ViroReal® Kit SARS-CoV-2 Multiplex

Manual

CE

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

REF	**DHUV02413**	Σ	**100**
REF	**DHUV02413x5**	Σ	**500**

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Index

Explanation of symbols

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

1. Intended use

ViroReal® Kit SARS-CoV-2 Multiplex 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) and of the RdRp gene of SARS-CoV-2 in patients with or without a suspected SARS-CoV 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.

2. Product description

ViroReal® Kit SARS-CoV-2 Multiplex detects the nucleocapsid protein gene (N gene) of SARS-CoV-2, SARS-CoV and SARS-related coronavirus (Sarbecovirus) and as well as the RdRp gene of SARS-CoV-2.

The multiplex approach allows universal detection of all so far known SARS coronavirus strains (Sarbecovirus) including SARS-CoV-2, and the discrimination between SARS-CoV-2 and the other Sarbecovirus strains.

The primer and probe designs chosen are, with the exception of one primer, not identical with designs published by the WHO.

The chosen design for the N gene includes a highly conserved region in all SARS coronavirus clusters of the N gene, which allows the universal detection of previously known Sarbecoviruses including SARS-CoV-2. Due to its universal character, it takes into account possible future mutations in the SARS-CoV-2 virus sequence. Because of its design, the test has the potential to detect new SARS corona viruses in the future. The N gene is detected in the fluorescence channel for FAM (530 nm).

For the RdRp gene, a sequence range highly specific for SARS-CoV-2 was chosen, which allows the specific detection of SARS-CoV-2. The RdRp gene is detected in the fluorescence channel for VIC (554 nm).

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 (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 Diagnostics), 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.

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 as the most frequent initial symptoms, later on it can lead to pneumonia. The coronavirus spreads mainly by droplet and contact transmission.

4. Contents of the Kit, Stability and Storage

		DHUV02413	DHUV02413x5	
Component	**Content**	**Quantity 100 reactions**	**Quantity 500 reactions**	**Storage**
SARS-CoV-2 Multiplex Assay Mix (green cap)	Primer and probe for virus detection (FAM, VIC) and RNA IPC detection (Cy5)	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 Multiplex Positive Control (red cap)	RNA Positive Control for the N gene and RdRp gene (à 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-CoV-2 Multiplex 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 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.

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

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 SARS-CoV-2 detection has been validated for swabs from the respiratory tract (see 11.6). Test performance with other specimen types has not yet been assessed.
- Testing of self-collected or healthcare provider-collected anterior and mid-turbinate nasal swabs is limited to patients with symptoms of COVID-19. 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 or Sarbecovirus infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or viral quantities below the assay sensitivity. The presence of PCR inhibitors may lead to invalid results.
- In general, sequence variability in the target region of previously unknown clinical subtypes may lead to false negative or less sensitive results. The universal test character of ViroReal® Kit SARS-CoV-2 Multiplex takes into account possible future mutations of the SARS-CoV-2 virus sequence and thus reduces the probability of false negative or less sensitive results due to mutations in the target region.
- Results should be interpreted in context of clinical and laboratory findings.

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 Multiplex Positive Control is 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. 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 10 µl of the Positive Control.

8.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	4.0 µl
	RNA Reaction Mix	5.0 µl
	SARS-CoV-2 Multiplex 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.

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. Close the plate with appropriate optical closing material.

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: Specific detection of SARS-CoV-2
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

9. Interpretation of PCR-data

For analysis of PCR results gained with ViroReal® Kit SARS-CoV-2 Multiplex, select fluorescence display options FAM and VIC channel for the virus target and Cy5 channel for the RNA IPC target. Samples with positive Cq-values are considered positive (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)).

Important: Please, also check amplification curves, not only Cq 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

	Cq FAM channel Viral target (N gene)	Cq VIC channel Viral target (RdRp gene)	Cq Cy5 channel RNA IPC target[2]	Interpretation	Action
Positive control	25-27	25-27	Negative	Valid	-
Positive control	Negative	Negative	Negative	Invalid	See 10.1
Positive control	25-27	Negative	Negative	Invalid	See 10.1
Positive control	Negative	25-27	Negative	Invalid	See 10.1
Positive control	25-27	25-27	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. If results of controls are not valid, the patient results cannot be interpreted.

Table 2: Interpretation of data with clinical samples

	Cq FAM channel Viral target (N gene)	Cq VIC channel Viral target (RdRp gene)	Cq Cy5 channel RNA IPC target	Interpretation	Action
Clinical sample	Negative	Negative	25-28[1]	Negative	-
Clinical sample	Positive	Positive	Positive/Negative[2]	Positive for SARS-CoV-2	-
Clinical sample	Negative	Positive	Positive/Negative[2]	Positive for SARS-CoV-2	-
Clinical sample	Positive[3]	Negative[3]	Positive/Negative[2]	Positive for Sarbecovirus[3]	-
Clinical sample	Negative	Negative	Negative	Invalid	See 10.5

[1] A positive signal excludes PCR inhibition. However, IPC Cq values should show comparable results among samples. A shift of Cq 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] If the sample is weak positive (approx. Cq > 33) in FAM channel and negative in VIC channel, there is a low virus concentration. In this case it is not possible to distinguish between SARS-CoV-2 or any other sarbecovirus.

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 master mix. 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 master mix. If no RNA IPC Target was added to master mix:
  → 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

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.

11. Specifications and performance evaluation

11.1. Kit performance

Performance of ViroReal® Kit SARS-CoV-2 Multiplex with an ABI® 7500 instrument is shown in Figure 2.

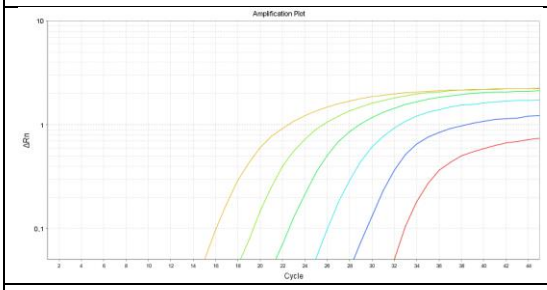
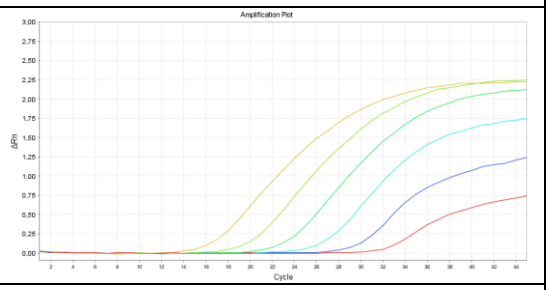
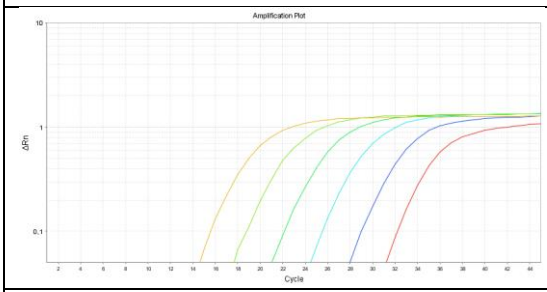
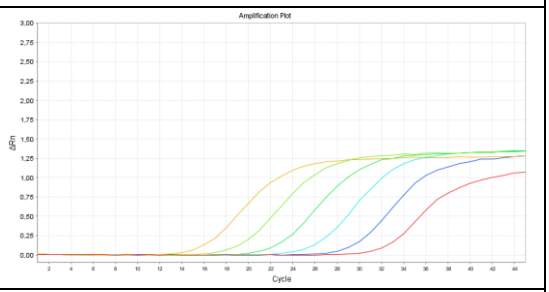
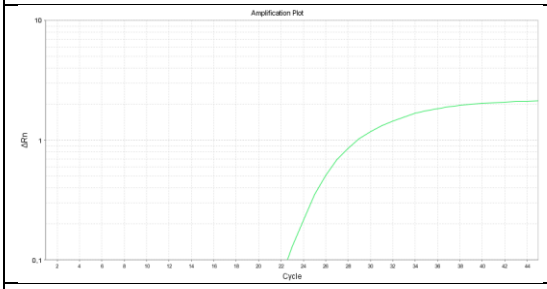
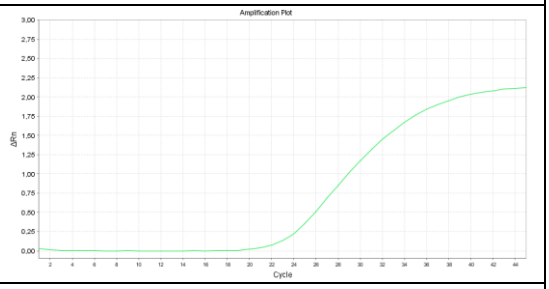
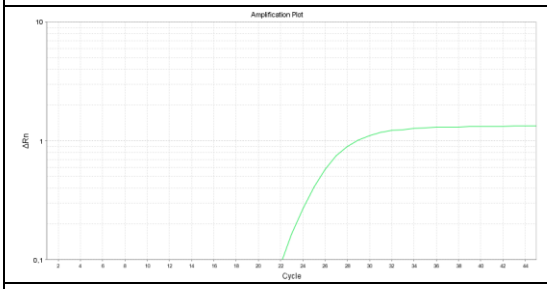
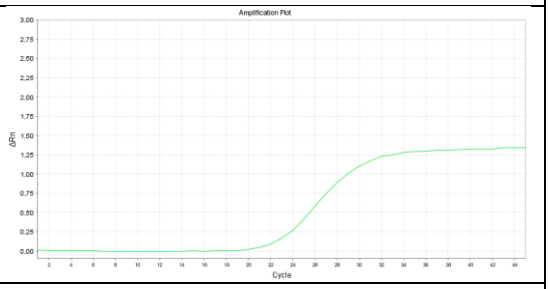
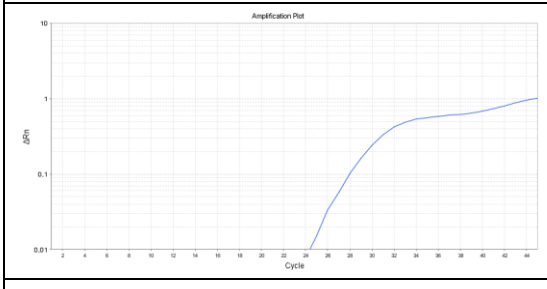
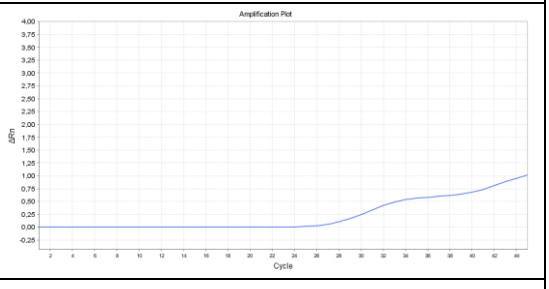
Logarithmic view	Linear view
	
FAM channel, 1:10 serial dilution of a SARS-CoV-2 RNA target	
	
VIC channel, 1:10 serial dilution of a SARS-CoV-2 RNA target	
	
FAM channel, SARS-CoV-2 RNA positive control	
	
VIC channel, SARS-CoV-2 RNA positive control	
	
Cy5 channel, Internal RNA positive control	

Figure 2 Performance of ViroReal® Kit SARS-CoV-2 Multiplex

ViroReal® Kit SARS-CoV-2 Multiplex was validated with the Applied Biosystems® (ABI) 7500 instrument (Thermo Fisher Scientific), Mx3005P® (Agilent), qTOWER^{3}G (Analytik Jena), MIC instrument (bio molecular systems) and LightCycler® 480 I (Roche).
To evaluate the performance of ViroReal® Kit SARS-CoV-2 Multiplex with different real-time PCR instrument systems, testing was performed using SARS-CoV-2 negative sputum extracts (QIAamp Viral RNA Mini Kit) that were spiked with different concentrations of AMPLIRUN® Coronavirus SARS-CoV-2 RNA control (Vircell) (100, 50, 25, 12.5, 6 and 3 copies/PCR reaction). Tested instruments were: Applied Biosystems® (ABI) 7500 instrument (Thermo Fisher Scientific), Mx3005P® (Agilent), qTOWER3G (Analytik Jena) and MIC instrument (bio molecular systems). Testing showed that the analytical sensitivity of the assay was similar, irrespective of the amplification platforms used (Table 3, Table 4).

Table 3 Testing of different real-time PCR instruments: detection of N gene

	Average Cq values			
	ABI® 7500	MIC	Mx3005P®	qTOWER^{3}G
100 copies	29.64	29.90	30.73	35.14
50 copies	30.69	31.04	32.03	34.98
25 copies	31.79	31.78	32.36	35.26
12.5 copies	32.44	31.20	35.11	38.05
6 copies	34.97	33.77	35.43	37.44
3 copies	33.97	34.44	36.47	43.90

Table 4 Testing of different real-time PCR instruments: detection of RdRp gene

	Average Cq values			
	ABI® 7500	MIC	Mx3005P®	qTOWER^{3}G
100 copies	30.26	32.35	32.54	33.14
50 copies	31.43	33.39	33.31	33.14
25 copies	31.92	33.72	34.45	33.54
12.5 copies	33.25	34.88	35.59	34.87
6 copies	34.17	35.45	36.31	34.58
3 copies	35.50	38.72	37.71	37.10

11.2. Limit of detection and linearity

ViroReal® Kit SARS-CoV-2 Multiplex was tested with the ABI® 7500 instrument with a 10-fold dilution series of a synthetic RNA representing a fragment of SARS-CoV-2. At least three target copies/reaction could be detected.

11.2.1. LoD

To determine LoD, sputum (sputasol added 1:1) was extracted (QIAamp Viral RNA Mini Kit) and the extract was spiked with a commercially available SARS-CoV-2 RNA (AMPLIRUN® Coronavirus SARS-CoV-2 RNA Control, Vircell, Order No. MBC137-R). The LoD was determined by testing different concentrations 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 13 copies/reaction for the N gene and 6 copies/reaction for the RdRp gene.

11.2.2. Linearity

For the N gene, the test shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.37 and a R^2 of 0.99 as shown in Figure 3.

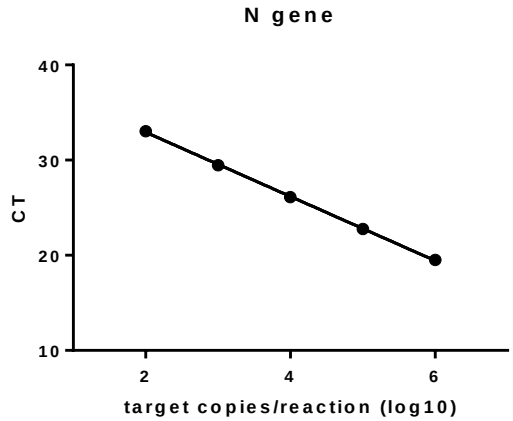


Figure 3 Ten-fold dilution series of SARS-CoV-2 RNA standard plotted against CT, FAM channel, N gene

For the RdRp gene, the test 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 4.

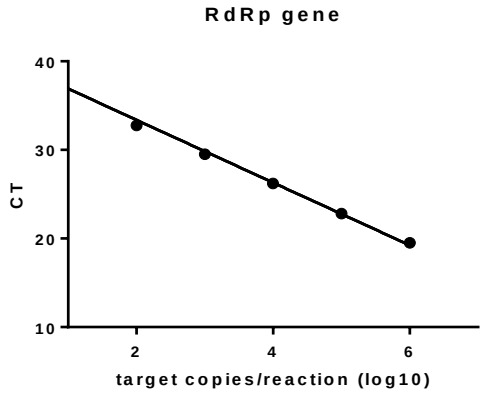


Figure 4 Ten-fold dilution series of SARS-CoV-2 RNA standard plotted against CT, VIC channel, RdRp gene

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-CoV-2 Multiplex was determined from 10-fold dilutions of an RNA (1.00E+06 to 1.00E+00 target copies/reaction) in three independent experiments performed on different days in quadruplicates.

For the N gene, precision standard replicate CV's ranged from 0.27% to 3.80%, with mean overall inter-assay precision equal to 1.04%.

For the RdRp gene, precision standard replicate CV's ranged from 0.05% to 5.75%, with mean overall inter-assay precision equal to 1.27%.

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-CoV-2 Multiplex was determined from the replicate runs above (11.3).

For the N gene, precision standard replicate CV's from replicate 1 ranged from 0.32% to 3.20%, with mean overall intra- assay precision equal to 1.07%.

For the RdRp gene, precision standard replicate CV's from replicate 1 ranged from 0.08% to 5.84%, with mean overall intra- assay precision equal to 1.55%.

11.5. Analytical specificity

ViroReal® Kit SARS-CoV-2 Multiplex detects the nucleocapsid protein gene (N gene) of SARS-CoV-2, SARS-CoV and SARS-related coronavirus (Sarbecovirus) as well as the RdRp gene of SARS-CoV-2. Other beta coronaviruses are not detected with this kit. Specificity is ensured by the selection of highly specific primers and probes. *In silico* validation of primers and probes was carried out with the basic local alignment tool (BLAST) against the NCBI database and with analyses in the GISAID database.

For the N gene, a highly conserved region in all SARS coronavirus clusters (SARS-CoV-2 as well as SARS-CoV and SARS-related bat coronavirus) was chosen as target region The selected primers and probe are intended to cover possible future mutations in the virus sequence and are with the exception of one primer not identical to the primers and probes published by the WHO. All data in the NCBI and GISAID database showed homology of the sequence region of primers and probe in all SARS coronavirus clusters. This approach allows universal detection of all so far known SARS coronavirus strains including SARS-CoV-2.

For the RdRp gene, a highly specific region for SARS-CoV-2 was chosen. The design of primers and the probe are not identical with designs published by the WHO. All data in the NCBI and GISAID database showed 100% homology in the sequence region of primer and probe for all SARS-CoV-2 published sequence entries. The test cross reacts with bat coronavirus RaTG13, which is the coronavirus most closely related to SARS-CoV-2. This approach allows the specific detection of all previously known SARS-CoV-2 strains.

Furthermore, specificity was validated by testing against respiratory viruses and SARS-CoV-2. A total of 22 ring trial samples from INSTAND e.V. were retrospectively analysed. All samples were correctly analysed with ViroReal® Kit SARS-CoV-2 Multiplex.

11.6. Diagnostic Evaluation

ViroReal® Kit SARS-CoV-2 Multiplex 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 Abbott 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 Multiplex has a sensitivity of approx. 893 copies/µl sputum (when 200 µl sputum are extracted).

For testing with ViroReal® Kit SARS-CoV-2 Multiplex, 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, 44 samples were positive with ViroReal® Kit SARS-CoV-2 Multiplex. Three samples, which were questionably and weak positive with the reference method (Cq>38), were negative with ViroReal® Kit SARS-CoV-2 Multiplex. One sample tested negative with the reference method showed a positive result with ViroReal® Kit SARS-CoV-2 Multiplex. Due to the high Cq value (40.9) this sample was considered to be a contamination. The remaining 46 samples were negative by both methods (Tables 5, 6).

The Cq value (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)) corresponds to the number of PCR cycles required until a positive signal is obtained and is therefore a measure of the virus concentration in the sample material. However, the Cq values are also depending on the instrument software and on the quality of swab samples.

Guidelines for interpretation of the Cq values can be found on the website of the Robert Koch Institute.

Table 5 Summary of data

		Reference		
		pos	**neg**	**Total**
ViroReal® Kit SARS-CoV-2 Multiplex	**pos**	**44**[1]	**1**[2]	**45**
	neg	**3**[3]	**46**	**49**
	Total	**47**	**47**	**94**

[1] Two samples positive for N gene or RdRp gene only, respectively
[2] Cq=40.9, regarded as contamination
[3] Questionably and weak positive with reference method

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

	Value
Sensitivity	93.6 %
Specificity	100 %
NPV	93.9 %
PPV	97.8 %

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
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)).