SARS-CoV-2 RT-PCR

Kit for the detection of Severe Acute Respiratory Syndrome-related Coronavirus 2 (SARS-CoV-2) by One-Step Real-Time RT-PCR

Please refer to the correct Instructions for use according to the format used:

Vitro S.A.
Calle Luís Fuentes Bejarano 60 Ed. Nudo Norte Local 3 41020 Sevilla (Spain)
Tel: +34 954 933 200. vitro@vitro.bio ; www.vitro.bio

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Kit for the detection of Severe Acute Respiratory Syndrome-related Coronavirus 2 (SARS-CoV-2) by One-Step Real-Time RT-PCR

REF **Ref. MAD-003941M**
Ref. MAD-003941M-OP
Ref. MAD-003941M-EX

100 determinations

For in vitro diagnostic use only
Directive 98/79/EC

Vitro S.A.
Calle Luís Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Sevilla (Spain).
Tel: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

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1 INTENDED USE

The **SARS-CoV-2 RT-PCR** kit is an in vitro diagnostics kit for the qualitative detection of the RNA of the Severe Acute Respiratory Syndrome-related Coronavirus 2, taking as starting sample the RNA from human clinical samples of different origin, such as, naso- and oropharyngeal swabs, bronchoalveolar lavages (BAL) and saliva. It is based on a multiplex one-step real-time RT-PCR assay, using primers and fluorescence-labeled probes specific for the gene targets N (independent regions N1 and N2) and E from SARS-CoV-2, following the methodology recommended by the WHO.

The kit is designed for the universal detection of SARS-like coronaviruses with the primers and probe sets of the E gene (Corman et al, 2020) and for the specific detection of SARS-CoV-2 with the primers and probe sets of the N gene (N2 probe from the CDC protocol).

Specific fluorescent primers and probes are also included for the simultaneous detection of the human RNaseP gene as internal quality control of the starting and amplification material. The detection channels of the different targets are:

Target	Fluorophore
N gene (independent regions N1 and N2)	FAM
E gene	ROX
RNaseP	JOE

Table 1. Detection channels for the different targets of the SARS-CoV-2 RT-PCR kit

Microbiological status: Non-sterile product.

2 INTRODUCTION

Coronaviruses are positive single-stranded RNA viruses, members of the Orthocoronavirinae subfamily within the Coronaviridae family (Nidovirales order). This subfamily encompasses four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus* according to their genetic structure. Alphacoronaviruses and betacoronaviruses infect only mammals and they usually cause acute respiratory tract infections in humans and gastroenteritis in animals. Up to the appearance of SARS-CoV-2, six *alpha- and betacoronaviruses* were described in humans. Four of them (HCoV-NL63, HCoV-229E, HCoV-OC43 and HKU1) cause a considerable number of mild upper respiratory tract infections in immunocompetent adults, but they can also cause a more serious symptomatology in children and geriatric patients with winter seasonality. The betacoronaviruses SARS-CoV and MERS-CoV, both emerging pathogens, provoked two outbreaks that caused severe epidemic respiratory infections on a global scale due to their morbidity and mortality. SARS-CoV-2betacoronavirus is the seventh isolated and characterized coronavirus able to cause infections in humans.

SARS-CoV-2 was first identified in China in December 2019 as a viral agent that causes respiratory tract infections with symptoms such as fever, dry cough and respiratory failure. In more severe cases, the infection can cause pneumonia, renal failure and death.

The transmission occurs by direct contact with infected people or through saliva, coughing or sneezing.

The rapid diagnosis of the SARS-CoV-2 infection is essential to stop the virus spread. In this context, real time RT-PCR is the most suitable technique for the detection of the virus due to its high sensitivity and specificity, and it is now a routine tool in medical laboratories.

3 COMPONENTS

The **SARS-CoV-2 RT-PCR** kit is commercialized as a ready-to-use Master Mix which includes all the necessary reagents to perform the real-time RT-PCR.

Furthermore, in order to avoid contamination with previous PCR products, the Mix contains the enzyme Uracil-DNA Glycosylase (Cod-UNG), which degrades PCR products containing dUTP.

A positive control (PC) and DNase/RNase-free DEPC-treated water to use with the negative controls (NTC), are supplied in the kit.

Components of the kit for 100 tests:

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003941M-100 (SARS-CoV-2 MMIX)	**MAD-003941-MIX (SARS-CoV-2 MMix)**	Reverse transcriptase, Hot Start DNA Polymerase, Uracil DNA glycosylase, primers, fluorescent probes, reaction buffer, dNTPs (dATP, dCTP, dGTP, dTTP, dUTP)	**2 vials with 50 tests/vial**
	MAD-DDW-DEPC (RNAse/DNAse free water)	---	**1 vial (200 µl)**
MAD-COV-2 (SARS-CoV-2 PC)		Synthetic non-infectious DNA containing part of the genome of SARS-CoV-2 and human DNA	**1 vial (100 µl)**

Table 2. Reagents supplied in the SARS-CoV-2 RT-PCR kit.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

- Disposable gloves.
- DNAse/RNAse-free filtering pipette tips.
- RNA extraction kit.
- Tube strips/plates/optical adhesive films specific for each equipment of Real-Time PCR

4.2 Equipment

- Laminar flow cabinet
- Microcentrifuge for tubes of 1.5ml.
- Microcentrifuges of PCR tube strips or 96-well plates.

- Vortex.
- Automatic micropipettes: P1000, P200, P20 and P2.
- Real Time-PCR instrument.

5 STORAGE AND STABILITY CONDITIONS

The **SARS-CoV-2 RT-PCR** kit must be transported and stored at -10 to -30 °C*. However, in addition to the recommended transport at -10 to -30 °C, it is also possible to carry out the transport at cooling temperature (2 °C - 8 °C) as long as the transit time does not exceed a maximum of ten days. In any case, once received, the kit must be stored at -10 to -30 °C.

The reaction mix **SARS-CoV-2 MMix** is sensitive to physical state changes and it has been proven that it supports up to seven freeze-thaw cycles. If a run is performed with a low number of samples, it is recommended to aliquot the reagent in advance. The mix contains fluorescent molecules and it must be kept away from direct light.

The positive control is sensitive to physical state changes and it must not undergo more than eight freeze-thaw cycles. It is advisable to handle the positive control vial separately from the clinical samples to avoid potential contamination which might yield false positives.

If stored at recommended temperature, the PCR reagents are stable until the expiration date indicated. The PCR reagents must be stored in areas free of DNA or PCR products contamination.

*A temperature indicator is included in the package to control the conditions during the shipment. In case the cold chain is interrupted, it is recommended to contact the manufacturer before using the reagents.

6 WARNINGS AND PRECAUTIONS

- Read the instructions for use before using this product.
- The kit must be handled by qualified technicians who are trained in molecular biology techniques applied to diagnosis.
- Do not use any component of the kit after the expiration date.
- The SARS-CoV-2 MMix must be thawed before use and handled on ice or cold plate and away from light. Mix the solutions by inverting the tubes several times without shaking in vortex. Then, centrifuge briefly.
- The positive control must be thawed at room temperature, mix well and centrifuge briefly before use.
- The safety and disposal precautions are described in the Safety Data Sheet of this product. This product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- The SARS-CoV-2 RT-PCR kit uses nucleic acids previously extracted and purified as starting material. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.
- General considerations to avoid RNA degradation with ribonucleases (RNases)

RNases are very stable enzymes, hard to inactivate, that get to degrade RNA quickly. The introduction of RNases in the test sample and the reagents used for the RT-PCR must be avoided by taking the following precautions:

- Work in a clean RNase-free area. The main RNase contamination source comes from skin and dust particles, which are bacterial and fungal carriers.
- Always use disposable gloves to prevent RNase contamination from the skin.
- Change the gloves frequently and keep the tubes closed.
- Use RNase-free tubes and pipette tips.
- Work quickly to avoid RNA degradation by residual and endogenous RNases during the whole preparation process of the sample to be amplified.

- **General considerations to avoid the contamination with PCR product:**

  The most important contamination source is usually the same amplified PCR product. Therefore, it is recommended to carry out the amplification and handling of the amplified products in a different area to the one where the RNA extraction and PCR preparation are performed. It is recommended to work in different pre- and post-PCR areas where the handling of the test RNA and preparation of the PCR tubes (pre-PCR), and the amplification and handling of the amplified products (post-PCR) are performed. These areas must be physically separated, and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, in order to avoid the contamination with previous PCR products, the enzyme *Uracil-DNA Glycosylase (Cod-UNG)*, which degrades the PCR products containing dUTP, is included in the kit.

  It is recommended to include negative amplification controls replacing the RNA sample with RNase/DNase-free water, in order to detect and control any possible contamination of the reagents with test samples or amplified products.

- **Waste disposal:**

  The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of wastes generated by this kit according to the European Law, specifically according to the *European Commission Decision of December 18 2014* amending decision 2000/532/CE on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTES GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW*
1. Liquid waste disposal	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, these wastes must be considered as "wastes whose storage and disposal is subjected to special requirements in order to prevent infection"
2. Perishable material (tubes, tips, etc.) 3. Any element that has been in contact with the starting genetic material	180103	"Wastes whose collection and disposal is subject to special requirements in order to prevent infection"
4. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers having residues of or contaminated by dangerous substances"

Table 3. Classification of wastes generated by this kit according to the European Legislation. *ELW: *European Legislation of Waste.*

*Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of materials.

7 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS

7.1 Sample collection

The **SARS-CoV-2 RT-PCR** kit has been validated for its use with purified genetic material from bronchoalveolar lavage, naso- and oropharyngeal swabs and saliva.

The samples from bronchoalveolar lavage are taken from hospital patients with a bronchoscope through the installation and subsequent aspiration of liquid from one or two pulmonary segments or sub-segments.

In the case of naso- and oropharyngeal swabs, the swab is introduced carefully into the posterior part of the nasal cavity or pharynx. The tip to be used must be of polyester, rayon or nylon, with a soft and flexible handle of plastic (swabs with tip of calcium alginate or cotton must not be used). Once inserted, the swab is held in the same place for about 10 seconds and, after that, it is placed in a dry sterile tube or preferably in a tube with transport medium (eg UTM universal transport medium) to preserve the integrity of the sample.

For collection of saliva samples it is recommended do not eat, drink, smoke or chew gum for 30 min before taking the sample. Collected specimens can be stabilized for transporting.

The samples are collected in a sterile recipient and kept at 2-8 °C for a maximum of 5 days. Once the samples are classified, or for prolonged storage, they are stored at -80 °C in order to preserve the viral viability. The nucleic acids extracted must be stored at -80 °C.

7.2 Nucleic acids extraction from bronchoalveolar lavages, naso- or oropharyngeal swabs and saliva

The **SARS-CoV-2 RT-PCR** kit has been tested with purified genetic material from human bronchoalveolar lavages, naso- and oropharyngeal swabs and saliva. This kit has been validated with starting genetic material from the following DNA/RNA purification kits and extraction equipment* from 200 µl of clinical sample and eluting in 100 µ of elution buffer (for purification with Opentrons, start with 92 µl of clinical sample and elute in 50 µl of elution solution):

EXTRACTION KITS	EXTRACTION EQUIPMENT
MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics)	MagNA Pure Compact Instrument. Version 1.1.2 (Roche Diagnostics)
QIAsymphony Certal Kits (Qiagen)	QIAsymphony SP (Qiagen)
RNeasy Mini QIAcube Kit (Qiagen)	QIAcube (Qiagen)
PureLink Viral RNA/DNA extraction mini kit (Invitrogen)	Manual system
Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega)	Maxwell® 16 (Promega)
NX48S – Viral NA Kit (Genolution)	Nextractor NX-48S (Genolution)
RNA/DNA viral extraction kit (Robot Opentrons) (Vitro, ref. MAD-003955M)	Opentrons OT-2

Table 4. Extraction kits and instruments used for the purification of DNA/RNA from clinical samples.

*Note: The system has not been validated with other DNA/RNA extraction systems. Therefore, if any other purification system is used, this must be previously verified.

8 PCR PROTOCOL

8.1 Preparation of the Reaction mix

The RT-PCR reaction is carried out in a final volume of 20 µl. Prepare the Master Mix as indicated below:

1. Thaw and homogenize SARS-CoV-2 MMix (do not use vortex), once it is thawed, centrifuge briefly.
2. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
SARS-CoV-2 MMix	12 µl
Sample	8 µl

3. Include a negative control by adding 8 µl of the water included in the kit.

4. Include a positive control by adding 8 µl of the positive DNA control SARS-CoV-2 PC included in the kit.
5. Centrifuge briefly to make sure there are no air bubbles in the wells and.

It is recommended to keep the MMix on cold plate during the preparation of the samples and do not freeze-thaw more than five times.

8.2 Set the real-time PCR instrument

In the instrument's software, enter the different targets and detection channels for each of them. Create the samples, the positive control (PC), the negative control (NTC) and allocate the positions of the samples in the PCR plate.

Set the real-time PCR instrument following the steps below:

PCR PROGRAM		
25 °C	5 min	1 cycle
50 °C	15 min	1 cycle
95 °C	5 min	1 cycle
95 °C	15 sec	45 cycles
56 °C*	40 sec	

Table 5. PCR program of the SARS-CoV-2 RT-PCR kit.

*The fluorescence data must be collected during the extension stage (*) by means of the FAM channels (N gene), ROX (E gene) and HEX, JOE or VIC (Internal Control).

This kit has been validated with the platforms:

- QuantStudio™ 3 Real-Time PCR System (Applied Biosystems)
- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- 7500 Real-Time PCR System (Applied Biosystems)
- StepOne Plus™ Real-Time PCR System (Applied Biosystems)
- StepOne™ Real-Time PCR System (Applied Biosystems)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- Rotor – Gene – Q (Qiagen)

Before using other thermal cyclers, it is recommended to verify the compatibility of the fluorochromes with the fluorescence detection channels of the instrument. The fluorochromes included in the kit are compatible with the most common real time PCR equipments in the market.

In the thermal cyclers Applied Biosystems 7500 Fast Real-Time PCR System, Applied Biosystems StepOne™ Real-Time PCR System, Applied Biosystems QuantStudio™ 3, QuantStudio™ 5 Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System, the option of the passive control ROX must be disabled. In the thermal cyclers Applied Biosystems QuantStudio™ 3, 5 Real-Time PCR System and Applied Biosystems

7500 Fast Real-Time PCR System, select Ramp Speed Standard in the menu "Select New Experiment/Advanced Setup/Experiment Properties".

9 INTERPRETATION OF RESULTS

Before analyzing the results of the clinical samples, it is required to follow the interpretation guide of the positive and negative controls as follows:

	RESULT	INTERPRETATION
Positive Control SARS-CoV-2	Signal for the channels FAM, ROX and JOE*	The control/reaction is correct
	No signal for FAM and/or ROX and/or JOE	Problem in the amplification: repeat analysis
Negative control	Signal for the channels FAM and/or ROX and/or JOE	Contamination, repeat analysis
	No signal	The control/reaction is correct

*The amplification signal must be determined by a rapid and steady increase in the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular background or increased background noise) (Fig 1).

The run is considered valid when correct results have been obtained for all the reaction controls.

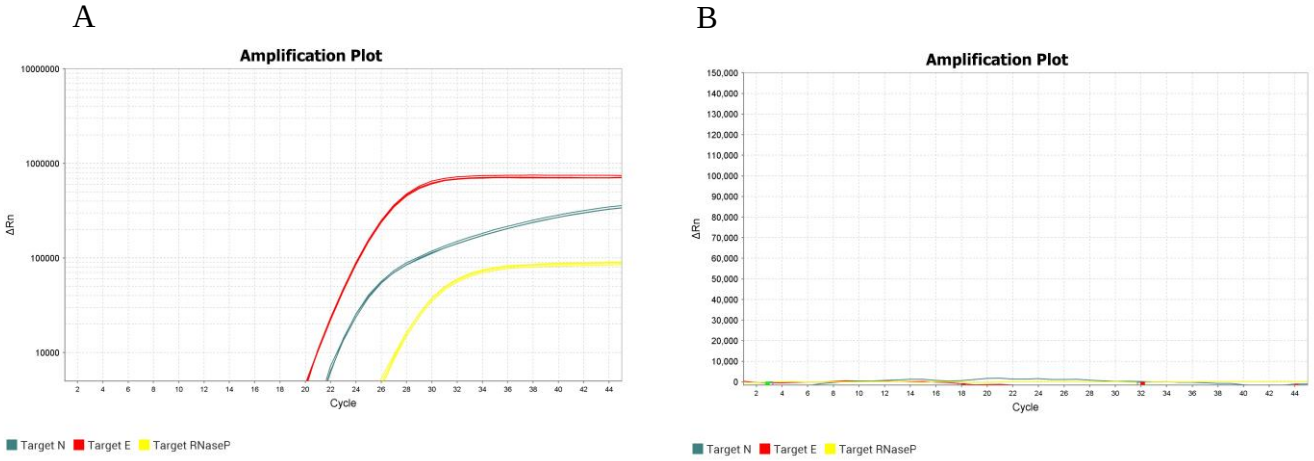

Figure 1: Graphs of amplification of the positive control (A) and a no template control (B). (Ct values for the PC: N (FAM) 21±2; E (ROX) 23 ±2; RNasaP (JOE) 20±2). Experiment performed in Applied Biosystems QuantStudio™ 3 Real-Time PCR System.

Once the run has been validated, interpret the results of the clinical samples according to the following table:

SARS-CoV-2 RT-PCR			INTERPRETATION
FAM (N gene)	ROX (E gene)	JOE (Internal control)	
Signal	Signal	Signal	Positive sample for SARS-CoV-2
		No signal	
No signal	No signal	Signal	Negative sample for SARS-CoV-2[(1)]
		No signal	Invalid sample, repeat analysis
Signal	No signal	Signal	Uncertain Results [(2)]
		No signal	Problems in the extraction or amplification: repeat analysis
No signal	Signal	Signal	Uncertain Results [(2)]
		No signal	Problems in the extraction or amplification: repeat analysis

[(1)] Negative or below the limit of detection of the kit.

[(2)] It is recommended to repeat the PCR or start from a new RNA extraction.
In a pandemic situation the results can be accepted as positive for SARS-CoV-2 if only one of the two genes E or N gives positive results.

It is recommended to use the default threshold line stablished automatically by the instrument. If required, the threshold line can be manually adjusted until it lies within the exponential phase of the fluorescence curves and above any background signal.

A sample is positive if the Ct value obtained is ≤38 and the internal control shows or not an amplification graph. Sometimes, it might occur that the internal control is not amplified correctly due to the presence of a high initial number of copies of target viral nucleic acid, which can cause a preferential amplification of the latter.

A sample is negative if an amplification curve is not detected over the threshold value, and if the internal control does show it. The inhibition of the PCR reaction can be excluded by the amplification of the internal control.

10 PERFORMANCE CHARACTERISTICS

10.1 Analytical sensitivity

The analytical sensitivity of the SARS-CoV-2 RT-PCR was determined by making six replicas of serial dilutions of synthetic fragments from each of the N and E genes at a known concentration.

It has been established that the SARS-CoV-2 RT-PCR kit has a limit of detection (LoD) of 10 copies/reaction for the N and E genes (Figure 2).

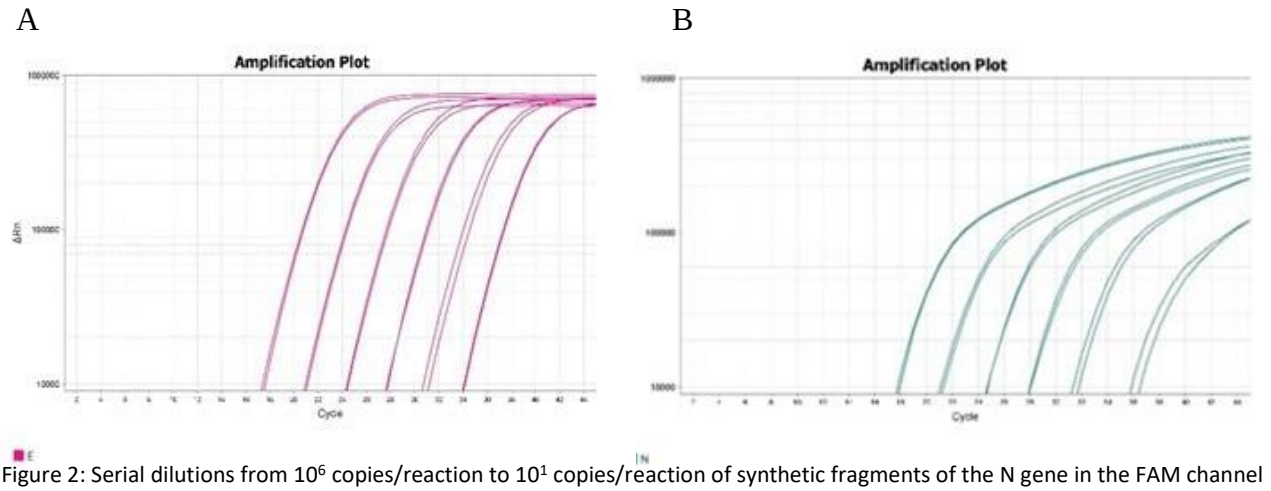

Figure 2: Serial dilutions from 10^6 copies/reaction to 10^1 copies/reaction of synthetic fragments of the N gene in the FAM channel (A) and E gene in the ROX channel (B).

The amplification efficiency for each of the targets was evaluated with six serial dilutions of a standard of SARS-CoV-2 from 10^6 copies/rxn. By adjusting the Cts data to a line, the amplification efficiency, R^2 and the slope were determined for each of the genes (fig 3).

The N gene showed an efficiency of 90.15%, an R^2 of 0,996 and a slope of -3.58. The E gene showed an efficiency of 99.95%, an R^2 of 0.999 and a slope of -3.32. The SARS-CoV-2 RT-PCR kit detects both genes at 10 copies/reaction.

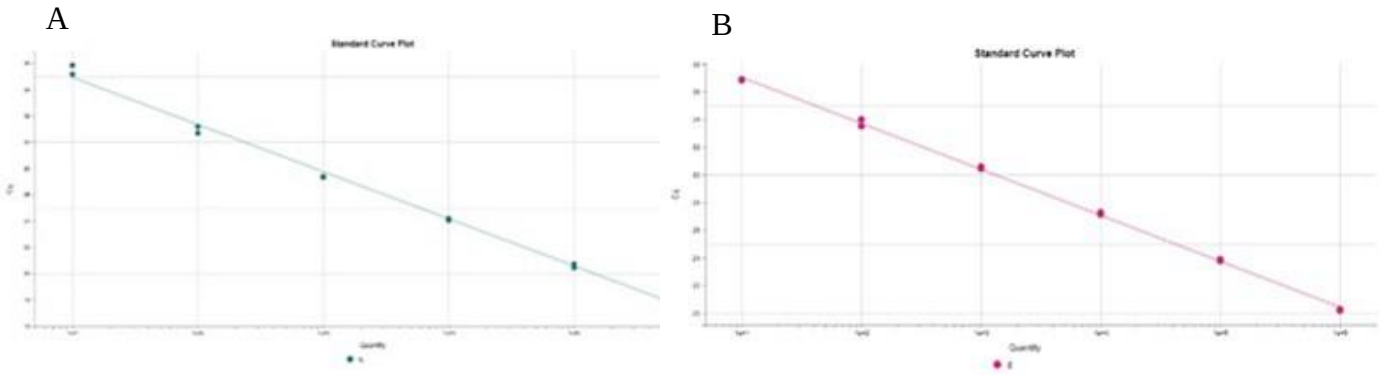

Figure 3: Calibration lines of the N gene in the FAM channel (A) and the E gene in the ROX channel (B) of a standard SARS-CoV-2.

10.2 Analytical specificity

The specificity of the test of the SARS-CoV-2 was confirmed by testing positive clinical samples for different microorganisms that represent the most common respiratory pathogens. No cross-reactions were detected with any of the following pathogens tested:

Cross-reactivity test	
Microorganism	**Results**
Adenovirus	Negative
Bordetella parapertussis	Negative
Bordetella pertusis	Negative
Candida albicans	Negative
Human bocavirus	Negative
Human coronavirus 229E	Negative
Human coronavirus HKU1	Negative
Human coronavirus NL63	Negative
Human coronavirus OC44	Negative
Human metapneumovirus	Negative
Human parainfluenza virus type 1	Negative
Human parainfluenza virus type 2	Negative
Human parainfluenza virus type 3	Negative
Human parainfluenza virus type 4	Negative
Human rhinovirus	Negative
Influenza A virus subtype H1N1	Negative
Influenza A virus subtype H3	Negative
Influenza B virus	Negative
Mycobacterium tuberculosis	Negative
Mycoplasma pneumoniae	Negative
Pseudomonas aeruginosa	Negative
Respiratory Syncytial Virus (RSV) A	Negative
Respiratory Syncytial Virus (RSV) B	Negative
Staphylococcus aureus	Negative
Streptococcus pneumoniae	Negative
Streptococcus pyogenes	Negative

10.3 Clinical sensitivity and specificity

The SARS-CoV-2 RT-PCR kit was tested with a panel of 40 respiratory samples from the biobank of the Spanish National Center of Microbiology (NCM-ISCIII). The sample set included 19 positive and 21 negative samples, which had been previously characterized by a reference method recommended by the WHO (Corman et al, 2020). The results obtained with the SARS-CoV-2 RT-PCR kit show a total of 19 positive and

21 negative samples, with 100% sensitivity and specificity.

SARS-CoV-2 RT-PCR kit was also validated with extracted RNA from 49 respiratory clinical samples from naso-and oropharyngeal swabs and bronchoalveolar lavages, the results were compared with those obtained with a reference molecular detection method. The results are summarized in the table below and show a 100% of sensitivity and specificity of the kit to detect SARS-CoV-2.

	Reference Method		
SARS-CoV-2 RT-PCR kit	**+**	**-**	**Total**
+	**35**	**0**	**35**
-	**0**	**14**	**14**
Total	**35**	**14**	**49**

11 LIMITATIONS OF THE TEST

1. The results of the test must be evaluated by a health care professional in the context of medical history, clinical symptoms, and other diagnostic tests.

2. This test can be used with different types of samples, although it has only been validated with RNA extracted from respiratory samples (naso- oropharyngeal swabs, bronchoalveolar lavage and saliva).

3. The correct performance of the test depends on the quality of the sample; the nucleic acid must be properly extracted from the clinical samples. Improper collection, storage and/or transport of samples can result in false negatives.

4. A low number of target copies below the detection limit can be detected, but the results may not be reproducible.

5. A positive test for SARS-CoV-2 does not exclude the possibility that other pathogens are present.

6. The test works within the genomic regions in which the probes have been designed. Due to the high variability of RNA, certain subtypes may not be detected. However, at the time of design, no mutations were observed in the target regions after alignment with all deposited SARS-CoV-2 sequences.

7. Negative results do not exclude SARS-CoV-2 infection and should not be used as the only method for the patient management decisions.

8. Negative results must be combined with patient's medical history, and epidemiological information.

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13 LABEL AND BOX SYMBOLS

IVD	Health product for in vitro diagnosis.		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions of use		Sufficient content for <n> assays
	Material safety data sheet		Keep away from sunlight

14 CHANGELOG

Date	Description
2020-11-09	• Inclusion of the section changelog. • Inclusion of the explanation of the pictogram of the Safety Sheet. • A note referring to a pandemic situation is included in section 9.
2020-12-03	• Cross-reactivity table in section 10.2 is updated.
2021-04-14	• A new sample is included in section 7.
2021-05-20	• Two new nucleci acid kits and extraction equipment are included.
2021-06-11	• A clarification on the quantity of starting sample for the extraction with Opentrons is included in section 7.2
2021-09-28	• Temperature is adjusted in section 5 Storage And Stability Conditions
2022-03-08	• Inclusion of the explanation of the pictogram “Keep away from sunlight”.
2023-01-24	• In section 1 "Intended Use" it is included that fluorescent primers and probes are used for the N target genes, with independent regions for N1 and N2.

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1 INTENDED USE

The **SARS-CoV-2 RT-PCR** kit is an *in vitro* diagnostics kit for the qualitative detection of the RNA of the Severe Acute Respiratory Syndrome-related Coronavirus 2, from RNA from human clinical samples of different origin, such as nasopharyngeal and oropharyngeal exudates, bronchoalveolar lavages (BAL) and saliva. It is based on the multiplex One-Step RT-PCR technique, using primers and fluorescent probes for the target genes N (independent regions N1 and N2) and E of the SARS-CoV-2 following the guidelines recommended by the WHO (11).

The kit is designed for the universal detection of SARS-like coronavirus through primers and probe of the E gene (Corman et al. 2020) and for the specific detection of SARS-CoV-2 through the primers and probe of the N gene (N2 probe described by the CDC).

Specific primers and fluorescent probe are included for the simultaneous detection of the human RNaseP gene as internal quality control of the starting and amplification material. The detection channels of the different targets are:

Target	Fluorophore
N gene (independent regions N1 and N2)	FAM
E gene	ROX
RNaseP	JOE

Table 1. Detection channels for the different targets of the SARS-CoV-2 RT-PCR kit

Microbiological status: Non-sterile product.

2 INTRODUCTION

Coronaviruses are single-stranded RNA virus belonging to the Orthocoronavirinae subfamily within the Coronaviridae family (Nidovirales order). This subfamily is divided into four categories: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus* and *Deltacoronavirus* according to their genetic structure. *Alpha* and *betacoronaviruses* infect only mammals and they usually cause acute respiratory tract infections in humans and gastroenteritis in animals. Up until the apparition of the SARS-CoV-2, six alpha and betacoronaviruses infecting humans were described. Four of them (HCoV-NL63, HCoV-229E, HCoV-OC43 and HKU1) cause a considerable number of mild upper respiratory tract infections in immunocompetent adults, but they can also cause a more serious symptomatology in children and geriatric patients with typically winter seasonality. The betacoronaviruses SARS-CoV and MERS-CoV, both emerging pathogens, provoked two outbreaks that caused severe epidemic respiratory infections on a global scale due to their morbidity and mortality. The betacoronavirus SARS-CoV-2 is the seventh isolated and characterized coronavirus able to cause infections in humans.

SARS-CoV-2 was first identified in China in December 2019 as a viral agent that causes acute respiratory tract infections with symptoms ranging from fever, dry cough and respiratory failure to more severe complications

Vitro S.A.
Calle Luís Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Sevilla (Spain).
Tel: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

such as pneumonia and renal failure leading to death. The transmission occurs by direct contact with infected people or through saliva droplets, coughing or sneezing.

The rapid diagnosis of the SARS-CoV-2 infection is essential to stop the virus spread. In this context, real time RT-PCR is the most suitable technique for the detection of the virus due to its high sensitivity and specificity, and it is now a routine tool in medical laboratories.

3 COMPONENTS

The **SARS-CoV-2 RT-PCR** kit is commercialized as a lyophilized Master Mix which includes all the necessary reagents to perform the real-time RT-PCR.

Furthermore, in order to avoid contamination with previous PCR products, the Mix contains the enzyme Uracil-DNA Glycosylase (Cod-UNG), which degrades PCR products containing dUTP.

A positive control (PC) and a reconstitution solution to reconstitute the lyophilized Master Mix and to include it in the negative controls (NTC) are supplied along with the RT-PCR Mix.

Components of the kit for 100 tests:

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003941M-100 (SARS-CoV-2 MMIX)	**MAD-003941-MIX* (SARS-CoV-2 MMix)**	Reverse transcriptase, Hot Start DNA Polymerase, Uracil DNA glycosylase, primers, fluorescent probes, reaction buffer, dNTPs (dATP, dCTP, dGTP, dTTP, dUTP)	**2 vials with 50 test/vial Lyophilized**
	MAD-LYO-SOL (Reconstitution solution)	---	**1 vial (1700 µl)**
MAD-COV-2 (SARS-CoV-2 PC)		Synthetic non-infectious DNA containing part of the genome of SARS-CoV-2 and human DNA	**1 vial (100 µl)**

Table 2. Reagents supplied in the SARS-CoV-2 RT-PCR kit

*__Before the first use__, open the glass vial containing the lyophilized Master Mix, shake the **Reconstitution Solution (MAD-LYO-SOL)** in vortex and hydrate the vial by adding **660 µl** of the Reconstitution Solution per vial.

The reconstituted mix is stable if stored at -20 ºC in the same glass vial by closing it with the stopper and the screw cap, for a maximum of 4 months. It is recommended do not freeze-thaw the vial once reconstituted more than five times.

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4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

- Disposable gloves.
- DNase/RNase-free filtering pipette tips.
- RNA extraction kit.
- Tube strips/plates/optical adhesive films specific for each equipment of Real-Time PCR

4.2 Equipment

- Laminar flow cabinet
- Microcentrifuge for tubes of 1.5ml.
- Microcentrifuges of PCR tube strips or 96-well plates.
- Vortex.
- Automatic micropipettes: P1000, P200, P20 and P2.
- Real Time-PCR instrument.

5 STORAGE AND STABILITY CONDITIONS

The **SARS-CoV-2 RT-PCR** kit must be transported at a temperature between 2 and 8 °C*, and long-term storage must be as indicated on the label for each of its components: SARS-CoV-2 MMIX (mastermix and reconstituent) at 2 and 8 °C and the SARS-CoV-2 PC (positive control) at -10 to -30°C.

Once reconstituted, the reaction mix **SARS-CoV-2 MMix** is stable for 4 months frozen stored at -10 to -30ºC and bears up to five freezing/thawing cycles. If a run is performed with a low number of samples, it is recommended to aliquot the reagent in advance. The mix contains fluorescent molecules and it must be kept away from direct light.

The positive control is sensitive to physical state changes and it must not undergo more than eight freeze-thaw cycles. It is advisable to handle the positive control vial separately from the clinical samples to avoid potential contamination which might yield false positives.

If stored at recommended temperature, the PCR reagents are stable until the expiration date indicated. The PCR reagents must be stored in areas free of DNA or PCR products contamination.

*A temperature indicator is included in the package to control the conditions during the shipment. In case the cold chain is interrupted, it is recommended to contact the manufacturer before using the reagents.

6 WARNINGS AND PRECAUTIONS

- Read the instructions for use before using this product.
- The kit must be handled by qualified technicians in molecular biology techniques applied to diagnosis.

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- Do not use any component of the kit after the expiration date.
- **The mix SARS-CoV-2 MMix must be reconstituted before first use.** Once reconstituted, it must be handled in ice or cold plate and away from light. Mix the solutions by inverting the tubes several times without shaking in vortex, and centrifuge briefly.
- The positive control must be thawed at room temperature, mixed well and centrifuged briefly before use.
- The safety and disposal precautions are described in the Safety Data Sheet of this product. This product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- The SARS-CoV-2 RT-PCR kit uses nucleic acids previously extracted and purified as starting material. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.
- General considerations to avoid RNA degradation with ribonucleases (RNases)

  RNases are very stable enzymes, hard to inactivate, that get to degrade RNA quickly. The introduction of RNases in the test sample and the reagents used for the RT-PCR must be avoided by taking the following precautions:

  - Work in a clean RNase-free area. The main RNase contamination source comes from skin and dust particles, which are bacterial and fungal carriers.
  - Always use disposable gloves to prevent RNase contamination from the skin.
  - Change the gloves frequently and keep the tubes closed.
  - Use RNase-free tubes and pipette tips.
  - Work quickly to avoid RNA degradation by residual and endogenous RNases during the whole preparation process of the sample to be amplified.

- **General considerations to avoid the contamination with PCR product**

  The most important contamination source is usually the same amplified PCR product. Therefore, it is recommended to carry out the amplification and handling of the amplified products in a different area to the one where the RNA extraction and PCR preparation are performed. It is recommended to work in different pre- and post-PCR areas where the handling of the test RNA and preparation of the PCR tubes (pre-PCR), and the amplification and handling of the amplified products (post-PCR) are performed. These areas must be physically separated and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, in order to avoid the contamination with previous PCR products, the enzyme *Uracil-DNA Glycosylase (Cod-UNG)*, which degrades the PCR products containing dUTP, is included in the kit.

  It is recommended to include negative amplification controls replacing the RNA sample with RNase/DNase-free water, in order to detect and control any possible contamination of the reagents with test samples or amplified products.

- **Waste disposal:**
  The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of wastes generated by this kit according to the European Law, specifically according to the European Commission Decision of December 18, 2014 amending decision 2000/532/EC on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTES GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW*
1. Liquid waste disposal	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, these wastes must be considered as "wastes whose storage and disposal is subjected to special requirements in order to prevent infection"
2. Perishable material (tubes, tips, etc.) 3. Any element that has been in contact with the starting genetic material	180103	"Wastes whose collection and disposal is subject to special requirements in order to prevent infection"
4. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers having residues of or contaminated by dangerous substances"

Table 3. Classification of wastes generated by this kit according to the European Legislation. *ELW: *European Legislation of Waste.*

*Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of materials.

7 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS

7.1 Sample taking

The **SARS-CoV-2 RT-PCR** kit has been validated for its use starting from purified genetic material from bronchoalveolar lavage, nasopharyngeal and oropharyngeal exudates and saliva.

The samples from bronchoalveolar lavage are taken from hospital patients with a bronchoscope through the installation and subsequent aspiration of liquid from one or two pulmonary segments or sub-segments.

In the case of nasopharyngeal and oropharyngeal exudates, these samples are taken with swabs. The swab is introduced carefully into the posterior part of the nasal cavity or the pharynx. The tip of the swab must be of polyester, rayon or nylon, with a soft and flexible handle of plastic (swabs with tip of calcium alginate

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or cotton must not be used). Once inserted, the swab is held in the same place for about 10 seconds and, after that, it is placed in a dry sterile tube, or preferably in a tube with transport medium (for example, Universal Transport Medium UTM) to preserve the integrity of the sample.

For collection of saliva samples it is recommended do not eat, drink, smoke or chew gum for 30 min before taking the sample. Collected specimens can be stabilized for transporting.

The samples are collected in a sterile recipient and kept at 2-8 °C for a maximum of 5 days. Once the samples are classified or for longer storage periods, they are stored at -80 °C in order to preserve the viral viability. The nucleic acids extracted must be stored at -80 °C.

7.2 Extraction of nucleic acids from bronchoalveolar lavages, nasopharyngeal and oropharyngeal exudates and saliva

The **SARS-CoV-2 RT-PCR** kit has been tested with purified genetic material from human bronchoalveolar lavages, naso- and oropharyngeal swabs and saliva. This kit has been validated with starting genetic material from the following DNA/RNA purification kits and extraction equipment* from 200 µl of clinical sample and eluting in 100 µ of elution buffer (for purification with Opentrons, start with 92 µl of clinical sample and elute in 50 µl of elution solution):

EXTRACTION KITS	EXTRACTION EQUIPMENT
MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics)	MagNA Pure Compact Instrument. Version 1.1.2 (Roche Diagnostics)
QIAsymphony Certal Kits (Qiagen)	QIAsymphony SP (Qiagen)
RNeasy Mini QIAcube Kit (Qiagen)	QIAcube (Qiagen)
PureLink Viral RNA/DNA extraction mini kit (Invitrogen)	Manual system
Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega)	Maxwell® 16 (Promega)
NX48S – Viral NA Kit (Genolution)	Nextractor NX-48S (Genolution)
RNA/DNA viral extraction kit (Robot Opentrons) (Vitro, ref. MAD-003955M)	Opentrons OT-2

Table 4. Extraction kits and instruments used for the purification of DNA/RNA from clinical samples.

*Note: The system has not been validated with other DNA/RNA extraction systems. Therefore, if any other purification system is used, this must be verified beforehand.

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8 PCR PROTOCOL

8.1 Preparation of the Reaction mix

Before the first use, open the glass vial containing the lyophilized Master Mix, shake the **Reconstitution Solution (MAD-LYO-SOL)** in vortex and reconstitute the vial by adding **660 µl** of that Reconstitution Solution per vial. Homogenize through 8 – 10 suctions with micropipettes. The liquid reconstituted mix must be used as follows:

1. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
SARS-CoV-2 MMix	12 µl
Sample	8 µl

2. Include a negative control by adding 8 µl of the Reconstitution Solution included in the kit.
3. Include a positive control by adding 8 µl of the positive DNA control SARS-CoV-2 PC included in the kit.
4. Centrifuge briefly to make sure there are no air bubbles in the wells.

Excess of reconstituted mix can be stored at -20 ºC in the same glass vial by closing with the stopper and the screw cap, for a maximum of 4 months to be used in subsequent assays.

It is recommended to keep the MMix on cold plate during the preparation of the samples and do not freeze-thaw the vial once reconstituted more than five times.

For the next uses, the RT-PCR reaction will be prepared as follows:

1. Thaw SARS-CoV-2 Mix and homogenize through several suctions-dispensations with micropipette (do not use vortex).
2. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
SARS-CoV-2 MMix	12 µl
Sample	8 µl

3. Include a negative control by adding 8 µl of the Reconstitution Solution included in the kit.
4. Include a positive control by adding 8 µl of the positive DNA control SARS-CoV-2 PC included in the kit.
5. Centrifuge briefly to make sure there are no air bubbles in the wells.

8.2 Configuration of the instrument for real-time PCR

Enter the different targets and detection channels for each of them in the instrument's software. Create the samples, the positive control (PC), the PCR targets (NTC) and allocate the positions of the samples in the PCR plate.

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Set the real-time PCR instrument following the steps below:

PCR PROGRAM		
25 °C	5 min	1 cycle
50 °C	15 min	1 cycle
95 °C	5 min	1 cycle
95 °C	15 sec	45 cycles
56 °C*	40 sec	
10 °C	∞	

Table 5. PCR program of the SARS-CoV-2 RT-PCR kit

*The fluorescence data must be collected during the extension stage (*) by means of the FAM (N gene), ROX (E gene) and HEX, JOE or VIC channels (Internal Control).

This kit has been validated with the platforms:

- QuantStudio™ 3 Real-Time PCR System (Applied Biosystems)
- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- 7500 Real-Time PCR System (Applied Biosystems)
- StepOne Plus™ Real-Time PCR System (Applied Biosystems)
- StepOne™ Real-Time PCR System (Applied Biosystems)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- Rotor – Gene – Q (Qiagen)

For its use in other platforms, it is recommended to verify the compatibility of the fluorochromes with the detection channels of each instrument. Although the fluorochromes included in the kit are compatible with the majority of the most-used real-time instruments available on the market.

In the thermal cyclers Applied Biosystems 7500 Fast Real-Time PCR System, Applied Biosystems StepOne™ Real-Time PCR System, Applied Biosystems QuantStudio™ 3, QuantStudio™ 5 Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System, the option of passive control ROX must be disabled.

In the thermal cyclers Applied Biosystems QuantStudio™ 3 or 5 Real-Time PCR System and Applied Biosystems 7500 Fast Real-Time PCR System, select Ramp Speed Standard in the menu “Select New Experiment/Advanced Setup/Experiment Properties”.

9 INTERPRETATION OF RESULTS

Before interpreting the results of the clinical samples, it is necessary to follow the interpretation guide of the positive and negative controls as in the table below:

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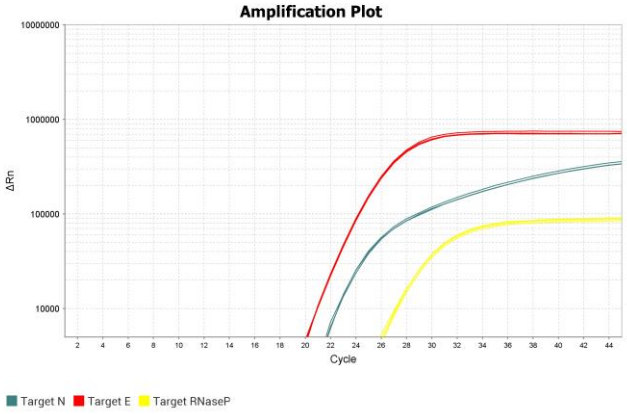
	RESULT	INTERPRETATION
Positive Control SARS-CoV-2	Signal for the channels FAM, ROX and JOE*	The control/reaction is correct
	No signal for FAM and/or ROX and/or JOE	Problem in the amplification: repeat analysis
Negative control:	Signal for the channels FAM and/or ROX and/or JOE	Contamination, repeat analysis
	No signal	The control/reaction is correct

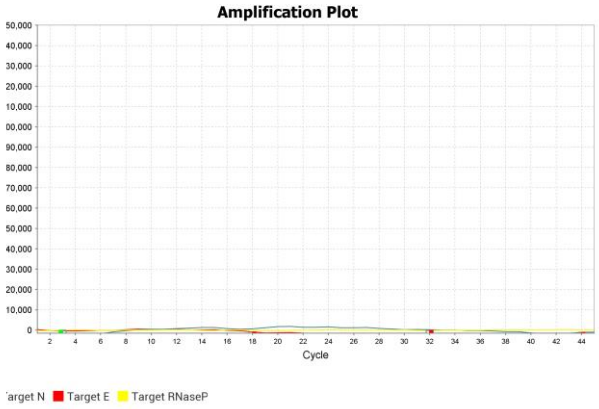
*The amplification signal must be determined by a rapid and steady increase in the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular background or increased background noise) (Fig 1).

The run is considered valid when adequate results have been obtained for all the reaction controls.

A



B



If the run has been validated, interpret the results of the clinical samples according to the following table:

SARS-CoV-2 RT-PCR			INTERPRETATION
FAM (N gene)	**ROX (E gene)**	**JOE (Internal control)**	
Signal	Signal	Signal	Positive sample for SARS-CoV-2
		No signal	
No signal	No signal	Signal	Negative sample for SARS-CoV-2[(1)]
		No signal	Invalid sample, repeat analysis
Signal	No signal	Signal	Uncertain Results [(2)]
		No signal	Problems in the extraction or amplification: repeat analysis

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No signal	Signal	Signal	Uncertain Results [2]
		No signal	Problems in the extraction or amplification: repeat analysis

[1] Negative or below the limit of detection of the kit.

[2] It is recommended to repeat the PCR or start from a new RNA extraction.
In a pandemic situation the results can be accepted as positive for SARS-CoV-2 if only one of the two genes E or N gives positive results.

It is recommended to use the automatic threshold adjustment made by the default software of each instrument and if necessary, the threshold can be adjusted manually ensuring that it falls within the exponential phase of the fluorescence curve and that the background noise is below the threshold line.

A sample is positive if the Ct value obtained is ≤38, although the internal control does not show an amplification graph. Sometimes, it might occur that the internal control is not amplified correctly due to the presence of a high initial number of copies of target viral nucleic acid, which can cause a preferential amplification of the latter.

A sample is negative if an amplification curve is not detected over the threshold value, and if the internal control does show it. The inhibition of the PCR reaction can be excluded by the amplification of the internal control.

10 PERFORMANCE CHARACTERISTICS

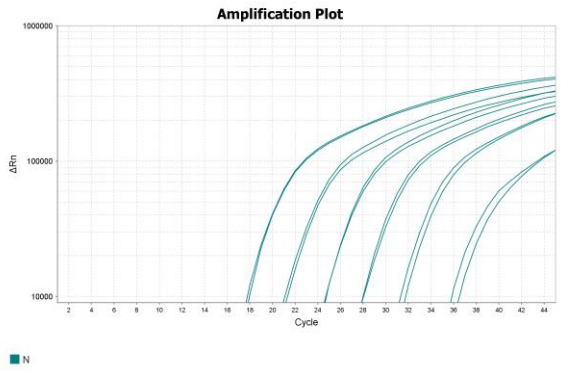
10.1 Analytical sensitivity

The analytical sensitivity of the SARS-CoV-2 RT-PCR kit was determined by making six replicas of serial dilutions of synthetic fragments from each of the N and E genes at a known concentration.

It has been established that the SARS-CoV-2 RT-PCR kit has a limit of detection of 10 copies/reaction for the N and E genes (Figure 2).

Vitro S.A.
Calle Luís Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Sevilla (Spain).
Tel: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

A



B

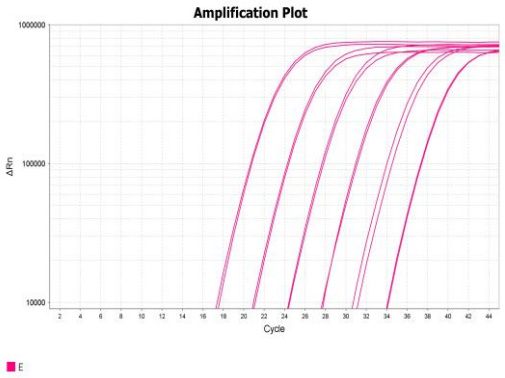
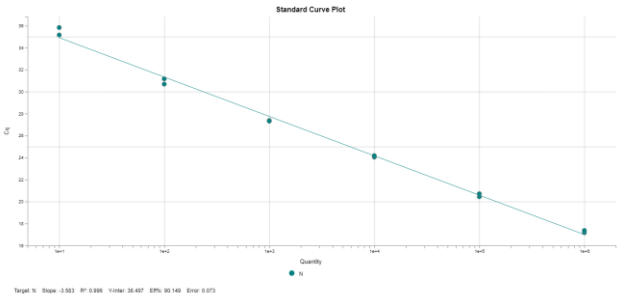


Figure 2: Serial dilutions from 10^6 copies/reaction to 10^1 copies/reaction of synthetic fragments of the N gene in the FAM channel (A) and E gene in the ROX channel (B).

The amplification efficiency for each of the targets was evaluated with six serial dilutions of a standard of SARS-CoV-2 from 10^6 copies/rxn. By adjusting the Cts data to a line, the amplification efficiency, R^2 and the slope were determined for each of the genes.

The N gene showed an efficiency of 90.15%, an R^2 of 0.996 and a slope of -3.58. The E gene showed an efficiency of 99,952%, an R^2 of 0.999 and a slope of -3.32. The SARS-CoV-2 RT-PCR kit detects both genes at 10 copies/reaction.

A



B

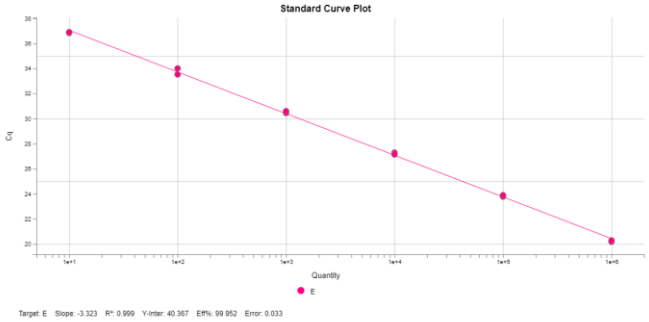


Figure 3: Calibration lines of the N gene in the FAM channel (A) and the E gene in the ROX channel (B) of a standard SARS-CoV-2.

10.2 Analytical specificity

The specificity of the test of the SARS-CoV-2 was confirmed by testing positive clinical samples for different microorganisms representing the most common respiratory pathogens. No cross-reactions were detected with any of the following pathogens tested:

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Calle Luís Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Sevilla (Spain).
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Cross-reactivity test	
Microorganism	**Results**
Adenovirus	Negative
Bordetella parapertussis	Negative
Bordetella pertusis	Negative
Candida albicans	Negative
Human bocavirus	Negative
Human coronavirus 229E	Negative
Human coronavirus HKU1	Negative
Human coronavirus NL63	Negative
Human coronavirus OC44	Negative
Human metapneumovirus	Negative
Human parainfluenza virus type 1	Negative
Human parainfluenza virus type 2	Negative
Human parainfluenza virus type 3	Negative
Human parainfluenza virus type 4	Negative
Human rhinovirus	Negative
Influenza A virus subtype H1N1	Negative
Influenza A virus subtype H3	Negative
Influenza B virus	Negative
Mycobacterium tuberculosis	Negative
Mycoplasma pneumoniae	Negative
Pseudomonas aeruginosa	Negative
Respiratory Syncytial Virus (RSV) A	Negative
Respiratory Syncytial Virus (RSV) B	Negative
Staphylococcus aureus	Negative
Streptococcus pneumoniae	Negative
Streptococcus pyogenes	Negative

10.3 Clinical sensitivity and specificity

The SARS-CoV-2 RT-PCR kit was evaluated with 40 anonymized respiratory samples from the National Center for Microbiology (NCM-ISCIII) biobank. This panel includes 19 positive samples and 21 negative samples, previously characterized according to the methodology recommended by the WHO and optimized at the NCM.

Vitro S.A.
Calle Luís Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Sevilla (Spain).
Tel: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

The results obtained with the SARS-CoV-2 RT-PCR kit show a total of 19 positive and 21 negative samples, with 100% sensitivity and specificity.

Another validation of the SARS-CoV-2 RT-PCR kit was performed with RNA extracted from 49 clinical samples of nasopharyngeal and oropharyngeal exudates and bronchoalveolar lavages. The results were compared with another RT-PCR reference method with CE-IVD marking. The results are summarized in the table below, showing 100% sensitivity and specificity of the kit to detect SARS-CoV-2.

	Reference method		
SARS-CoV-2 RT-PCR kit	**+**	**-**	**Total**
+	**35**	**0**	**35**
-	**0**	**14**	**14**
Total	**35**	**14**	**49**

11 LIMITATIONS OF THE TEST

1. The results of the test must be evaluated by a healthcare professional in the context of medical history, clinical symptoms, and other diagnostic tests.

2. This test can be used with different types of samples, although it has only been validated with RNA extracted from respiratory samples (nasopharyngeal and oropharyngeal smear, bronchoalveolar lavages and saliva).

3. The correct functioning of the test depends on the quality of the sample; the nucleic acid must be properly extracted from the clinical samples. Improper collection, storage and/or transport of samples can result in false negatives.

4. A low number of target copies below the detection limit can be detected, but the results may not be reproducible.

5. A positive test for SARS-CoV-2 does not exclude the possibility that other pathogens are present in the clinical sample.

6. The test works within the genomic regions in which the probes have been designed. Due to the high variability of RNA, certain subtypes may not be detected. However, at the time of design, no mutations were observed in the target regions after alignment with all deposited SARS-CoV-2 sequences.

7. A negative result of the test does not exclude that there is an infection with SARS-COV-2 and it should not be used as the sole diagnostic method to establish a treatment or patient management regime.

Vitro S.A.
Calle Luís Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Sevilla (Spain).
Tel: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

8. A negative result of the test must be analyzed in the context of medical history of the patient and epidemiology.

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13 LABEL AND BOX SYMBOLS

IVD	Health product for in vitro diagnosis.		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions of use		Sufficient content for <n> assays
	Material safety data sheet		Keep away from sunlight

14 CHANGELOG

Date	Description
2020-11-09	• Inclusion of the section changelog. • Inclusion of the explanation of the pictogram of the Safety Sheet. • A note referring to a pandemic situation is included in section 9.
2020-12-03	• Cross-reactivity table in section 10.2 is updated.
2021-04-14	• A new sample is included in section 7.
2021-05-20	• Two new nucleci acid kits and extraction equipment are included.
2021-06-11	• A clarification on the quantity of starting sample for the extraction with Opentrons is included in section 7.2
2021-09-28	• Temperature is adjusted in section 5 Storage And Stability Conditions.
2022-03-08	• Inclusion of the explanation of the pictogram “Keep away from sunlight”.
2023-01-24	• In section 1 "Intended Use" it is included that fluorescent primers and probes are used for the N target genes, with independent regions for N1 and N2.

Vitro S.A.
Calle Luís Fuentes Bejarano nº 60. Ed. Nudo Norte Local 3. 41020 Sevilla (Spain).
Tel: +34 954 933 200. vitro@vitro.bio; www.vitro.bio