ViroReal® Kit
SARS Coronavirus & Influenza A/B

Manual

CE

IVD For *in vitro* diagnostic use

REF	DHUV02513	Σ	100
REF	DHUV02513x5	Σ	500

ingenetix GmbH
Arsenalstr. 11
1030 Vienna, Austria
T +43 (0)1 36 198 0 198
F +43 (0)1 36 198 0 199
office@ingenetix.com
www.ingenetix.com

Distributed by

Index

DRAFT

1. Intended use ... 3
2. Product description ... 3
3. Pathogen information ... 3
4. Contents of the Kit, Stability and Storage ... 4
5. Additionally required materials and devices ... 4
6. Precautions and safety information ... 5
7. Limitations ... 5
8. Preparation of samples and real-time PCR ... 6
8.1. Internal RNA Positive Control (RNA IPC) ... 6
8.2. Positive Control ... 6
8.3. Pipetting scheme ... 6
8.4. Programming of the temperature profile ... 7
9. Interpretation of PCR-data ... 8
10. Troubleshooting ... 9
10.1. No virus specific signal with positive control and with IPC ... 9
10.2. Virus specific signal with negative control ... 9
10.3. Virus specific signal with negative control of extraction ... 9
10.4. IPC specific signal with negative control and positive control ... 10
10.5. No signal with IPC and no virus specific signal with sample ... 10
11. Specifications and performance evaluation ... 11
11.1. Kit performance ... 11
11.2. Limit of detection and linearity ... 13
11.2.1. LoD ... 13
11.2.2. Linearity ... 13
11.3. Precision inter-assay ... 14
11.4. Precision intra-assay ... 14
11.5. Analytical specificity ... 14
11.6. Diagnostic evaluation ... 14
12. References ... 16
13. Revision history ... 16

DRAFT

Explanation of symbols

	Batch code		Use by
	Catalogue number		Manufacturer
	Contains sufficient for <n> tests		Store at
	This product fulfills the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices		For *in vitro* diagnostic use
	Corrosion, GHS05		Exclamation mark, GHS07

1. Intended use

ViroReal® Kit SARS Coronavirus & Influenza A/B is an *in vitro* diagnostic test, based on one-step reverse transcription real-time PCR, for the detection 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 of patients with or without a suspected SARS-CoV or influenza virus infection. Proper specimens are samples from the upper and lower respiratory tract (throat rinsing fluid, nasopharyngeal and oropharyngeal swabs, anterior nasal swab and mid-turbinate nasal swab specimens, nasopharyngeal wash/aspirate and nasal aspirates, sputa and BAL). Testing of self-collected or healthcare provider-collected anterior and mid-turbinate nasal swabs is limited to patients with symptoms of COVID-19 or influenza.

2. Product description

ViroReal® Kit SARS Coronavirus & Influenza A/B 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.

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 changes 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 (530 nm).

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 (554 nm). A distinction between influenza A virus and influenza B virus is not possible.

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. 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. ViroReal® Kit SARS Coronavirus & Influenza A/B was validated with the Applied Biosystems® (ABI) 7500 instrument (Thermo Fisher Scientific), Mx3005P® (Agilent), MIC instrument (bio molecular systems) and LightCycler® 480 I (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.

The test is based on one-step reverse transcription real-time PCR (RT-PCR). A specific RNA sequence of the pathogen genome is transcribed into cDNA and amplified in a one-step PCR. The generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows a sequence-specific detection of PCR amplificates

Ingenetix ViroReal®, BactoReal®, MycoReal, PanReal, ParoReal and SeptiReal 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

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

DRAFT

as the most frequent initial symptoms, later on it can lead to pneumonia. The coronavirus spreads mainly by droplet and contact transmission. In most cases a mild course of infection is observed, while more severe courses are observed in about 15%-20%, with a mortality rate of up to 3%.

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. Contents of the Kit, Stability and Storage

		DHUV02513	DHUV02513x5	
Component	**Content**	**Quantity 100 reactions**	**Quantity 500 reactions**	**Storage**
SARS-CoV-2 & SARS+ RNA IPC-3 Assay Mix (green cap)	Primers and probes for SARS-CoV-2&SARS coronavirus detection (FAM) and RNA IPC detection (Cy5)	1 x 100 µl	1 x 500 µl	-15°C to -25°C
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	-15°C to -25°C
RNA IPC Target (orange cap)	Target for RNA IPC	1 x 100 µl	1 x 500 µl	-15°C to -25°C
SARS-CoV-2 Positive Control (red cap)	RNA Positive Control for SARS-CoV-2 (10^3 copies/µl)	1 x 120 µl	1 x 300 µl	-15°C to -25°C
Influenza A Positive Control (red cap)	RNA Positive Control for influenza A virus (10^3 copies/µl)	1 x 120 µl	1 x 300 µl	-15°C to -25°C
RNA Reaction Mix (white cap)	RNA Reaction Mix	1 x 500 µl	5 x 500 µl	-15°C to -25°C
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	3 x 1000 µl	-15°C to -25°C

The components of ViroReal® Kit SARS Coronavirus & Influenza A/B are stable until the expiry date stated on the label. Repeated freeze/thaw cycles should be avoided. Protect kit components from light.

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 Reaction 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.

5. Additionally required materials and devices

- Reagents and devices for RNA-extraction
- Nuclease-free water
- Disposable powder-free gloves
- Sterile filter pipette tips
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM, VIC and Cy5 channel
- Appropriate optical 96-well reaction plates or reaction tubes with optical closing material recommended by the manufacturer of the real-time PCR instrument

DRAFT

Real-time PCR instruments validated with ViroReal® Kit SARS Coronavirus & Influenza A/B:

- Applied Biosystems® (ABI) 7500 instrument (Thermo Fisher Scientific)
- Mx3005P® (Agilent)
- MIC instrument (bio molecular systems)
- LightCycler® 480 I (Roche)

6. Precautions and safety information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostics.
- The real-time PCR instrument should be serviced and cleaned regularly.
- Clean benches and devices periodically.
- Use sterile filter pipette tips.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective disposable powder-free gloves when handling kit reagents and specimens.
- Use separated workspaces for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate workspaces and ensure workflow in the laboratory from pre- to post-PCR.
- Be careful when handling samples and positive control to avoid cross contamination. Change gloves after handling of samples or positive control. Store positive or potentially positive material separately from reagents.
- Prevent contamination of work equipment and reagents with DNA/RNA, nuclease or amplification products by good laboratory practice.
- Quality of RNA has a profound impact on the test performance. Ensure that the used RNA extraction system is compatible with reverse transcription real-time PCR technology.
- For a valid interpretation of results, a negative control must be included during RNA-extraction (e.g. extraction of water instead of sample material) and tested per PCR-run, in order to exclude false-positive results due to contamination with virus RNA during extraction.
- Optional, also include a negative control per PCR-run (nuclease-free water instead of sample).
- Please note the expiry date of the kit.
- Repeated thawing and freezing of kit components should be avoided. Protect kit components from light. **Caution:** RNA IPC Target is stored in RNA stabilizer which contains Guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.

7. 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 from the respiratory tract (see 11.6). Test performance with other specimen types has not yet been assessed.
- Regarding COVID-19, testing of self-collected or healthcare provider-collected anterior and mid-turbinate nasal swabs is limited to patients with symptoms. Please refer to FDA's FAQs on Diagnostic Testing for SARS-CoV-2 for additional information, for example.
- 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 kit 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.

DRAFT

8. Preparation of samples and real-time PCR

Swabs can be collected with swab material proper for PCR (e.g. sterile polyester or rayon swabs with aluminium or plastic shaft, not provided) and put into 1 ml isotonic saline solution (NaCl 0.9%, not provided). Do not freeze samples prior to extraction. Extract RNA from 140-200 µl sample (depending on the extraction method) and elute in 50 µl.
Extract samples with an RNA extraction system compatible with reverse transcription real-time PCR technology. A negative control of the RNA extraction must always be included (e.g. extraction of water instead of sample material).
Make sure that at least one extraction negative control, as well as one positive control (red cap) and optional one negative control (water) are included per PCR run.

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.
Best use RNA immediately after extraction and keep it on ice. Alternatively, use RNA stored at -20°C to -80°C and avoid prolonged exposure to room temperature, thaw on ice and immediately refreeze the RNA.
If the extracted RNA is stored at -80°C, it should remain stable for 3-6 months, at -20°C for 3-6 weeks, at 4°C for 3-5 hours and at room temperature for 0.5-1 minute.

8.1. Internal RNA Positive Control (RNA IPC)

An Internal RNA Positive Control system containing the RNA IPC assay and the RNA IPC Target excludes false-negative results due to inhibition of reverse transcription real-time PCR.

RNA IPC Target (approx. 6x10^5 target copies/µl) is stored in RNA stabilizer which contains Guanidinium thiocyanate/Triton X-100. This stabilizer crystallizes by repeated freeze/thaw cycles and can be dissolved again by briefly warming up to approx. 50°C.

→ For control of RNA extraction and reverse transcription real-time PCR (exclusion of PCR inhibition), the RNA IPC Target must be added during extraction. Spike 1 µl of undiluted RNA IPC Target into the sample material after the lysis buffer was added, then continue the extraction procedure. **Caution:** The RNA IPC Target must not be added directly to the sample material.

→ As alternative (not recommended): For the only control of the reverse transcription real-time PCR, 1 µl of freshly 1:500 diluted RNA IPC Target (approx. 1200 target copies) per reaction has to be added to the master mix. **Caution:** The RNA IPC Target must not be added undiluted to the master mix.

8.2. Positive Control

SARS-CoV-2 Positive Control 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.

8.3. 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+ RNA 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	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 has to be adjusted accordingly.

DRAFT

Not recommended - if RNA IPC Target has not already been added during extraction: add 1 µl freshly 1:500 diluted IPC Target per reaction to the Master Mix. In this case, the IPC monitors the reverse transcription real-time PCR only. **Caution:** undiluted RNA IPC Target inhibits the PCR reaction.

For preparation of RT real-time PCR, dispense 10 µl aliquots of prepared Master Mix into the plate wells and then add 10 µl of RNA sample per well. At last, pipet the Positive Control.

8.4. Programming of the temperature profile

Further information on programming the real-time PCR instrument can be found in the respective operator's manual. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

Sample Volume: 20 µl

Temperature Profile:

Program 1	Program 2	Program 3
Cycles: 1 Analysis: None **Reverse Transcription**	Cycles: 1 Analysis: None **Polymerase Activation**	Cycles: 45 Analysis: Quantification Acquisition at 60°
50°C 15 min	95°C 20 sec	95°C 5 sec 60°C 30 sec

For ABI® 7500 instrument
Ramp speed: Without "fast cycling" parameter

Note: This temperature profile can be used for all ViroReal®, BactoReal®, MycoReal, PanReal, ParoReal and SeptiReal kits on all real-time PCR instruments.

Detection channels:

FAM-TAMRA: Universal detection of SARS-CoV-2 or SARS-CoV (Sarbecovirus)
VIC-NONE: Detection of influenza A virus and influenza B virus
Cy5-NONE: Detection of IPC
Passive reference dye, if required: ROX (e.g. for ABI® 7500 instrument)

cobas z 480 Analyzer (Roche):
FAM: Excitation at 465 nm, Emission at 510 nm
VIC: Excitation at 540 nm, Emission at 580 nm
Cy5: Excitation at 610 nm, Emission at 670 nm
Detection format: 3 Color Hydrolysis Probe
Passive reference dye: None

LightCycler® 480 II (Roche):
FAM: Excitation at 465, Emission at 510 nm
VIC: Excitation at 533, Emission at 580 nm
Cy5: Excitation at 618, Emission at 670 nm
After analysis of Cy5 channel, a color compensation for FAM and VIC has to be selected from the Roche database.
Detection format: 3 Color Hydrolysis Probe
Passive reference dye: None

MIC Instrument (bio molecular systems):
FAM: Green, **VIC:** Yellow, **Cy5:** Red
Passive reference dye: None

DRAFT

9. Interpretation of PCR-data

For analysis of PCR results gained with ViroReal® Kit SARS Coronavirus & Influenza A/B, select fluorescence display options FAM channel for SARS Coronavirus target, VIC channel for the influenza A or B virus target and Cy5 channel for the RNA IPC target. Samples with positive Ct/Cp-values are considered positive.

Important: Please, also check amplification curves, not only Ct-values. Samples should be inspected both in logarithmic and linear scale view and compared with the negative control. Adjust the Threshold, if necessary.
Table 1 shows the criteria for valid controls. Table 2 shows interpretation of data with clinical samples.

Table 1: Criteria for valid controls, IPC Target was added during extraction

	Ct FAM channel SARS Coronavirus	Ct VIC channel Influenza A / B	Ct Cy5 channel RNA IPC[2]	Interpretation	Action
Positive control	26-28	26-28	Negative	Valid	-
Positive control	Negative	Negative	Negative	Invalid	See 10.1
Positive control	26-28	Negative	Negative	Invalid	See 10.1
Positive control	Negative	26-28	Negative	Invalid	See 10.1
Positive control	26-28	26-28	Positive	Invalid	See 10.4
Extraction negative control	Negative	Negative	25-28	Valid	-
Extraction negative control	Negative	Negative	Negative	Invalid	See 10.1
Extraction negative control	Positive	Positive	25-28	Invalid	See 10.3
Extraction negative control	Positive	Negative	25-28	Invalid	See 10.3
Extraction negative control	Negative	Positive	25-28	Invalid	See 10.3
Negative control[1]	Negative	Negative	Negative	Valid	-
Negative control[1]	Positive	Positive	Negative	Invalid	See 10.2
Negative control[1]	Negative	Positive	Negative	Invalid	See 10.2
Negative control[1]	Positive	Negative	Negative	Invalid	See 10.2
Negative control[1]	Negative	Negative	Positive	Invalid	See 10.4

[1] Optional
[2] If the RNA IPC Target has been added directly to the Master Mix, all samples must be positive in Cy5 channel

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted.

DRAFT

Table 2: Interpretation of data with clinical samples

	Ct FAM channel SARS Coronavirus	Ct VIC channel Influenza A / B	Ct 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 10.5

[1] A positive signal excludes PCR inhibition. However, IPC Ct-values should show comparable results among samples. A shift of Ct- values can indicate a partial inhibition of PCR.
[2] High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the RNA IPC.
[3] Positive for SARS-CoV-2, SARS-CoV or SARS-related coronavirus (Sarbecovirus)

10. Troubleshooting

10.1. No virus specific signal with positive control and with IPC

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 8. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 8. Preparation of real-time PCR) and repeat the PCR, if necessary.
- The RNA IPC Target was added undiluted directly to the mastermix. The PCR reaction is therefore inhibited.
  → Freshly dilute RNA IPC Target 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 1:500 diluted RNA IPC Target has to be added to the mastermix. If no RNA IPC Target was added to mastermix:
  → Freshly dilute RNA IPC Target and repeat PCR extraction.
- For control of RNA extraction and PCR inhibition, the RNA IPC Target must be added during extraction. If no RNA IPC Target was added to lysis buffer during extraction:
  → Repeat PCR extraction.

10.2. 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 the positive control at last.
  → Make sure that workspace and instruments are decontaminated at regular intervals.

10.3. Virus specific signal with negative control of extraction

- A contamination occurred during extraction.
  → Repeat extraction and RT-PCR using new reagents.
  → Make sure that workspace and instruments are decontaminated at regular intervals.
  → See also 10.2

DRAFT

10.4. IPC specific signal with negative control and positive control

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

10.5. No signal with IPC and no virus specific signal with sample

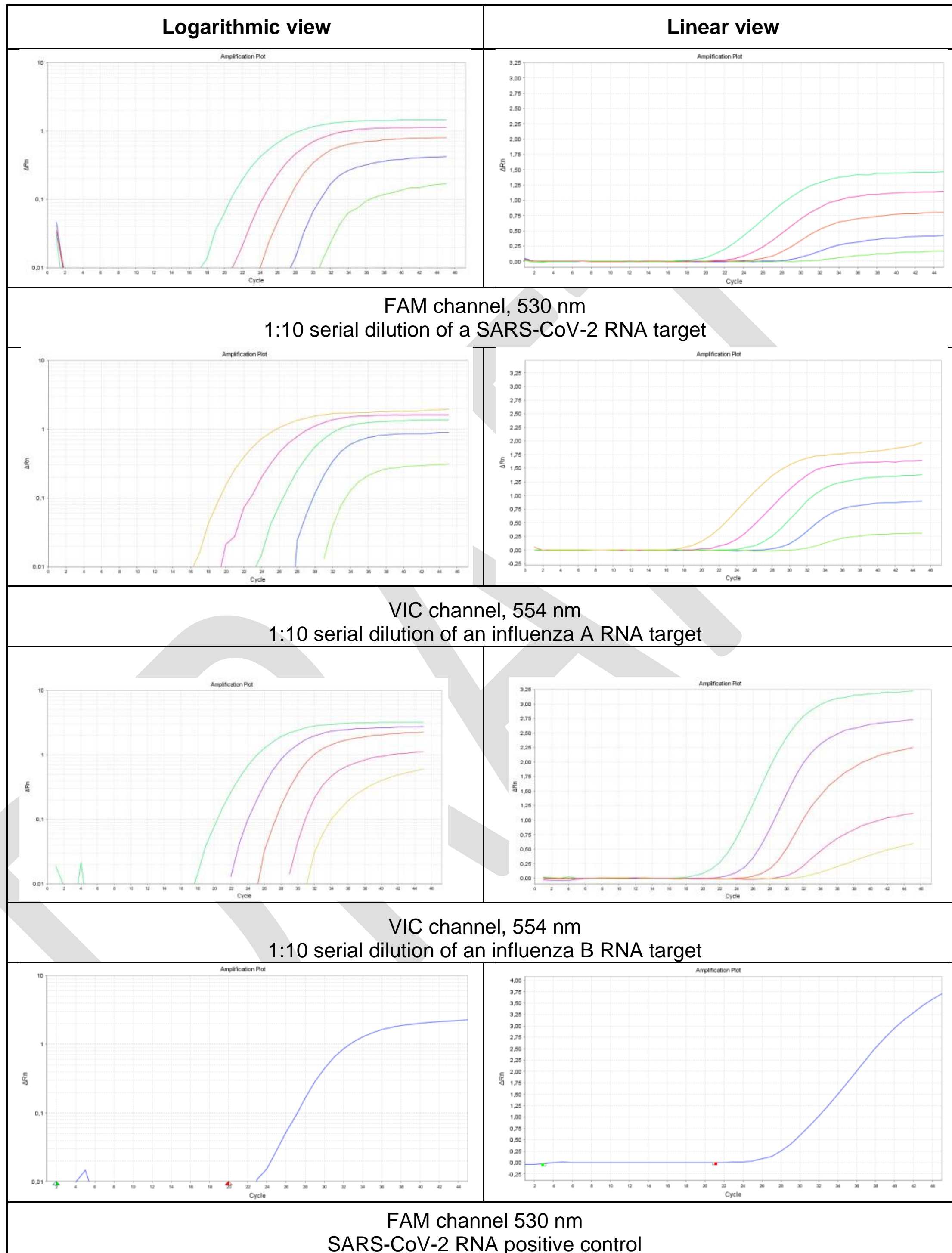
- PCR reaction was inhibited. No interpretation can be made.
→ Make sure that you use a recommended method for RNA isolation and stick closely to the manufacturer's instructions.
→ If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of RNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of H_2O).
- Incorrect PCR conditions.
→ Check the RT-PCR conditions and repeat the RT-PCR, if necessary.

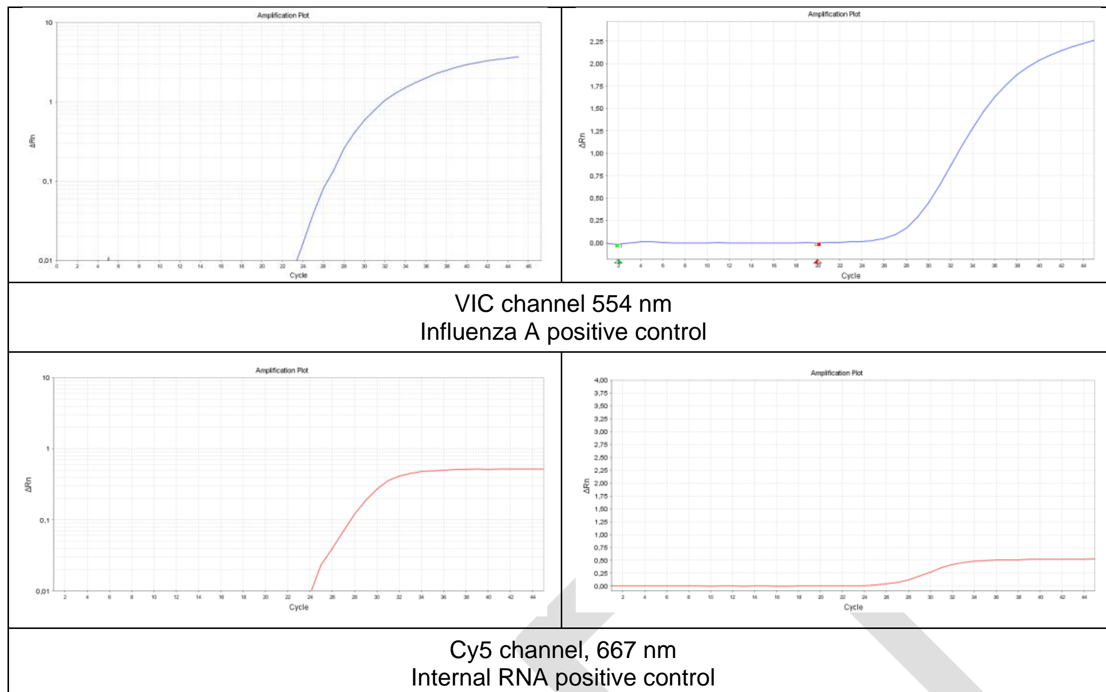
DRAFT

11. Specifications and performance evaluation

11.1. Kit performance

Performance of ViroReal® Kit SARS Coronavirus & Influenza A/B with an ABI® 7500 instrument is shown in Figure 2.





VIC channel 554 nm
Influenza A positive control

Cy5 channel, 667 nm
Internal RNA positive control

Figure 2 Performance of ViroReal® Kit SARS Coronavirus & Influenza A/B

To evaluate the performance of ViroReal® Kit SARS Coronavirus & Influenza A/B 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 3.

Table 3: Testing of different real-time PCR instruments

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

DRAFT

11.2. Limit of detection and linearity

ViroReal® Kit SARS Coronavirus & Influenza A/B was tested 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.

11.2.1. LoD

The LoD 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 detection limit (LoD95: number of copies, which are positively detected in 95% of cases) is 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 effect sensitivity (data not shown).

11.2.2. Linearity

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 (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 (σ) and coefficient of variation (CV %) were calculated.

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 a R^2 of 0.96 as shown in Figure 3.

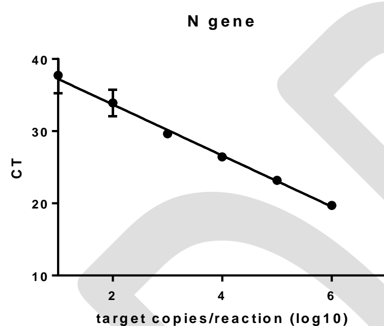


Figure 1 Ten-fold dilution series of SARS-CoV-2 RNA standard plotted against CT, FAM channel, N gene

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 4.

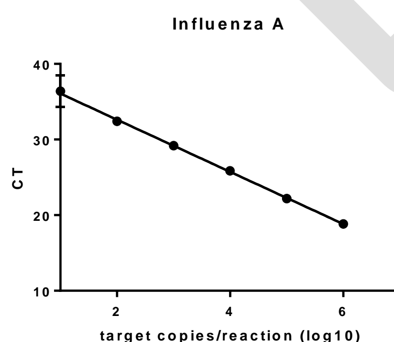


Figure 4 Ten-fold dilution series of influenza A RNA standard plotted against CT, VIC channel

DRAFT

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 5.

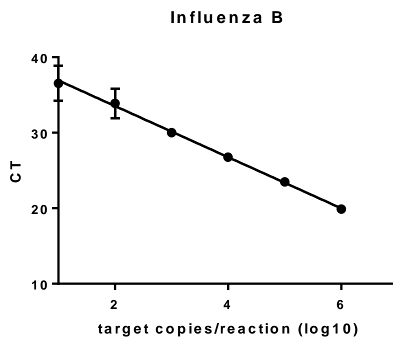


Figure 5 Ten-fold dilution series of influenza B RNA standard plotted against CT, VIC channel

11.3. Precision inter-assay

Inter-assay precision is defined as the reproducibility of a sample between assay runs. The inter-assay precision of ViroReal® Kit SARS Coronavirus & Influenza A/B was determined 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%.

11.4. Precision intra-assay

Intra-assay precision is defined as the reproducibility of a sample within an assay run. Intra-assay precision of ViroReal® Kit SARS Coronavirus & Influenza A/B was determined 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%.

11.5. Analytical specificity

Specificity is ensured by the selection of highly specific primers and probes. ViroReal® Kit SARS Coronavirus & Influenza A/B detects 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 changes 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

DRAFT

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 retrospectively analysed. All samples were correctly analysed by ViroReal® Kit SARS Coronavirus Influenza A/B.

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). All samples were correctly analysed negative by ViroReal® Kit SARS Coronavirus Influenza A/B.

11.6. Diagnostic evaluation

A serial dilution of a SARS-CoV-2 positive RNA swab extract was tested by an external service provider (AGES, Vienna) in triplicates with ViroReal® Kit SARS Coronavirus & Influenza A/B and with an in-house test, see Table 3. Furthermore, ViroReal® Kit SARS-CoV-2 Multiplex was tested by an external service provider (AGES, Vienna) on 94 swab samples from the respiratory tract collected from patients suspected to suffer from COVID-19. Extraction was performed with the MagNA Pure Compact using the Total Nucleic Acid Isolation Kit (Roche). Analyses were performed in single reactions on the LightCycler® 480 I instrument (Roche).
In house test (reference method): results were compared with results of a real-time PCR test detecting the E gene of SARS-CoV-2, according to the recommendations of the WHO: Detection of 2019 novel coronavirus (2019-nCoV) (Victor M Corman et al., 2020).
Fifteen out of the 94 samples were positive with both methods. One sample was positive with ViroReal® Kit SARS-CoV-2 & SARS only (Cp-value: 35.5). The remaining samples were negative with both methods, see Table 5.

Table 3 Comparison of test results of a serial dilution of a SARS-CoV-2 positive RNA extract in triplicates

	E gene (reference method)		**N gene ViroReal® Kit** SARS Coronavirus & Influenza A/B	
Swab extract dilution	Ct value (mean)	σ	Ct value (mean)	σ
1:100				
1:1000				
1:10,000				
1:100,000				
1:1,000,000				

Table 4 Diagnostic evaluation of ViroReal® Kit SARS Coronavirus & Influenza A/B

		Reference		
		pos	**neg**	**Total**
ViroReal® Kit SARS Coronavirus & Influenza A/B	**pos**			
	neg			

DRAFT

	Total			

Table 5 Diagnostic performance

	Value	95% CI
Sensitivity		
Specificity		
NPV		
PPV		
Prevalence		

12. 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

13. Revision history

Revision	Date	Description

DRAFT

Distributed by