480 I (Roche Diagnostics) and MIC instrument (bio molecular systems).

13.2 Limit of detection, LoD95%

Method: ViroReal® Kit SARS Coronavirus & Influenza A/B was tested with SARS-CoV-2 RNA (AMPLIRUN® Coronavirus SARS-CoV-2 RNA control) and with synthetic RNA representing a fragment the influenza A virus or influenza B virus, respectively.
The LoD95% (LoD95%: number of copies, which are positively detected in 95% of cases) was determined by testing different RNA concentrations around the detection limit in 20 replicates. It was then calculated with a non-linear (logistic) curve fit using Graph Pad Prism Software.
Furthermore, the simultaneous detection of low amounts of SARS-CoV-2 RNA (E+3, E+2 and E+1 copies/PCR reaction) in the presence of high amounts of influenza A or influenza B virus RNA (E+6 copies/PCR reaction) was tested.

Result: At least three target copies/reaction of SARS-CoV-2 and at least 10 copies of influenza A virus or influenza B virus could be detected. The LoD95% is 12 copies/reaction for SARS-CoV-2, 15 copies/reaction for the influenza A and 28 copies/reaction for the influenza B.
Results showed that multiplex detection does not affect sensitivity (data not shown).

13.3 Linearity and dynamic measuring range

Method: Linearity of ViroReal® Kit SARS Coronavirus & Influenza A/B was determined by a 10-fold dilution series of synthetic RNAs of SARS-CoV-2, of influenza A virus and influenza B virus (10^6 – 10^1 target copies/reaction).

Result: For SARS-CoV-2, the assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.54 and an R^2 of 0.96 as shown in Figure 2.
For influenza A, the assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.40 and a R^2 of 0.99 as shown in Figure 3.
For influenza B, the assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.34 and a R^2 of 0.96 as shown in Figure 4.

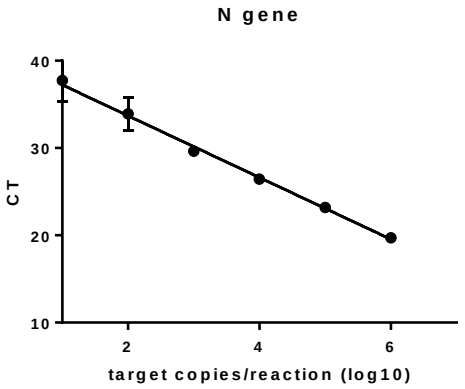


Figure 2 Ten-fold dilution series of SARS-CoV-2 RNA standard plotted against Cq, FAM channel

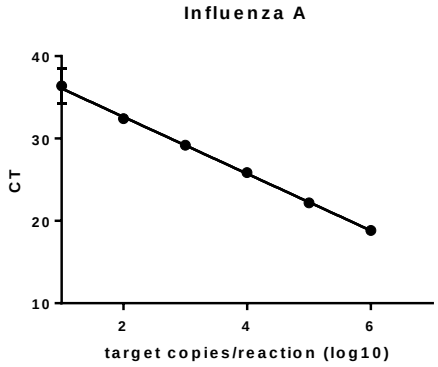


Figure 3 Ten-fold dilution series of influenza A RNA standard plotted against Cq, VIC channel

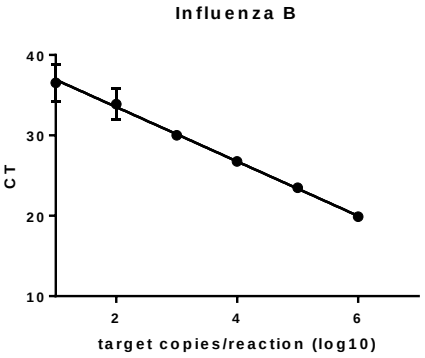


Figure 4 Ten-fold dilution series of influenza B RNA standard plotted against Cq, VIC channel

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 – 10^2 target copies/reaction) of RNA.

Result:
For SARS-CoV-2 the mean values of the coefficients of variation (CV%) are 0.95% for intra-assay precision, 2.7% for inter-assay precision and 3.25% for inter-lot precision.
For influenza A the mean values of the coefficients of variation (CV%) are 0.38% for intra-assay precision, 0.64% for inter-assay precision and 0.77% for inter-lot precision.
For influenza B the mean values of the coefficients of variation (CV%) are 1.24% for intra-assay precision, 2.81% for inter-assay precision and 2.83% for inter-lot precision.

13.5 Analytical specificity

ViroReal® Kit SARS Coronavirus & Influenza A/B detects the nucleocapsid protein gene (N gene) of SARS-CoV-2, of SARS-CoV and SARS-related coronaviruses (sarbecoviruses), as well as the matrix protein gene of Influenza A virus and the hemagglutinin protein gene of Influenza B virus. Other beta coronaviruses or influenza viruses are not detected with this test.

Method BLAST analysis: Specificity is ensured by the selection of highly specific primers and probes. In silico validation of primers and probes was performed using the Basic Local Alignment Search Tool (BLAST) in the NCBI database and sequence analyses in the GISAID database. It was checked for potential

homologies to currently published sequences. Analytical specificity is ensured by selecting highly specific primers and probes.
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 the target region. The primers and probe chosen are intended to cover possible future mutations of the viral sequence and are not identical to the primers and probes published by WHO. All data in the NCBI database showed homology of the sequence region of primers and probe in all SARS coronavirus clusters. This approach allows universal detection of all SARS coronavirus strains known to date, including SARS-CoV-2.
For the matrix protein gene of influenza A virus and for the hemagglutinin protein gene of influenza B virus, a highly conserved region in known influenza A and B virus strains was chosen as the target region. It is not possible to distinguish between influenza A virus and influenza B virus. Due to sequence heterogeneity within the target region, some subtypes of influenza A virus may not be detected or may be detected with lower sensitivity.

Method testing inclusivity: A total of 21 ring trial samples containing three negative samples, six influenza A virus positive samples, eight influenza B virus positive samples and three SARS-CoV-2 positive samples from INSTAND e.V. were retrospectively analysed.

Result: All samples were correctly analysed.

Method testing exclusivity: Furthermore, specificity was determined by testing against respiratory viruses. A total of 13 ring trial samples from INSTAND e.V. were retrospectively analysed (Enterovirus, Adenovirus, RSV, MPV, Rhinovirus, MERS, HCoV 229E, HCoV NL63, HCoV NL63, HCoV OC43).

Result: All samples were correctly analysed as negative.

13.6 Diagnostic evaluation

Method: ViroReal® Kit SARS Coronavirus Influenza A/B was tested by an external service provider (AGES, Austrian Agency for Health and Food Safety, Vienna) on 94 swab samples from the upper respiratory tract of patients with signs and symptoms of COVID-19. The test panel included 47 samples tested positive (range from highly positive to weak positive with Cq values in the range from 17-41) and 47 samples tested negative for SARS-CoV-2 with the RealTime SARS-CoV-2 assay (Abbott) using the Alinity m or the m2000 real-time PCR instrument. The Abbott RealTime SARS-CoV-2 assay detects the N and RdRp gene of SARS-CoV-2 in FAM channel and is one of the most sensitive tests on the market with a sensitivity of 100 copies/µl sputum. ViroReal® Kit SARS Coronavirus & Influenza A/B has a sensitivity of approx. 893 copies/µl sputum (when 200 µl sputum are extracted).
For testing with ViroReal® Kit SARS Coronavirus Influenza A/B, 200 µl samples were extracted with the KingFisher instrument and were eluted in 60 µl. Analyses were performed in single reactions on the LightCycler® 480 I instrument (Roche) with 5 µl RNA sample volume.

Result: Of the 47 samples positive with the reference method, 43 samples were positive with ViroReal® Kit SARS Coronavirus Influenza A/B also. Four samples, which were questionably and weak positive with the reference method (Cq>38), were negative with ViroReal® Kit SARS Coronavirus Influenza A/B. The remaining 47 samples were negative by both methods (Tables 5, 6).

Table 5 Summary of data

	Reference			
		pos	**neg**	**Total**
ViroReal® Kit SARS Coronavirus & Influenza A/B	**pos**	**43**	**0**	**43**
	neg	**4[1]**	**47**	**51**
	Total	**47**	**47**	**94**

[1] Questionably and weak positive with reference method

Table 6 Results of clinical validation compared with Abbott RealTime SARS-CoV-2 assay

	Value
Sensitivity	91.5 %
Specificity	100 %
NPV	92.2 %
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. 9. Samples with positive Cq-values are considered positive (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)).
1.2	01 May 2023	**Change of the company address** **5. Contents, stability and storage** Kit version 1.1: Changed filling volume of DNA IPC Target and Positive Control in DHUV02513 Changed filling volume of Positive Control in DHUV02513x5 Positive control contains both SARS-CoV-2 and influenza A RNA The RNA IPC-Target is not stored in RNA/DNA Stabilizer anymore and was renamed to IPC-Target (RNA). SARS-CoV-2&SARS+RNAIPC Assay Mix was renamed to SARS-CoV-2 & SARS + IPC3 Assay Mix **7. Precautions and safety instructions** Update to this section. Inclusion of additional information on sample collection and storage, post-collection sample processing, sample storage conditions, transport and storage conditions of purified RNA. Added recommended measures to avoid RNA contamination. **9. Sample preparation** Update of paragraph on quality control for DNA extraction and PCR inhibition. Note that the IPC target (RNA) must be diluted for Cq values <27 prior to extraction. **10. Preparation of real-time PCR** Dilution of IPC target (RNA) was changed from 1:500 to 1:100. **11. Interpretation of PCR data** Table 1 and Table 2: Update **13. Specification and evaluation of the test performance** Update.

Note:
Any serious incident that has occurred in relation to the device 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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