ViroReal® Kit RT-LAMP SARS-CoV-2

Instructions for use

CE

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

REF	**DHUV02610**	Σ	**100**
REF	**DHUV02610x5**	Σ	**500**

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Explanation of symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Store at
CE	This product fulfils 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 RT-LAMP SARS-CoV-2 is an *in vitro* diagnostic test, based on RT-LAMP, for the detection of RNA of a part of the ORF1ab region of SARS-CoV-2. This RT-LAMP based test is used as a screening tool for samples from patients of all age groups with and without suspected COVID-19 disease.

The test is suitable for the detection of SARS-CoV-2 of persons who are in an infectious stage. Samples with an RNA concentration of approximately 20,000 copies per ml or more (equivalent to approximately 100 copies per RT-LAMP reaction, which corresponds to a real-time PCR Cq value of approx. 31) can be reliably detected. The results of ViroReal® Kit RT-LAMP SARS-CoV-2 represent a snapshot of the infection status of the tested person. Persons with symptoms similar to COVID-19 should remain in quarantine. Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Persons with a positive or questionably positive test result should be further checked by real-time PCR (gold standard).

The test is intended for use in point-of-care settings (e.g., doctor's offices, hospitals, urgent care centers and emergency rooms). The RT-LAMP reaction takes between 30-53 minutes, depending on the (real-time) PCR device. Samples must be collected by a healthcare provider when testing individuals under the age of 14.

This test can either be used with native specimens (without prior extraction) or with extracted RNA.

- Proper **native specimens** are oropharyngeal swabs (posterior throat swabs) in 2 ml of isotonic saline solution (NaCl 0.9%), solely. There is no need for RNA extraction, because virus inactivation and lysis occur during the isothermal amplification step.
  Caution: Other native specimens or transport media are not suitable.
- Suitable purified (extracted) test materials are samples from the upper respiratory tract (throat rinsing fluid, nasopharyngeal and oropharyngeal swabs, nasopharyngeal wash/aspirate and nasal aspirates). This test material must be prepared with a suitable RNA extraction procedure before testing with ViroReal® Kit RT-LAMP SARS-CoV-2.

  In the case of deep respiratory tract infections, the testing of nasopharyngeal or oropharyngeal samples alone is not suitable for the exclusion of SARS-CoV-2 disease, since in this phase of the disease only samples from the lower respiratory tract are positive in the PCR.

ViroReal® Kit RT-LAMP SARS-CoV-2 is a qualitative test and not suitable for virus quantification.

2. Product description

ViroReal® Kit RT-LAMP SARS-CoV-2 detects RNA of a part of the ORF1ab of SARS-CoV-2.

The test is based on one-step RT-LAMP technology (reverse transcription loop-mediated isothermal DNA amplification). For this purpose, a reverse transcriptase is used to transcribe a specific RNA region of the pathogen genome into cDNA. The cDNA is then amplified in a reaction at a constant temperature using six DNA oligonucleotides and a strand displacing DNA polymerase.

After amplification, a visual check of the color change in the reaction tubes must be performed (colorimetric detection of amplification by change of color from red to yellow, based on the pH indicator dye used in the LAMP reaction), which allows the test to be used in a conventional block PCR device. The drop in pH value is due to the large amount of generated DNA.

Alternatively, a real-time PCR device can be used for detection of the RT-LAMP reaction. In this case, the generated DNA amplificate can be detected by means of an intercalating fluorescent dye (contained in the reaction mix of the kit) by amplification and melting curves. The amplification is detected in the fluorescence channel for SYBR Green/FAM. This test is compatible with real-time PCR instruments which detect fluorescence in FAM 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), Gentier (Labomedic), LightCycler® 480 II (Roche Diagnostics), cobas z 480 Analyzer (Roche)).

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 main route of SARS-CoV-2 transmission is via respiratory uptake of virus particles (droplets or smaller aerosols).

4. Contents of the Kit, Stability and Storage

		DHUV02610	DHUV02610x5	
Component	**Content**	**Quantity 100 reactions**	**Quantity 500 reactions**	**Storage**
RT-LAMP SARS-CoV-2 Super Mix (green cap)	Primer + intercalating dye for virus detection + Triton X-100	1 x 500 µl	5 x 500 µl	-15°C to -25°C
RT-LAMP SARS-CoV-2 Positive Control (red cap)	RNA Positive Control (10^3 copies/µl)	1 x 300 µl	1 x 300 µl	-15°C to -25°C
RT-LAMP RNA/DNA Reaction Mix (white cap)	Reaction Mix	1 x 1000 µl	5 x 1000 µl	-15°C to -25°C
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	1 x 1000 µl	-15°C to -25°C

RT-LAMP SARS-CoV-2 Super Mix: The added Triton X-100 leads to lysis of the virus envelope at 63°C. In addition, it contains an intercalating fluorescent dye, enabling detection through amplification curves and melting curves on a real-time PCR device.

RT-LAMP RNA/DNA Reaction Mix: The reaction mix provided with the kit has been designed for reliable, high-sensitivity RT-LAMP. It features inhibitory enzyme activity at room temperature (Warm-Start) and allows for fast, visual colorimetric detection of amplification.

The components of ViroReal® Kit RT-LAMP SARS-CoV-2 are stable until the expiry date stated on the label. Repeated freeze/thaw cycles should be avoided. Protect kit components from light. The components can be stored for one week at 4°C.

5. Additionally required materials and devices

- Optional: Reagents and devices for RNA-extraction
- Disposable powder-free gloves
- Sterile filter pipette tips
- Conventional block-PCR instrument with appropriate reaction plates or reaction tubes with closing material recommended by the manufacturer of the PCR instrument
- Alternatively: Real-time PCR instrument which is able to detect fluorescence in SYBR Green/FAM 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 or RT-LAMP 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.
  Remark: If SARS-CoV-2 is not cultivated, biosafety level 2 (BSL-2) is applicable. The WHO guidelines recommend the initial handling of samples (before virus inactivation) in a tested microbiological safety cabinet or in closed apparatus.
- Wear protective disposable powder-free gloves when handling kit reagents and specimens.
- Use separate areas for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate areas 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 equipment and reagents with DNA/RNA, nuclease or amplification products by good laboratory practice.
- When performing RNA-extraction: Quality of RNA has a profound impact on the test performance. Ensure that the used RNA extraction system is compatible with RT-LAMP 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.
- Always include a negative control per RT-LAMP run (nuclease-free water instead of sample).
- Please note the expiry date of the kit.
- Repeated freeze/thaw cycles of kit components should be avoided. Protect kit components from light.
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.
- Do not open RT-LAMP reactions after amplification in order to prevent carry-over contamination leading to false positives.
- **Caution:** the RT-LAMP RNA/DNA Reaction Mix contains Triton X-100 (see MSDS, www.ingenetix.com).

7. Limitations

- Optimal performance of this test requires appropriate specimen collection, transport, storage and preparation of the samples. Proper native specimens used without prior RNA extraction are oropharyngeal swabs in 0.9% NaCl only. Other native specimens are not suitable and lead to false positive or negative results.
  Remark: If extracted RNA from patients with or without suspected SARS-CoV-2 infection is used as a template in this diagnostic test, proper specimens are samples from the upper respiratory tract. Be sure to use an appropriate RNA extraction procedure.
- Samples with an RNA concentration of approx. 20,000 copies per ml (corresponds to approx. 100 copies per RT-LAMP reaction and a real-time PCR Cq value of approx. 31) can be reliably detected. Patients with a lower viral load are most likely not infectious (Wölfel et al., 2020 and recommendation of Robert Koch-Institute).
- A negative test result does not exclude the possibility of a SARS-CoV-2 infection, because test results may be affected by improper specimen collection, PCR inhibition, technical error, specimen mix-up, unfavourable time (related to the course of the disease) or viral quantities below the assay sensitivity. A negative result does not rule out a beginning infection with a low viral load.
- The test does not contain an internal positive control system for controlling of RNA extraction as well as for detection of possible RT-LAMP inhibition. The presence of 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.
- Results should be interpreted in accordance with clinical and laboratory findings.

8. Preparation of samples and RT-LAMP PCR

Do not freeze native samples prior to extraction – storage a 4°C to 8°C is recommended. Samples should be analysed on the same day.

This test can either be used with native (not extracted/purified) oropharyngeal swabs or with extracted RNA from other specimens.

- Proper **native specimens** are oropharyngeal swabs in 2 ml of isotonic saline solution (NaCl 0.9%), solely. There is no need for RNA extraction, because virus inactivation and lysis occur during the isothermal amplification step. Swabs are stored either dry or in 0.9% NaCl. Suitable swabs: polyester or rayon swabs with aluminium or plastic shaft, such as Copan 503C Floq Swabs or Raucotupf (not provided in the kit). Swabs are put into 2 ml isotonic saline solution (NaCl 0.9%, not provided) and shaked gently.
  Caution: Other native samples and transport media (such as eSWabs) are not suitable as they cause inhibition or immediate orange coloration of the reaction.
- Suitable purified (extracted) test materials are samples from the upper and lower respiratory tract (throat rinsing fluid, nasopharyngeal and oropharyngeal swabs, nasopharyngeal wash/aspirate and nasal aspirates, sputa and BAL). This test material must be subjected to appropriate RNA extraction before testing with ViroReal® Kit RT-LAMP SARS-CoV-2. Elute RNA with nuclease-free water.
- Make sure that at least one negative control (water or NaCl 0.9% instead of sample material), as well as one positive control (red cap) are included per PCR run. In case of RNA extraction, include an extraction negative control.
- Mix RT-LAMP RNA/DNA Reaction Mix 2 to 3 times by inverting the tube to obtain a homogeneous solution. All kit components have to be completely thawed at room temperature before set-up of the Master Mix. After thawing, the individual components are mixed and then placed on ice.
- **Important:** Reclose the RT-LAMP RNA/DNA Reaction Mix and RT-LAMP SARS-CoV-2 Super Mix immediately after use. The pH indicator contained in the mixes will change from red to orange under the influence of CO_2 in the air. The RT-LAMP SARS-CoV-2 Super Mix is not buffered and changes its colour in case of repeated opening.

8.1. Positive Control

RT-LAMP SARS-CoV-2 Positive Control is an *in vitro* synthesized RNA fragment with a concentration of 10^3 copies/µl. Ensure a homogenous solution by gently mixing. To avoid freeze/thaw cycles, it can also be temporarily stored at 4°C.
→ As positive control, use 5 µl of the Positive Control (red cap) in the last pipetting step.

8.2. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	RT-LAMP RNA/DNA Reaction Mix	10.0 µl
	RT-LAMP SARS-CoV-2 Super Mix	5.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of RT-PCR	Master Mix	15.0 µl
	Sample (RNA)*	5.0 µl
	Total volume	**20.0 µl**

*5 µl sample can be used. When using a volume other than 5 µl, the volume has to be adjusted with nuclease-free water.

Important: close the tube with the prepared Master Mix immediately after pipetting. The finished Master Mix in the PCR tubes is stable for one day in the refrigerator or on ice, protect from light.
Do not leave the PCR tubes or the PCR plate open for a longer period of time after aliquoting the master mix, but pipette the samples and controls immediately and close afterwards. The pH indicator contained in the master mix leads to a color change from red to orange under the influence of CO_2 in the air and thus to false positive results during colorimetric evaluation.

→ For preparation of RT-LAMP, dispense 15 µl aliquots of prepared Master Mix into the plate wells and then add 5 µl of sample (or extracted RNA) per well.

At last, pipet the Positive Control. Close the plate with appropriate (optical) closing material.

Important: After closing the reaction tubes a visual check of the reaction tubes must be performed in order to ensure that there is no color change from red to yellow after adding the sample. Swabs showing a change of color from red to yellow before the RT-LAMP reaction has been started have an acidic pH value and have therefore to be interpreted as invalid. A new swab has to be taken from the respective person.

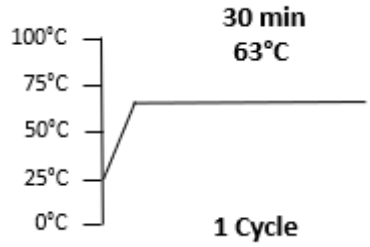
8.3. Programming of the temperature profile

Further information on programming the PCR instrument can be found in the respective operator's manual.

8.3.1. Programming of Block-PCR instrument

Sample Volume 20 µl

Temperature Profile

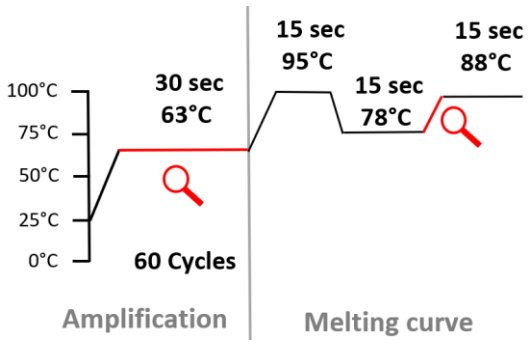


The amplification takes 30 min at 63°C

8.3.1. Alternative: Programming of real-time PCR instrument

Sample Volume 20 µl

Temperature Profile



The amplification takes 30 min at 63°C (30 sec x 60 cycles with fluorescence data acquisition).

The melting curve analysis is performed from 78°C to 88°C with fluorescence data acquisition and takes between 5-13 minutes, depending on the real-time PCR device.

Detection channels

SYBR Green-NONE (FAM channel): Detection of SARS-CoV-2
Passive reference dye: None

MIC Instrument (bio molecular systems):
FAM: Green

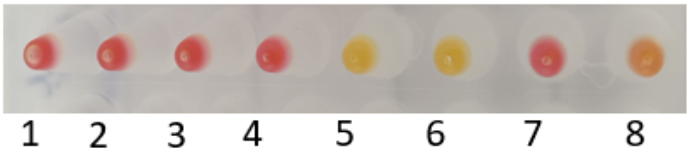
9. Interpretation of RT-LAMP data

- **Important:** Do not open RT-LAMP reactions after amplification in order to prevent contamination with DNA amplificate leading to false positives.
- After amplification, a visual check of the reaction tubes must be performed (colorimetric detection of amplification by change of color from red to yellow, based on the pH indicator dye used in the LAMP reaction), which allows the test to be used in conventional block PCR devices. The drop in pH value is due to the large amount of generated DNA.

Figure 1 Examples for positive and negative samples

- Beyond a colorimetric detection, the generated DNA amplificate can be detected by means of the intercalating fluorescent dye via amplification curves and melting curves using a real-time PCR instrument.
- 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, assessment of patient results is not possible.
- A negative result does not rule out a beginning infection with a low viral load below the detection limit.
- The test does not contain an internal positive control system to control RNA extraction and the detection of possible RT-LAMP inhibition. Inhibitors can therefore lead to a false negative result.
- If RNA was eluted with elution buffer instead of nuclease-free water, color change of positive samples from red to orange might occur.
- Weak positive samples show a color change from red to orange. Analysis thereof should be repeated.
- Positive test results should be double-checked by the gold standard real-time PCR.

9.1. Interpretation of RT-LAMP data generated by the block-PCR instrument



1: negative (red)
2: negative (red)
3: negative (red)
4: negative (red)
5: positive (yellow)
6: positive (yellow)
7: negative (red)
8: weak/questionably positve (orange)

Figure 2 Examples for positive, weak/questionably positive and negative samples

Table 1 shows the criteria for valid controls. Table 2 shows interpretation of data with clinical samples.

Table 1: Criteria for valid controls

	Colour of tube	Interpretation	Action
Positive control	Yellow	Valid	-
Positive control	Red/Orange	Invalid	See 10.1
Negative control	Red	Valid	-
Negative control	Yellow/Orange	Invalid	See 10.2

Table 2: Interpretation of data with clinical samples

	Colour in tube	Interpretation	Action
Clinical sample	Red	Negative	-
Clinical sample	Yellow	Positive for SARS-CoV-2	-
Clinical sample	Orange	Questionably positive for SARS-CoV-2	See 10.3
Clinical sample	Yellow **before** RT-LAMP	Invalid	See 10.4

9.2. Alternative: Interpretation of RT-LAMP data generated by a real-time PCR instrument

Besides a colorimetric detection of results by eye, the RT-LAMP reaction can be monitored by means of an intercalating fluorescent dye (contained in the reaction mix of the kit) via amplification curves and melting curves using a real-time PCR device.

- For analysis of results, select fluorescence display options FAM (fluorescence channel for SYBR Green).
- Please, also check amplification curves, not only Cq values (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)).
- Samples should be inspected both in logarithmic and linear scale view and compared with the negative control. Adjust the threshold, if necessary.
- The amplification plot and melting curve of a sample needs to be interpreted in context of the Cq and Tm [°C] values of the negative (NTC) and positive control.
- Positive samples must show a change of colour from red to yellow in the reaction tube, an exponential amplification curve and a melting curve with a melting point (Tm) of 80°-85°C. The Tm may vary slightly from sample-to-sample. Reaction specificity is ensured by the post-amplification melting-curve analysis.
- The Cq values of the RT-LAMP amplification curves output by the real-time PCR device do not correspond to conventional Cq values of a real-time PCR. They cannot be used for quantification but reflect the amplification time. A Cq value of two corresponds to one minute of amplification.

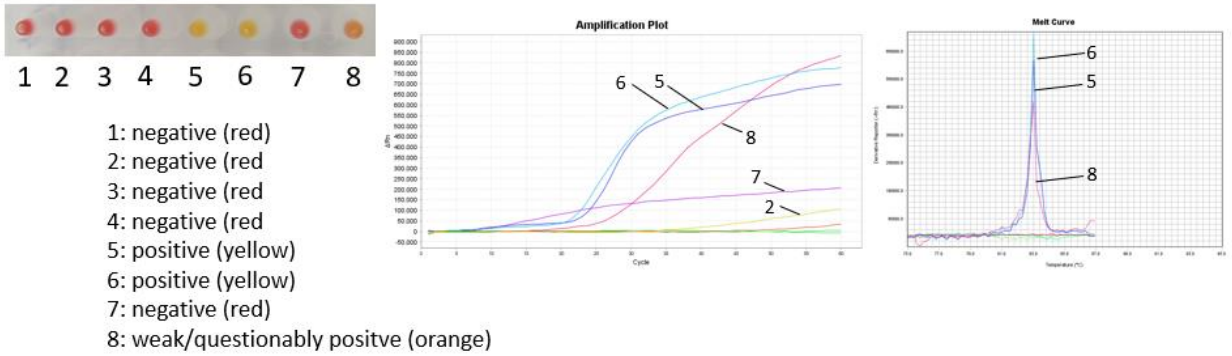


Figure 3 Examples for positive, weak/questionably positive and negative samples

Table 3: Criteria for valid controls

	Cq FAM channel	Tm	Colour in tube	Interpretation	Action
Positive control	Positive	80°- 85°C	Yellow	Valid	-
Positive control	Positive	80°- 85°C	Red or Orange	Invalid	See 10.1
Positive control	Negative	-	Yellow or Red	Invalid	See 10.1
Negative control	Negative	-	Red	Valid	-
Negative control	Negative	-	Yellow or Orange	Invalid	See 10.2
Negative control	Positive	80°- 85°C	Yellow or Orange	Invalid	See 10.2

Table 4: Interpretation of data with clinical samples

	Cq FAM channel	Tm	Colour in tube	Interpretation	Action
Clinical sample	Negative	-	Red	Negative	-
Clinical sample	Positive	80°- 85°C	Yellow	Positive	-
Clinical sample	Positive	-	Orange	Questionably positive	See 10.3
Clinical sample	Positive	80°- 85°C	Red	Invalid	See 10.5
Clinical sample	Negative	-	Yellow	Invalid	See 10.6
Clinical sample	Negative	-	Yellow **before** LAMP	Invalid	See 10.4

10. Troubleshooting

10.1. No virus specific signal with positive control

- Incorrect programming of the temperature profile or wrong detection channels on the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 8. Preparation of RT-LAMP) and verify detection channels.
- Incorrect configuration of the RT-LAMP reaction.
  → Check your work steps (see 8. Preparation of RT-LAMP) and repeat the RT-LAMP, if necessary.
- RNA in the sample may be degraded.
- No Positive Control was added.
  → Repeat RT-LAMP in case all clinical samples are negative.

10.2. Virus specific signal with negative control

- A contamination occurred during preparation of the RT-LAMP.
  → Repeat the RT-LAMP 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. Sample shows color change from red to orange

- If the RNA was eluted with elution buffer instead of nuclease-free water during extraction, positive samples may change color from red to orange instead to yellow.
  → Check the extraction procedure.
- Sample is weak/questionably positive.
  → Repeat the analysis

10.4. Color change after addition of the samples

- Ensure that no color change occurred after adding the samples before the RT-LAMP reaction. Samples that show a color change from red to yellow before the RT-LAMP reaction starts must be interpreted as invalid.
  → Repeat sample collection of the respective person.

10.5. Amplification curve and Tm with sample, but sample is red

- Invalid data, sample might be weak positive.
  → Repeat the analysis

10.6. No virus amplification curve and Tm with sample, but sample is yellow or orange

- Incorrect detection channels for sample.
  → Verify detection channels
  No interpretation is possible.

  Important: RT-LAMP RNA/DNA Reaction Mix and RT-LAMP SARS-CoV-2 Super Mix must be resealed immediately after use. After adding the sample to the master mix, a visual inspection of the reaction tubes must be performed to ensure that no color change from red to yellow occurs after the sample has been added, see section 8.2.

11. Specifications and performance evaluation

11.1. Kit performance

Performance of ViroReal® Kit RT-LAMP SARS-CoV-2 with an ABI® 7500 instrument is shown in Figure 4.

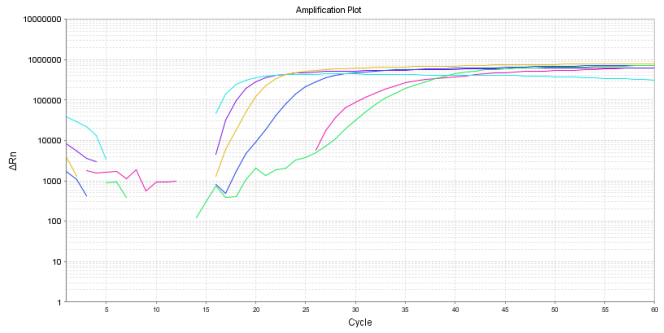
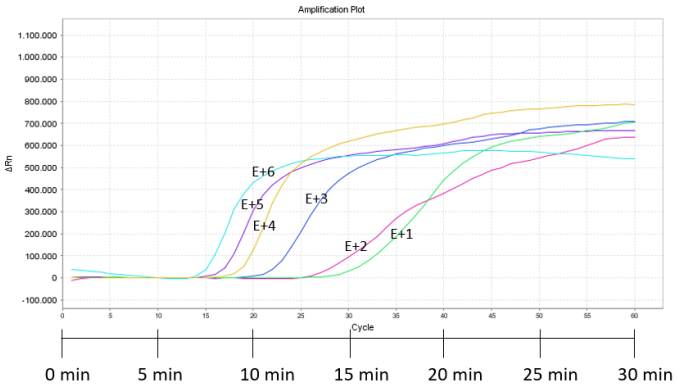
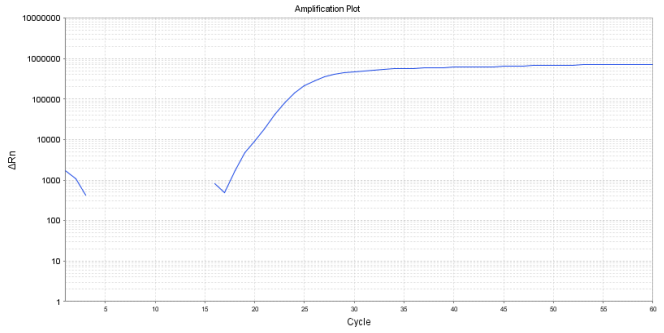
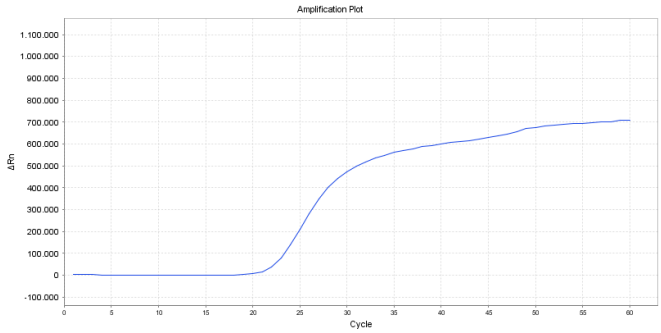
Logarithmic view	Linear view
	
FAM channel, 1:10 serial dilution of a SARS-CoV-2 RNA target	
	
FAM channel, RT-LAMP SARS-CoV-2 RNA positive control	

Figure 4 Performance of ViroReal® Kit RT-LAMP SARS-CoV-2

To evaluate the performance of ViroReal® Kit RT-LAMP SARS-CoV-2 with different (real-time) PCR instruments, testing was performed with different concentrations of AMPLIRUN® Coronavirus SARS-CoV-2 RNA control (Vircell) (150, 100, and 75 copies/reaction) and with different concentration of an *in vitro* synthesized RNA fragment (E+6 to E+1 copies/reaction).
Tested instruments were: Applied Biosystems® (ABI) 7500 instrument (Thermo Fisher Scientific), Mx3005P® (Agilent), MIC instrument (bio molecular systems) and GeneAmp® PCR System 9700 (Thermo Fisher Scientific). Testing showed that the analytical sensitivity of the assay was similar, irrespective of the amplification platforms used (Table 5).

Table 5 Testing of different (real-time) PCR instruments

	Average Cq values and color in reaction tubes (Y=yellow, R=red)			
	ABI® 7500	MIC	Mx3005P®	GeneAmp® PCR System 9700
E+6 copies synthetic RNA	16.2 (Y)	20.5 (Y)	19.0 (Y)	(Y)
E+6 copies synthetic RNA	16.1 (Y)	18.9 (Y)	17.9 (Y)	(Y)
E+5 copies synthetic RNA	18.4 (Y)	37.5 (Y)	20.4 (Y)	(Y)
E+5 copies synthetic RNA	18.3 (Y)	23.4 (Y)	20.2 (Y)	(Y)
E+4 copies synthetic RNA	20.0 (Y)	26.4 (Y)	22.6 (Y)	(Y)
E+4 copies synthetic RNA	20.1 (Y)	26.0 (Y)	22.7 (Y)	(Y)
E+3 copies synthetic RNA	24.3 (Y)	Negative (R)	25.9 (Y)	(Y)
E+3 copies synthetic RNA	22.3 (Y)	28.7 (Y)	26.1 (Y)	(Y)
E+2 copies synthetic RNA	35.8 (Y)	35.2 (Y)	29.7 (Y)	(Y)
E+2 copies synthetic RNA	32.8 (Y)	32.9 (Y)	29.0 (Y)	(Y)
E+1 copies synthetic RNA	37.6 (Y)	38.0 (Y)	37.6 (Y)	(O)
E+1 copies synthetic RNA	34.0 (R)	Negative (R)	37.57 (Y)	(O)
150 copies AmpliRun RNA	25.3 (Y)	34.8 (Y)	24.8 (Y)	(Y)
150 copies AmpliRun RNA	25.6 (Y)	33.4 (Y)	29.7 (Y)	(Y)
150 copies AmpliRun RNA	30.5 (Y)	32.2 (Y)	27.0 (Y)	(Y)
150 copies AmpliRun RNA	29.3 (Y)	33.8 (Y)	26.7 (Y)	(Y)
100 copies AmpliRun RNA	33.5 (Y)	34.7 (Y)	28.8 (Y)	(Y)
100 copies AmpliRun RNA	25.7 (Y)	32.3 (Y)	26.6 (Y)	(O)
100 copies AmpliRun RNA	28.7 (Y)	34.3 (Y)	30.3 (Y)	(Y)
100 copies AmpliRun RNA	26.6 (Y)	37.5 (Y)	31.9 (Y)	(Y)
75 copies AmpliRun RNA	26.8 (Y)	35.1 (Y)	29.4 (Y)	(Y)
75 copies AmpliRun RNA	40.4 (Y)	32.7 (Y)	30.4 (Y)	(O)
75 copies AmpliRun RNA	28.5 (Y)	37.0 (Y)	29.8 (Y)	(Y)
75 copies AmpliRun RNA	Negative (R)	35.4 (Y)	27.8 (Y)	(R)

11.2. Limit of detection and precision

ViroReal® Kit RT-LAMP SARS-CoV-2 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 ten target copies/reaction could be detected.

11.2.1. LoD

To determine LoD, a SARS-CoV-2 negative oropharyngeal swab was put in 2 ml of isotonic saline. Four µl of the saline was then 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 (150, 100 and 50 copies/reaction) in 20 replicates. The detection limit (LoD95: number of copies, which are positively detected in 95% of cases) is 100 copies/reaction.

11.2.2. Inter-assay precision

Inter-assay precision is defined as the reproducibility of a sample between assay runs. The inter-assay precision of ViroReal® Kit RT-LAMP SARS-CoV-2 was determined from 10-fold dilutions of a synthetic RNA (E+6 to E+3 target copies/reaction) in three independent experiments in triplicates and quadruplicates. The arithmetic mean ($\bar{x}$), the standard deviation (σ) and the coefficient of variation (CV %) between the replicate runs were calculated.

The test shows inter-assay precision with a mean overall inter-assay precision equal to 2.61% in the range from E+6 to E+3 target copies/reaction.

11.2.2. Intra-assay precision

Intra-assay precision is defined as the reproducibility of a sample within an assay run. The arithmetic mean ($\bar{x}$), the standard deviation (σ) and the coefficient of variation (CV %) of the replicates were calculated.

Precision standard replicate CV's ranged from 1.92% to 4.33%, with mean overall intra- assay precision equal to 2.82% % in the range from E+6 to E+3 target copies/reaction.

11.2.3. Inter-Lot precision

Inter-Lot variability was determined from two different lots of ViroReal® Kit RT-LAMP SARS-CoV-2 Supermix and tested with 10-fold dilutions of an RNA (1.00E+06 to 1.00E+01 target copies/reaction). The arithmetic mean ($\bar{x}$), the standard deviation (σ) and the coefficient of variation (CV %) of the triplicate analyses were calculated.

The test shows inter-lot precision with a mean overall inter-assay precision equal to 8.7% in the range from E+6 to E+3 target copies/reaction.

11.3. Compatibility with different RNA extraction methods

Furthermore, the compatibility of ViroReal® Kit RT-LAMP SARS-CoV-2 with the QIAamp® Viral RNA Mini Kit and the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (using the KingFisher Flex extraction instrument) was validated. Extraction was performed from SARS-CoV-2 negative sputum and oropharyngeal swabs 2 ml of isotonic saline. Volumes of 140 µl – 150 µl sample material were extracted and eluted in 60 µl nuclease-free water or 150 µl elution buffer, respectively.
Four µl of the RNA extracts were then spiked with a commercially available SARS-CoV-2 RNA (AMPLIRUN® Coronavirus SARS-CoV-2 RNA Control in different concentrations (150, 100 and 50 copies/reaction). Samples were then analysed with the ABI® 7500.

Testing showed that the analytical sensitivity of the assay was comparable, irrespective of the extraction platform used, see Table 6. Nevertheless, RNA extracts of the sputum showed a shift in Cq values and a color change from red to yellow-orange, indicating that this sample material partially inhibits the RT-LAMP reaction. Furthermore, the color of the reaction in tubes containing the RNA extracts obtained by the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit showed change from red to orange, indicating that the elution buffer of the extraction kit influences RT-LAMP reaction.

Table 6 Testing of two different extraction methods

	Average Cq values and color in reaction tubes (Y=yellow, YO=yellow-orange, O=orange)	
	QIAamp® Viral RNA Mini Kit	MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit
Extracted sputum + 150 target copies/reaction	43.4 (YO)	38.4 (O)
Extracted sputum + 100 target copies/reaction	43.0 (YO)	38.9 (O)
Extracted sputum + 75 target copies/reaction	48.0 (YO)	38.5 (O)
Extracted swab + 150 target copies/reaction	28.1 (Y)	27.2 (O)
Extracted swab + 100 target copies/reaction	26.9 (Y)	29.7 (O)
Extracted swab + 75 target copies/reaction	29.7 (Y)	29.6 (O)
150 target copies/reaction	26.9 (Y)	
100 target copies/reaction	28.1 (Y)	
75 target copies/reaction	35.5 (Y)	

11.4. Specificity

Analytical specificity is ensured by the selection of specific primers. A conserved region of the ORF1ab of SARS-CoV-2 was selected as target region. In *silico* validation of the primers was performed with the basic local alignment tool (BLAST) in the NCBI database and with sequence analyses in the GISAID database. It was checked for potential homologies to currently published sequences.

Primer sequences were matched with 59,000 published genomes of SARS-CoV-2 (as of September 16, 2020). A variant (C2416T, frequency 937/59600, 1.572%) is described in the 5´ section of the BI primer.

ViroReal® Kit RT-LAMP SARS-CoV-2 specifically detects the ORF1ab region of SARS-CoV-2. Other beta-corona viruses are not detected with this test.

Specificity was determined by testing against respiratory viruses and SARS-CoV-2. A total of 20 ring trial samples from INSTAND e.V. were retrospectively analysed. All samples were correctly analysed with ViroReal® Kit RT-LAMP SARS-CoV-2. Data were compared to results obtained with the appropriate virus-specific ViroReal® Kits (Table 7).

Table 7 Viral isolates tested

		ViroReal® Kit RT-LAMP SARS-CoV-2		Specific ViroReal® Kit
Sample No.	**Sample Name**	**Cq FAM channel**	**Color in reaction tube**	**Cq**
1	370055_Influenza B Virus Massachusetts 2/2012, seasonal	Negative	Red	24.7
2	370056_Influenza A Virus California 7/2009, H1N1, pdm 09	Negative	Red	26.4
3	370057_Influenza A Virus Victoria 361/2011, H3N2, seasonal	Negative	Red	26.3
4	370059_Influenza B Virus Brisbane/20/2008, seasonal	Negative	Red	24.3
5	372043_Enterovirus D68	Negative	Red	23.4
6	372044_Coxsackie-Virus B3	Negative	Red	28.9
7	371041_Adenovirus AdV 11	Negative	Red	23.8
8	359021_RSV A	Negative	Red	23.0
9	359023_RSV A	Negative	Red	22.6
10	359022_RSV B	Negative	Red	19.3
11	385005_MPV A	Negative	Red	31.9
12	385009_MPV A	Negative	Red	27.3
13	385011_MPV A	Negative	Red	30.3
14	393009_Rhinovirus A, type 56	Negative	Red	24.7
15	393012_Rhinovirus A, type 49	Negative	Red	20.5
16	393011_Rhinovirus A, type 56	Negative	Red	30.9
17	340067_MERS CoV	Negative	Red	24.4
18	340068_HCoV 229E	Negative	Red	25.4
19	340072_HCoV NL63	Negative	Red	26.7
20	340074_HCoV OC43	Negative	Red	25.3
21	Negative extraction control	Negative	Red	Negative

11.5. Diagnostic evaluation

The diagnostic evaluation was performed externally (Clinic Donaustadt, Vienna and Austrian Armed Forces) including 392 native oropharyngeal swabs from patients with and without suspected COVID-19. Oropharyngeal swabs were collected with a cotton swab and swirled in 2 ml isotonic saline solution.

As a reference method or gold standard, nasopharyngeal swabs from the respective individuals were tested with two in-house real-time PCR tests detecting the E and N genes of SARS-CoV-2 (Corman et al., 2020 and CDC protocol with N1 primers, respectively).

The study included 110 SARS-CoV-2 infected patients who were hospitalized at the Clinic Donaustadt. The onset of symptoms in many patients had already occurred a few days earlier and as a result, many samples showed a relatively low viral load. Real-time PCR and RT-LAMP analyses were performed in single runs on the MX3000P (Agilent).

Furthermore, 282 recruits of the Austrian Armed Forces were screened for Covid-19. Real-time PCR and RT-LAMP analyses were performed on the LightCycler® 480 (Roche Diagnostics).

From a total of 392 samples, 78 samples were positive in real-time PCR (gold standard) with either one or both genes. Twenty samples out of these 78 samples showed a Cq value ≤31 in the real-time PCR, 19 thereof

were positive in the RT-LAMP. The remaining 58 real-time positive samples showed a Cq value >31 in the real-time PCR, 11 thereof were positive in the RT-LAMP.

Previously unpublished results from diagnostic testing at the Robert Koch Institute (RKI) show that the loss of cultivability in cell culture was correlated with an RNA amount of <250 copies/5 µl RNA eluate as determined by real-time PCR. This RNA concentration corresponded to a Cq-value >30 in the test system used. Thus, the infectivity of patients with real-time PCR Cq-values from approx. 31 onwards can be classified as low. Nevertheless, a beginning infection with a still low virus load cannot be excluded.

The intended use of ViroReal® Kit RT-LAMP SARS-CoV-2 is the detection of SARS-CoV-2 in persons who are in an infectious stage. Therefore, when evaluating the clinical data, those 58 real-time positive samples with a Cq-value >31 were excluded from statistical analysis, see Table 8 and Table 9.

Samples with an RNA concentration of approx. 20,000 target copies per ml (approx. 100 copies per RT-LAMP reaction, corresponding to a real-time PCR Cq value of approx. 31) can be detected with ViroReal® Kit RT-LAMP SARS-CoV-2 with a sensitivity of 95% and a specificity of 99%.

Table 8 Diagnostic evaluation of ViroReal® Kit RT-LAMP SARS-CoV-2

		Reference		
		pos	**neg**	**Total**
ViroReal® Kit RT-LAMP SARS-CoV-2	**pos**	**19**	**3**	**22**
	neg	**1**	**311**	**312**
	Total	**20**	**314**	**334**

Table 9 Diagnostic performance

	Value	95% CI
Sensitivity	95.00%	75.13% to 99.87%
Specificity	99.04%	97.23% to 99.80%
NPV	99.70%	97.87% to 99.95%
PPV	86.36%	67.15% to 95.15%
Prevalence	5.99%	3.70% to 9.10%
Accuracy	98.80%	96.96% to 99.67%

12. References

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