ViroReal® Assay Influenza A/B

Only for use in combination with ViroReal® Kit SARS-CoV-2 & SARS

Instructions for Use

CE

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

REF **RTGM001RV** Σ **100**

REF **RTGM002RV** 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® Assay Influenza A/B is a non-automated IVD test, based on one-step reverse transcription real-time PCR (RT-PCR), for the detection of RNA of the matrix protein gene of influenza A virus and of the hemagglutinin gene of influenza B virus.

This test is only intended for use in combination with ViroReal® Kit SARS-CoV-2 & SARS, which detects the N gene of SARS-CoV-2, SARS-CoV and SARS-related coronavirus (Sarbecovirus).

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® Assay Influenza A/B detects RNA of the matrix protein gene of influenza A virus and of the hemagglutinin gene of influenza B virus. These two genes are detected in the fluorescence channel for VIC (554 nm). A distinction between influenza A virus and influenza B virus is not possible.

ViroReal® Kit SARS-CoV-2 & SARS detects the N gene of SARS-CoV-2, SARS-CoV and SARS-related coronavirus (Sarbecovirus). The N gene is detected in the fluorescence channel for FAM (530 nm). The primer and probe design chosen is not identical with the WHO design. ViroReal® Kit SARS-CoV-2 & SARS is intended to cover possible future mutations in the virus sequence, therefore a highly conserved region in all SARS coronavirus clusters of the N gene was chosen as target region. This approach allows universal detection of all so far known SARS-CoV strains including SARS-CoV-2 and SARS-like CoV without discriminating between strains.

An internal RNA positive control (RNA IPC) is detected in Cy5 channel and is used as RNA extraction as well as RT-PCR inhibition control (component of ViroReal® Kit SARS-CoV-2 & SARS). The target for the RNA IPC is added to the sample during extraction.

This test is compatible with real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC and Cy5 channel (e.g. ABI® 7500 instrument (Thermo Fisher Scientific), QuantStudio™ 5, QuantStudio™ 7 (Thermo Fisher Scientific), Mx3005P® (Agilent), qTOWER^{3}G (Analytik Jena), MIC instrument (bio molecular systems), LightCycler® 480 II (Roche), cobas z 480 Analyzer (Roche)).

When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR is recommended. 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 have been optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in the same run.

3 Pathogen information

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.

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 is a beta coronavirus that emerged in Wuhan, China in December 2019. The virus is responsible for the disease COVID-19 (corona virus disease 2019). Fever, cough and breathing difficulties are described as the most frequent initial symptoms, later on it can 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 Contents of ViroReal® Assay Influenza A/B order no. RTGM001RV and RTGM002RV

Component	Content	RTGM001RV	RTGM002RV	Storage
		Quantity 100 reactions	Quantity 500 reactions	
Influenza A/B Assay Mix (purple cap)	Primers and probes for influenza A virus and influenza B virus detection (VIC)	1 x 100 µl	1 x 500 µl	-25 to -15 °C
Influenza A Positive Control (red cap)	RNA Positive Control for influenza A virus (10^3 copies/µl)	1 x 300 µl	1 x 500 µl	-25 to -15 °C

Table 2 Contents of ViroReal® Kit SARS-CoV-2 & SARS order no. DHUV02313

		DHUV02313	
Component	**Content**	**Amount 100 reactions**	**Storage**
SARS-CoV2&SARS + IPC3 Assay Mix (green cap)	Primers and probes for detection of - Sarbecovirus incl. SARS-CoV-2 (FAM) - RNA IPC (Cy5)	1 x 100 µl	-25 to -15 °C
IPC-Target (RNA) (orange cap)	Target for RNA IPC (internal RNA positive control system)	1 x 200 µl	-25 to -15 °C
SARS-CoV-2 Positive Control (red cap)	RNA Positive Control (1000 copies/µl)	1 x 300 µl	-25 to -15 °C
RNA Reaction Mix (white cap)	PCR reaction mix for RNA amplification	1 x 500 µl	-25 to -15 °C
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-25 to -15 °C

Table 3 Order no. DHUV02313x5 and DHUV02313x50

		DHUV02313x5	DHUV02313x50	
Component	**Content**	**Amount 500 reactions**	**Amount 5000 reactions**	**Storage**
SARS-CoV2&SARS + IPC3 Assay Mix (green cap)	Primers and probes for detection of - Sarbecovirus incl. SARS-CoV-2 (FAM) - RNA IPC (Cy5)	1 x 500 µl	1 x 5 ml	-25 to -15 °C
IPC-Target (RNA) (orange cap)	Target for RNA IPC (internal RNA positive control system)	1 x 500 µl	1 x 5 ml	-25 to -15 °C
SARS-CoV-2 Positive Control (red cap)	RNA Positive Control (1000 copies/µl)	1 x 500 µl	1 x 1 ml	-25 to -15 °C
RNA Reaction Mix (white cap)	PCR reaction mix for RNA amplification	5 x 500 µl	1 x 25 ml	-25 to -15 °C
Nuclease-free water (blue cap)	Nuclease-free water	3 x 1000 µl	1 x 25 ml	-25 to -15 °C

RNA Reaction Mix: The Master Mix provided with ViroReal® Kit SARS-CoV-2 & SARS 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

- ViroReal® Kit SARS-CoV-2 & SARS (order no. DHUV02313, DHUV02313x5 or DHUV02313x50)
- 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

- Optimal performance of this test requires 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.
- For this test highly specific primers and probes have been selected. However, false-negative or less sensitive results might be obtained due to sequence heterogeneity within the target region of not yet described clinical subtypes.
- Results should be interpreted in context of clinical and laboratory findings.

9 Preparation of samples

ViroReal® Assay Influenza A/B in combination with ViroReal® Kit SARS-CoV-2&SARS 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® Assay Influenza A/B and ViroReal® Kit SARS-CoV-2&SARS. 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)

<u>For automated extraction recommended</u>

- Mag-Bind® Viral RNA XPress KIT (Omega Bio-tek)

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) provided with ViroReal® Kit SARS-CoV-2&SARS 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, 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.
- **Positive Control**
  SARS-CoV-2 Positive Control (provided with ViroReal® Kit SARS-CoV-2 & SARS) is an *in vitro* synthesized RNA fragment with a concentration of 10^3 copies/µl.
  Influenza A Positive Control is an *in vitro* synthesized RNA fragment with a concentration of 10^3 copies/µl.
  Both positive controls have to be stored at -20°C. Ensure a homogenous solution by gently mixing, do not vortex. To avoid freeze/thaw cycles, it can also be temporarily stored at 4°C if used several times on the same day.
  → As positive control, use 5 µl of the SARS-CoV-2 Positive Control and 5 µl of the Influenza A Positive Control together in one plate well.

10.1 Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water* (ViroReal® Kit SARS-CoV-2 & SARS)	3.0 µl
	RNA Reaction Mix (ViroReal® Kit SARS-CoV-2 & SARS)	5.0 µl
	SARS-CoV-2 & SARS + RNA IPC-3 Assay Mix (ViroReal® Kit SARS-CoV-2 & SARS)	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 has to 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 more than 1 µl diluted IPC-Target (RNA) per reaction 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% 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 4 shows the criteria for valid positive and negative controls. Table 5 shows interpretation of data with clinical samples.

11.1 Controls

Table 4 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 5.

Table 5 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

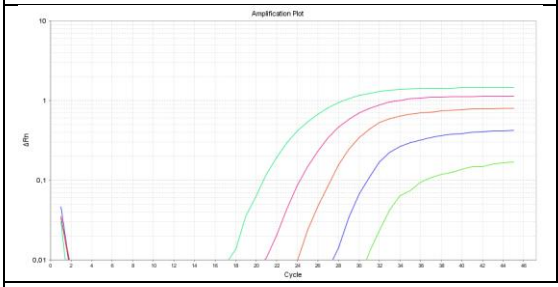
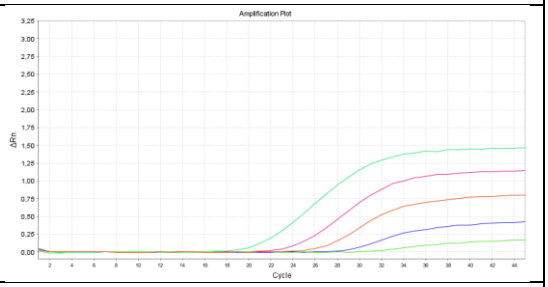
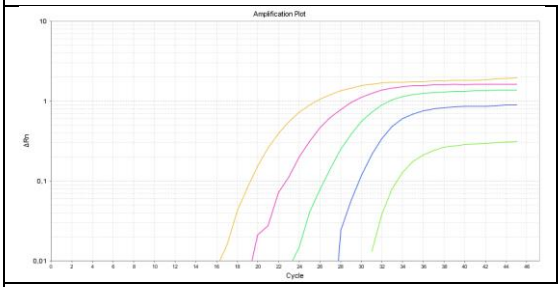
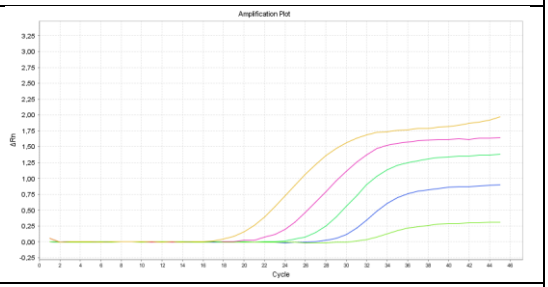
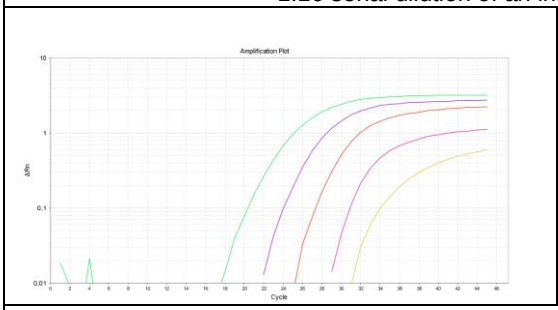
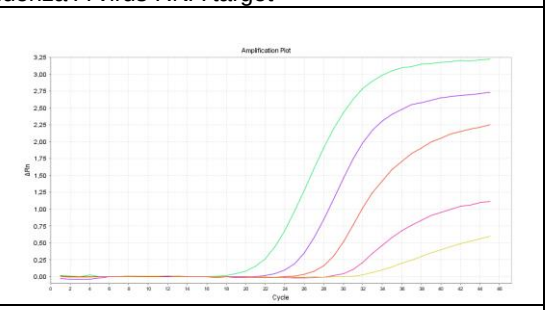
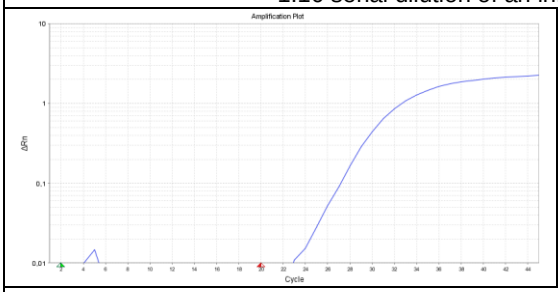
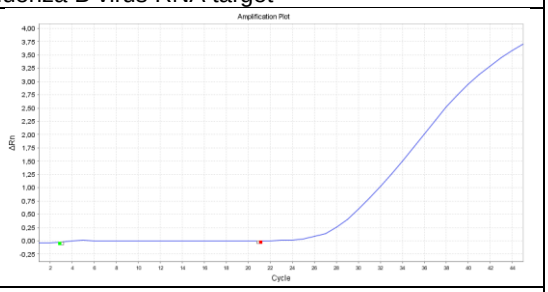
- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- Incorrect RT-PCR conditions.
  → Check the RT-PCR conditions and repeat the RT-PCR, if necessary.
- The RNA might be degraded.
- If IPC-Target (RNA) was added during extraction:
  - 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® Assay Influenza A/B in combination with ViroReal® Kit SARS-CoV-2 & SARS with an ABI® 7500 instrument 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	

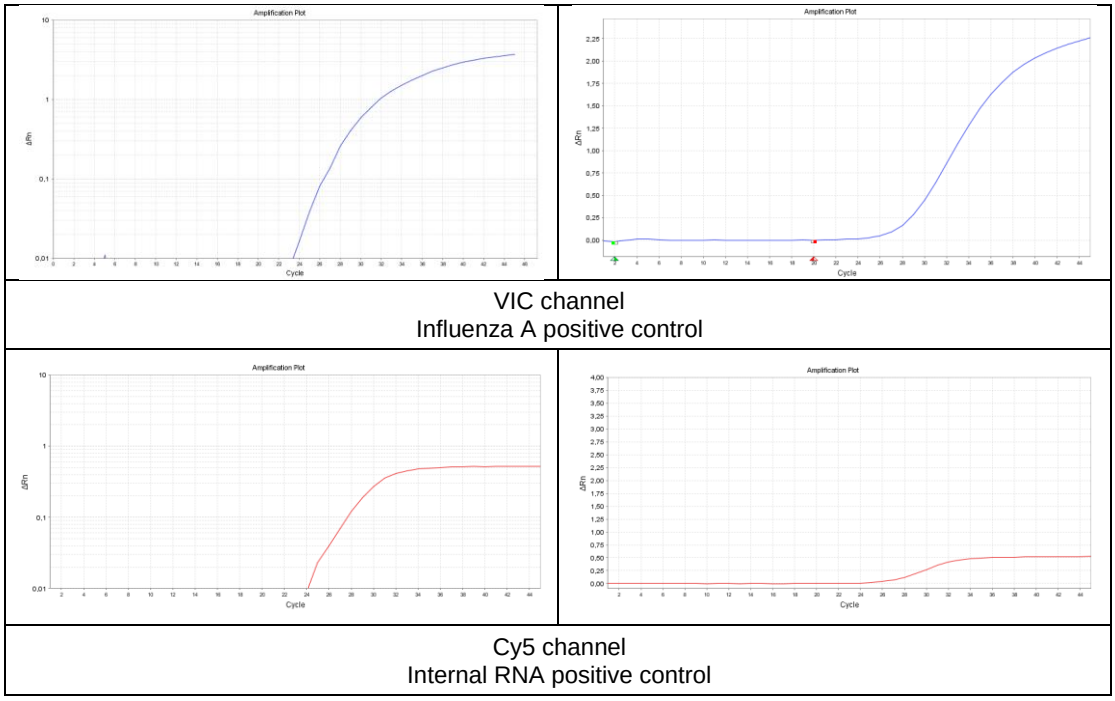


Figure 1 Test performance

ViroReal® Assay Influenza A/B was validated in combination with ViroReal® Kit SARS-CoV-2 & SARS with the ABI® 7500 instrument (Thermo Fisher Scientific), Mx3005P® (Agilent), LightCycler® 480 I (Roche Diagnostics) and MIC instrument (bio molecular systems).
To evaluate the performance with different real-time PCR instrument systems, testing was performed using different concentrations of AMPLIRUN® Coronavirus SARS-CoV-2 RNA control (100, 50, 25, 12.5, 6 and 3 copies/PCR reaction) and different concentrations of synthetic influenza A and B virus RNA, respectively (200, 100, 50, 25, 15 copies/PCR reaction). Testing showed that the analytical sensitivity of the assay was comparable, irrespective of the amplification platforms used, see Table 6.

Table 6 Testing of different real-time PCR instruments

	Average Cq values			
	ABI® 7500	MIC	Mx3005P®	LightCycler® 480 I
100 copies SARS-CoV-2 RNA	31.37	32.56	32.06	31.62
50 copies SARS-CoV-2 RNA	32.70	33.26	32.90	33.44
25 copies SARS-CoV-2 RNA	33.72	35.51	34.02	35.15
12.5 copies SARS-CoV-2 RNA	35.28	36.93	36.69	39.36
6 copies SARS-CoV-2 RNA	35.33	41.51	39.00	38.73
3 copies SARS-CoV-2 RNA	36.97	37.70	38.69	Negative
200 copies Influenza A RNA	31.97	33.21	31.79	32.99
100 copies Influenza A RNA	32.96	34.58	33.00	34.21
50 copies Influenza A RNA	34.40	35.94	34.54	36.18
25 copies Influenza A RNA	35.08	38.73	34.98	37.4
15 copies Influenza A RNA	34.55	36.36	35.09	37.47
200 copies Influenza B RNA	32.99	32.84	32.31	33.89
100 copies Influenza B RNA	34.18	33.91	33.10	35.10
50 copies Influenza B RNA	34.77	35.37	34.38	38.82
25 copies Influenza B RNA	36.44	Negative	35.74	40.98
15 copies Influenza B RNA	35.28	36.05	34.34	37.60

13.2 Limit of detection, LoD95%

ViroReal® Assay Influenza A/B was tested in combination with ViroReal® Kit SARS-CoV-2 & SARS with the ABI® 7500 instrument with 10-fold dilution series of a synthetic RNA representing a fragment of SARS-CoV-2, the influenza A virus or influenza B virus, respectively. At least 3 target copy/reaction of SARS-CoV-2, 10 target copy/reaction of influenza A and 10 target copy/reaction of influenza B could be detected.

The LoD95% (LoD95: number of copies, which are positively detected in 95% of cases) was determined by testing different RNA concentrations of SARS-CoV-2 (AMPLIRUN® Coronavirus SARS-CoV-2 RNA control), of influenza A virus and influenza B virus (synthetic RNA fragments) in 20 replicates. It was then calculated with a non-linear (logistic) curve fit using Graph Pad Prism Software. The LoD95% 12 copies/reaction for SARS-CoV-2, 15 copies/reaction for the influenza A and 28 copies/reaction for the influenza B.
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, results showed that multiplex detection does not affect sensitivity (data not shown).

13.3 Linearity

Linearity of ViroReal® Assay Influenza A/B was determined in combination with ViroReal® Kit SARS-CoV-2 & SARS by a 10-fold dilution series of synthetic RNAs of SARS-CoV-2, of influenza A virus and influenza B virus (1.00E+06 to 1.00E+00 target copies/reaction, diluted in nuclease-free water). Mean values were determined. Arithmetic mean ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV %) were calculated.

For SARS-CoV-2, the combination of tests shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.54 and a R^2 of 0.96 as shown in Figure 2.

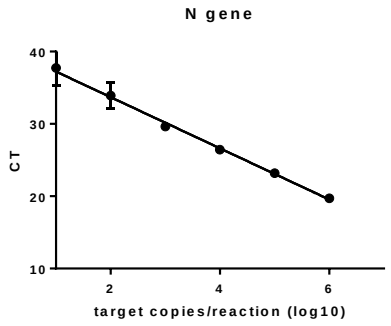


Figure 2 Ten-fold dilution series of SARS-CoV-2 RNA standard plotted against CT, FAM channel

For influenza A, the combination of tests 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.

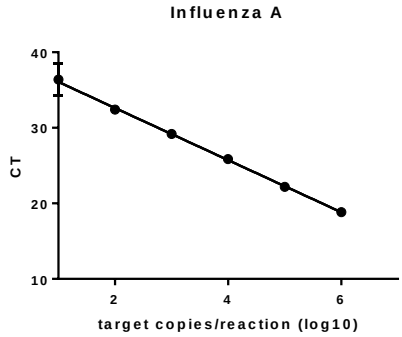


Figure 3 Ten-fold dilution series of influenza A RNA standard plotted against CT, VIC channel

For influenza B, the combination of tests 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.

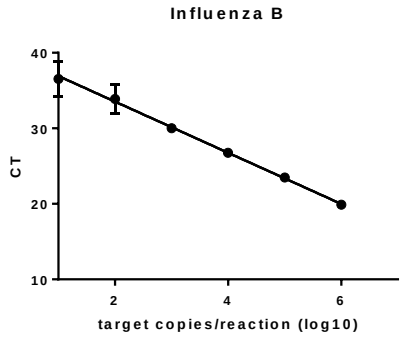


Figure 4 Ten-fold dilution series of influenza B RNA standard plotted against CT, VIC channel

13.4 Precision inter-assay

Inter-assay precision is defined as the reproducibility of a sample between assay runs. The inter-assay precision of ViroReal® Assay Influenza A/B was determined in combination with ViroReal® Kit SARS-CoV-2 & SARS from 10-fold dilutions of the synthetic RNAs (1.00E+06 to 1.00E+02 target copies/reaction) in three independent experiments performed on different days in quadruplicates. The arithmetic mean ($\bar{x}$), the standard deviation (σ) and the coefficient of variation (CV %) between the replicate runs were calculated.
For SARS-CoV-2, precision standard replicate CV's ranged from 1.40% to 5.43%, with mean overall inter-assay precision equal to 2.70%.
For influenza A, precision standard replicate CV's ranged from 0.22% to 1.29%, with mean overall inter-assay precision equal to 0.64%.
For influenza B, precision standard replicate CV's ranged from 0.98% to 7.27%, with mean overall inter-assay precision equal to 2.81%.

13.5 Precision intra-assay

Intra-assay precision is defined as the reproducibility of a sample within an assay run. Intra-assay precision of ViroReal® Assay Influenza A/B was determined in combination with ViroReal® Kit SARS-CoV-2 & SARS from the replicate runs above. The arithmetic mean ($\bar{x}$), the standard deviation (σ) and the coefficient of variation (CV %) of the replicates were calculated.
For SARS-CoV-2, precision standard replicate CV's from replicate 1 ranged from 0.20% to 3.98%, with mean overall intra- assay precision equal to 0.95%.
For influenza A, precision standard replicate CV's from replicate 1 ranged from 0.19% to 0.80%, with mean overall intra- assay precision equal to 0.38%.
For influenza B, precision standard replicate CV's from replicate 1 ranged from 0.20% to 5.00%, with mean overall intra- assay precision equal to 1.24%.

13.6 Analytical specificity

Specificity is ensured by the selection of highly specific primers and probes. ViroReal® Assay Influenza A/B detects in combination with ViroReal® Kit SARS-CoV-2 & SARS the N gene of SARS-CoV-2 as well as SARS-CoV and SARS-related bat coronavirus (universal detection of Sarbecovirus), the N gene of other coronaviruses are not detected. A highly conserved region in all SARS coronavirus clusters of the N gene was chosen as target region. The selected primer and probes should cover possible future mutations in the virus sequence and are therefore not identical with the primer and probes suggested by the WHO. *In silico* validation of primers and probes was carried out with the basic local alignment tool (BLAST) against the NCBI database and with comparative sequence analyses in the GISAID database. All the data in the two databases showed homology in the sequence region of primer and probes throughout all SARS coronavirus clusters. This approach allows specific and universal detection of all so far known SARS coronavirus strains including SARS-CoV-2 without discriminating between strains.

For the matrix protein gene of the influenza A virus and for the hemagglutinin protein gene of the influenza B virus a highly conserved region in known influenza A and B virus strains was chosen as target region. *In silico* validation of primers and probes was carried out with the basic local alignment tool (BLAST) against the NCBI database. Influenza A virus and influenza B virus cannot be differentiated with this test. False-negative or less sensitive results might be obtained due to sequence heterogeneity within the target region of some subtypes.

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 analysed, retrospectively. All samples were correctly analyzed by ViroReal® Assay Influenza A/B in combination with ViroReal® Kit SARS-CoV-2 & SARS.

Furthermore, specificity was determined by testing against respiratory viruses. A total of 13 ring trial samples from INSTAND e.V. were analysed, retrospectively (Enterovirus, Adenovirus, RSV, MPV, Rhinovirus, MERS, HCoV 229E, HCoV NL63, HCoV NL63, HCoV OC43). All samples were correctly analyzed negative by ViroReal® Assay Influenza A/B in combination with ViroReal® Kit SARS-CoV-2 & SARS.

13.7 Diagnostic Evaluation

ViroReal® Assay Influenza A/B was tested in combination with ViroReal® Kit SARS-CoV-2 & SARS 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 weakly 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-CoV-2 & SARS has a sensitivity of approx. 893 copies/µl sputum (when 200 µl sputum are extracted).

For testing, 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 for SARS-CoV-2 with the reference method, 43 samples were positive with ViroReal® Assay Influenza A/B + ViroReal® Kit SARS-CoV-2 & SARS. Four samples, which were questionably and weak positive with the reference method (Cq>38), were negative with ViroReal® Assay Influenza A/B + ViroReal® Kit SARS-CoV-2 & SARS. The remaining 47 samples were negative by both methods (Table 7, Table 8).

Table 7 Summary of data

		Reference		Total
		pos	neg	
ViroReal® Assay Influenza A/B + ViroReal® Kit SARS-CoV-2 & SARS	**pos**	**43**	**0**	**43**
	neg	**4[1]**	**47**	**51**
	Total	**47**	**47**	**94**

[1] Questionably and weak positive with reference method

Table 8 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	09 May 2023	**Change of the company address** **5. Contents, stability and storage** Changed filling volume of Positive Control The RNA IPC-Target provided with ViroReal® Kit SARS-CoV-2 & SARS is not stored in RNA/DNA Stabilizer anymore and was renamed to IPC-Target (RNA). **7. Precautions and safety instructions** General 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** General update 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 4 and Table 5: 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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