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Real SARS-CoV-2 PLUS
Test for the detection of Coronavirus SARS-CoV-2

FOR PROFESSIONAL USE ONLY
PLEASE READ THESE INSTRUCTIONS CAREFULLY BEFORE USING THE TEST

INTRODUCTION

- Intended use

The Real SARS-CoV-2 PLUS test is designed for the qualitative detection using the real-time PCR technique of SARS-CoV-2 Coronavirus, which causes CoVid19 disease. To obtain this, primers and probes are used that allow the amplification and detection of a highly specific region of the E gene, specific coronaviruses type sarcovirus (SARS-CoV-1 and SARS-CoV-2), and of a region of orf1ab gene and N gene, specific of SARS-CoV-2.
Real SARS-CoV-2 PLUS has been validated for use with respiratory samples, such as nasopharyngeal swabs, nasal swabs, oropharyngeal swabs and saliva, as well as with nucleic acids extracted from those samples.
It is a product intended for specialized users to be used as an aid in the diagnosis of CoVid19 infection, caused SARS-CoV-2. The product can be used for any indication involved with virus detection: initial diagnosis, infection monitoring, and contact control.
Take also under consideration other parameters such as symptoms or clinical history when giving the final diagnosis.

General information

COVID-19 (acronym for "coronavirus disease 2019") is an infectious disease caused by the SARS-CoV-2 virus, a sarcovirus-type coronavirus first detected in the Chinese city of Wuhan, Hubei province, in December 2019. The infection courses with respiratory symptoms similar to those from flu (fever, fatigue, aching muscles), which can become serious and even prove fatal in some cases.

·Intended test population

This product is intended for use on the general population, as any individual is susceptible to infection by SARS-CoV-2.
The product is mainly intended for use with respiratory samples from patients with symptoms of viral respiratory infection compatible with COVID-19 infection.
Furthermore, the test can also be used to monitor patients already diagnosed with COVID-19 and to evaluate after contact with people infected with SARS-CoV-2.

·Disease or infection incidence among the intended test population

The SARS-CoV-2 sarcovirus has caused a worldwide pandemic since December 2019. The incidence is constantly changing, with major variations depending on the season, geographical location, and vaccination completion status. Since its appearance and up to November 2021, over 250 million infected people have been reported, with mortality close to 5 million persons.

- Characteristics of the virus/bacterium and its infection

Orthocoronavirinae, more commonly known as Coronaviruses, are a subfamily of single-stranded RNA viruses belonging to the Coronaviridae family. They get their name from a spiked crown characteristic seen around the surface of the virus and include phylogenetically similar virus genogroups with a helically symmetrical nucleocapsid with membrane, the virions of which can be between 50 and 200 nm in diameter. Its genetic material is the longest amongst RNA viruses, with genomes between 26 and 32 kilonucleotides.
The Orthocoronavirinae subfamily is subdivided into Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus. The *Alphacoronavirus* genus includes subgroups 1a and 1b, the most representative members of which are the human coronaviruses HCoV-229E and HCoV-NL63. The *Betacoronavirus* genus includes several subgroups, the most prominent being subgroups 2a and 2b, which include human coronaviruses OC43, HKU1, SARS CoV and MERS CoV. The *Gammacoronavirus* genus includes all avian coronaviruses identified up to 2009. The 2019-20 pneumonia outbreak in Wuhan, China has led to the discovery of a new coronavirus, listed as SARS-CoV-2 by the WHO. To date, thirty-nine species of coronaviruses have been registered, several of them recently studied because until now they have not been previously identified in humans.
Coronaviruses are viruses that normally cause mild to moderate upper respiratory tract disease, such as the common cold, with symptoms such as a runny nose, cough, sore throat, and fever. Sometimes, however, they can also cause more serious illnesses, such as pneumonia, bronchitis, bronchiolitis, Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS).
COVID-19 (acronym of the English "*coronavirus disease 2019*"), is an infectious disease caused by the SARS-CoV-2 virus. It was first detected in the Chinese city of Wuhan, Hubei province, in December 2019 when a group of people were reported with pneumonia of unknown cause, mainly linked to shellfish wholesale market workers. The number of cases increased rapidly, reaching practically every country in the world in three months, first being declared a health emergency (30-01-2020) and then a pandemic (11-03-2020) by the World Health Organization. It produces symptoms similar to the flu, which include fever, cough, dyspnea, myalgia, and fatigue. Sudden loss of smell and taste has also been observed. In severe cases it is characterised by producing pneumonia, acute respiratory distress syndrome, sepsis and septic shock, causing death in around 3% of people infected. As of today, there is no specific treatment; the main therapeutic measures are to relieve symptoms and maintain vital functions.

RATIONALE

The Real SARS-CoV-2 PLUS kit is an "in vitro" diagnostic test, based on real-time PCR technology, for the qualitative detection and differentiation of the RNA specific to SARS-CoV-2.
Whether using a direct respiratory sample or nucleic acids extracted from the respiratory sample, the Real SARS-CoV-2-PLUS kit procedure involves two steps:

* If using a direct respiratory sample: a1) respiratory sample lysate and b) reverse transcription, amplification and detection of fluorescence (RT-PCR)
* If using nucleic acids: a2) nucleic acid extraction and b) reverse transcription, amplification and detection of fluorescence (RT-PCR)

a1) Respiratory sample lysate
The kit includes a lysis reagent, which in conjunction with heat treatment, helps to split the virus and release genetic material for its detection using RT-PCR.
The stability of the reagents was confirmed by up to 5 freeze/thaw cycles.
a2) Nucleic acid extraction
Reagents not included in the kit.
See "SAMPLES" section.

b) Reverse transcription, amplification and detection by probes labelled with fluorophores

The Real SARS-CoV-2 PLUS kit includes the reagents necessary to perform, in a single step, the reverse transcription of the RNA to its complementary DNA, the amplification of conserved and specific regions of the E, orf1ab and N genes from SARS-CoV-2 and its subsequent detection by using specific fluorescent probes.

For detection, the kit takes advantage of the 5' exonuclease activity of DNA polymerase. During amplification, the enzyme hydrolyses the probe that is attached to its complementary DNA sequence, thereby separating the fluorophore and quencher from the probe, leading to an increase in fluorescence signal proportional to the amount of target DNA.

The increase in fluorescence is monitored throughout the different amplification cycles in real-time with PCR equipment.

The kit includes the reagents necessary to perform the reverse transcription, amplification and simultaneous detection of a fragment of RNA from the human GAPDH gene, which is used as a valid internal control for taking samples, as well as reverse transcription and amplification.

Amplification detection of E gene is performed on the ROX (or Texas channel, in the absence of the former), of orf1ab gene on the FAM channel, of N gene on the Cy5 channel, and the internal GAPDH control on the HEX (VIC or JOE channel, in the absence of the former) (depending on the Real Time equipment used, select the appropriate detection channel).

KIT CONTENTS

Real SARS-CoV-2 PLUS		Kit 48 tests	Kit 96 tests
Master mix	MASTER MIX COV-2 PLUS	0.53 ml	1.04 ml
Probes/Primers	PRIMER COV-2 PLUS	0.265 ml	0.525 ml
Positive control	COV-2- POS CONTROL	1 Unit	1 Unit
Solution of rehydration of controls	REHYDRATION SOL	0.065 ml	0.065 ml
Lysis buffuer	REAG LYSIS	1.1 ml	2 x 1.1 ml
Instructions for use	DO-09051041	1 Unit	1 Unit

1) The information about the composition of the reagents is indicated in the material safety data sheet of the product. You may request a copy of the MSDS through the email address msds@operon.es
2) Attention, the content of the kit guarantees a maximum of 4 total uses, with a minimum of 12 samples for analysis (48 tests format) or 24 samples (96 tests format).
3) This instructions for use apply to any commercial reference of the product: 5.239.XXX.88.000

MATERIALS NOT INCLUDED IN THE KIT

The following additional material is required when using the kit:
1. PCR microtubes compatible with real-time PCR equipment.
2. Micropipettes and micropipette tips (sterile or UV-irradiated and ideally with a filter)
3. Centrifuge.
4. Disposable powder-free gloves.

REQUIRED EQUIPMENTS FOR KIT DEVELOPMENT

The following thermocyclers have validated with the kit: CFX96 Real-Time PCR Detection System (BioRad); QuantStudio 5 Real-Time PCR System (Applied Biosystems); Gentier 96 Real-Time PCR (Tianlong) and Gentier 48 E Real-Time PCR (Tianlong). The MIC qPCR Cycler (Bio Molecular Systems), PowerGene 9600 Plus real-time PCR system (Attila Biosystems) and LightCycler 96 (Roche) have also been used successfully in different external evaluations.

PRECAUTIONS

1. Only use the reagents *in vitro*.
2. Strictly follow the instructions provided. Modifying any of the prescribed **steps or temperatures** may severely affect the test results.
3. This is a molecular biology product. Every required precaution to avoid reagent contamination and/or degradation of nucleic acids during the process must be observed. The user has to wear lab coat and gloves during all the process, changing them as frequently as needed, and maintain a deep cleaning of the working area. All the materials and reagents used must be free of ADN/ARN/DNAses/RNAses, etc. The use of filtered pipette tips is recommended for PCR preparation to prevent aerosol contamination.
4. A one-way workflow is recommended. It should start in the nucleic acid extraction area, followed by the pre-PCR area (reaction mixture preparation for PCR and addition of nucleic acid) and lastly the post-PCR area (amplification and detection). To maintain the unidirectional work flow, each of the areas must exclusively have all the necessary resources to carry out the technique (nuclease-free water, centrifuges, vortex, micropipettes, tips for micropipettes, vials, etc.).
5. Molecular biology procedures require trained staff to avoid the risk of incorrect results due to nucleic acid degradation or possible contamination in the amplification process.
6. Regular cleaning of the equipment used, mainly micropipettes and work surfaces, is recommended in order to minimise the risk of reagent contamination.
7. Store the kit components as indicated in the instructions.
8. Do not exchange components from kits with different lot numbers.
9. Do not use kit components after the expiration dates.
10. If the package is broken, the product can still be used providing none of the components have been damaged.
11. In case of breakage or alteration of the primary packaging, discard the test.
12. The used product should be discarded in compliance with current legislation.
13. Patient samples and its vials must always be treated as potentially infectious. Environmental and safety standards must be adhered to.
14. Do not discard the external box of the kit until its content has been totally used. The external box contains essential information regarding the EC marking and component lots.
15. Any serious incident related to the product must be reported to the manufacturer and to the competent authority of the Member State in which the user and/or the patient are established.

STORAGE

The components of the Real SARS-CoV-2 kit must be stored at -20 °C and protected from light. The kit can be shipped at 2-8 °C provided the shipping time is less than 1 week.
Repeated thawing and freezing of reagents should be avoided as this could impact product performance. The stability of the reagents was confirmed by up to 5 freeze/thaw cycles.
The expiry date of all the reagents is printed on the label.
The expiration and storage conditions of the reagents once opened is the same as that of the original reagents.
The kit specifications are maintained provided the test is stored and manipulated in the indicated conditions.

SAMPLES

The Real SARS-CoV-2 PLUS kit can be used for analysing both the direct respiratory sample and the nucleic acids extracted from those respiratory samples. Specifically, its use has been validated with the following specimens: nasopharyngeal swabs, nasal swabs, oropharyngeal swabs and saliva.
When directly analysing nasopharyngeal/ oropharyngeal/ nasal swabs, the kit is only compatible for use when they are stored in non-inactivating transport media. **DO NOT use swabs in inactivating media**, as they affect the RT-PCR reagents.
When analysing nucleic acids (RNA or RNA+DNA), the kit is compatible with all types of swabs, whether stored in an inactivating or non-inactivating transport media.
For swabs, use only sterile, synthetic fibre swabs with a plastic rod. Do not use calcium alginate swabs or those with a wooden rod. Ensure to obtain sufficient sample, without causing bleeding. Immediately store the swab in a sterile tube with a virus-suitable transport method and store under the medium manufacturer's recommended conditions.
In the case of saliva, directly collect 0.2-0.5 ml of saliva into a sterile tube or tube adapted for saliva sampling (eg SimplOFy, Oasis Diagnostics, USA). Sampling saliva is recommended first thing in the morning or after at least 1-2 hours of fasting.
Sample stability can depend on the transport medium in which they are found; please consult the manufacturer's usage instructions as required. Evaluations undertaken in Operon S.A. provided evidence for sample stability of up to at least 1 month when stored at 2-8 °C, -20 °C, or -70 °C. All the same, we recommend storing the samples at 2-8 °C if they are going to tested within one day, or at -70 °C for longer storage periods.

Nucleic acid samples
For the extraction of RNA (or nucleic acids) use approved extraction procedures that have been shown to generate quality RNA, such as one of the following: the bioMérieux NucliSens systems, QIAamp Viral RNA Mini kit, QIAamp MinElute Virus Spin kit or Rneasy Mini kit (Qiagen), Roche MagNAPure Compact RNA isolation kit, Roche MagNAPure Compact Nucleic Acid isolation kit, Invitrogen ChargeSwitch Total RNA Cell Kit, etc (kits validated and recommended by the CDC).
The quality and concentration of the extracted RNA/nucleic acids essential for the kit to function correctly. Any inhibitors in the sample or a low RNA/nucleic acids concentration could limit the test's sensitivity.
During the kit's development, with the Real SARS-CoV-2 PLUS kit satisfactory results were observed with the QIAamp MinElute Virus Spin kit (Qiagen) kit and the Nucleic acid extraction kit (spin column)(XABT, Beijing Applied Biological Technologies Co, Ltd), Maxwell 16 Viral Total Nucleic Acid Purification Kit (in automatic extractor Maxwell) and GSD NovaPrime RNA Extraction AE1 (Eurofins Technologies, in automatic extractor KingFisher Flex System).
Evaluations undertaken in Operon S.A. provided evidence for RNA stability of up to at least 1 month when stored at 2-8 °C, -20 °C, or -70 °C. All the same and whenever possible, we recommend storing RNA samples at -70 °C, or failing that, at -20 °C.

INTERFERING SUBSTANCES

The effect of substances that may be present in the respiratory sample have been evaluated, with evidence for a lack of interference with the Real SARS-CoV-2-PLUS test by the following medicinal products or substances at the indicated concentrations: 4% capillary blood, 5 mg/ml mucin (mucin from bovine submaxillary glands, Type I-S, Sigma), 1,5 mg/ml benzocaine (Hurricaine throat spray), 5% Normomar saline gel, 15% Disneumon pernasal (5 mg/ml phenylephrine), 2.5% Nebulicina (0.5 mg/ml oxymetazoline), 0.05 mg/ml mometasone furoate (Nasonex 50 µg spray), 1% Flixonase (fluticasone propionate 50 µg spray), 50 mg/ml Strepsil, 5% Cusicrom Fuerte (sodium cromoglycate 40 mg/ml), 5 mg/ml Oseltalmivir (Tamiflu 30 mg/capsule), 4 µg/ml Tobramycin (Tobradex 3 mg/ml), 1 mg/ml amoxicillin (Amoxicilina Normon), 100 µg/ml Biotin, 10% Alkalol, 15% Propol 2, 2.2 mg/ml Ibuprofen Normon, 1 mg/ml Paracetamol Cinfa, 5 mg/ml Zanamivir, 1 mg/ml Ribavirin, 1 mg/ml acetylsalicylic acid (Aspirin 500 mg), 10% Budesonide Nasal Aldo-Unión.
Any inhibitor remaining in the final RNA would be detected due to a lack of amplification of the control gene (GAPDH) during the PCR.

REAL SARS-COV-2 PLUS PROCEDURE

1.- Reconstitution of the dry positive control

To be carried out only on first use of the kit.
Attention: this reagent contains a high copy number of viral RNA as well as human RNA. It should be handled carefully in a cabinet dedicated exclusively to nucleic acid handling, to avoid contamination problems from PCR reactions.
* Centrifuge the vial for 2 minutes at 6,000 x g.
* Add 25 µl of rehydration solution to each one of the positive and negative control, give a centrifuge pulse, and allow them to rehydrate at room temperature for 15 minutes.
* Vortex softly and centrifuge briefly at 6,000 x g.
* Store at -70 °C, or at -20 °C if that is not possible, until use.

2.- Sample lysate (only in case of direct respiratory sample testing)

* Allow the lysis reagent to thaw. Gently vortex mix and briefly centrifuge to ensure all the content is at the bottom of the tube.
* Add 20 µl of lysis reagent to a vial (for PCR, for example).
* Mix the sample well and add 20 µl to the lysis reagent. Gently mix by using the pipette and incubate: 5 min. at 95 °C + 5 min. at 4 °C (in a thermal cycler or thermal block for example). Store at 2-8 °C until required to add to the PCR reaction (in a chilled test-tube rack for example). Store at -20 °C prior to use if not testing immediately.

3.- Preparation of RT-PCR reaction

* Thaw the PCR reagents and the samples for testing (if applicable). Vortex and centrifuge briefly, to ensure that all contents are at the bottom of the tubes.
* Prepare the required PCR tubes based on the number of RNAs to be amplified.
Use vials suitable for the optical system of the Real Time equipment to be used. In addition to the sample vials, one vial will be required for the blank and one for the positive control.
* Add to each PCR tube: 10 µl of PCRMIX + 5 µl of PRIMER.
If various RNA samples are to be amplified, prepare a common mixture with all the reagents is recommended to finally add 15 µl of mixture. For example:

No. PCRs	Master MIX	PRIMER
1	10 µl	5 µl
8	90 µl	45 µl
16	180 µl	90 µl

The mixtures containing all PCR reagents should always be prepared in excess to compensate for the loss of volume that takes place during the pipetting process

Attention, to avoid unwanted contamination, it is recommended to carry out this preparation in a PCR workstation or clean area dedicated exclusively to the preparation of PCR reactions.
* Add 5 µl of nucleic acid/lysated sample to each tube (one sample per vial) and mix gently. For lysated direct samples, briefly mixing using a vortex or micropipette immediately prior to adding the sample is recommended to ensure it is homogeneous. In the case of a blank, add 5 µl DEPC-treated water, in the case of the positive, add 5 µl of rehydrated positive control.
Attention, to avoid unwanted contamination, it is recommended to add the RNA/nucleic acids in a workstation dedicated exclusively to the manipulation of nucleic acids. It is recommended to follow the following order of addition: blank (1st), samples and positive control (always last).
A blank (DEPC-treated water) and positive control included in the kit should always be included in each assay.
* Seal the vials with a cap suitable for the optical system of the Real Time equipment to be used.
* Centrifuge to eliminate possible bubbles that could affect the fluorescence reading.
* Insert the vials in the Real Time thermal cycler and proceed to amplification using the following parameters:
Reaction volume= 20 µl.
Amplification program:

Cycles	Step	Time	Temperature
1	Reverse transcription	20 min	48 °C
1	Initial denaturation	2 min	95 °C
45	Denaturation	5 sec	95 °C
	Hybridization/Elongation (Fluorescence reading)	30 sec	60 °C

Fluorescence data should be collected at the hybridization/elongation stage in the following channels:

ROX/TEXAS	FAM	Cy5	HEX/JOE/ VIC
E gene	Orf1ab	N gene	GAPDH

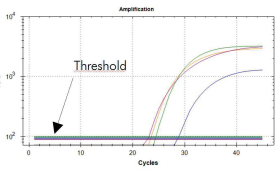
NOTE: for basic information on the preparation and programming of the different real-time PCR instruments, see the user manual for each instrument.

4.- Interpretation of results

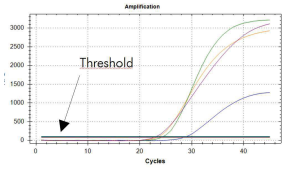
Once the reaction is complete, save and analyse the data with the proprietary software of the Real Time thermal cycler used and following the manufacturer's instructions.

The results of each target (E gene, orf1ab, N gene and GAPDH) must be analysed independently, adjusting the threshold value manually in each case. This threshold must be adjusted so that it is above any background signal.

Logarithmic fit



Linear fit



For the result to be valid, the acceptance criteria are the following:

Ct values	ROX/TEXAS (E gene)	FAM (orf1ab)	Cy5 (N gene)	HEX/JOE/VIC (GAPDH)
Blank	No signal	No signal	No signal	No signal
Positive control	≤ 35**	≤ 35**	≤ 35**	≤ 30*

* usually, 20-25 Cts ** usually, 25-30 Cts

After confirming that the results obtained for the reaction controls are valid, the results for the different samples will be analysed.

RESULTS

• For each gen, the result obtained is interpreted as **POSITIVE** if the reaction generates a fluorescence growth curve that crosses the threshold with a Ct value equal or under 40 cycles in the reading channel (ROX/TEXAS for E gene, FAM for orf1ab gene and Cy5 for N gene).
The result obtained is interpreted as **NEGATIVE** if the reaction generates a fluorescence growth curve that crosses the threshold with a Ct value greater than 40 cycles, or if the reaction does not generate a fluorescence growth curve, in channels ROX/TEXAS for E gene, FAM for orf1ab and Cy5 for N gene.
* A sample is to be considered **POSITIVE** for SARS-CoV-2 if it is positive for 2 or 3 viral genes.
The internal control of amplification (HEX/VIC/JOE channel, GAPDH) may or may not show a fluorescence growth curve, since the presence of a high amount of viral RNA could cause preferential amplification of it.
* A sample is to be considered **NEGATIVE** for SARS-CoV-2 if it is negative for the 3 viral genes or positive for only one of them (generally with high Ct values).
In this case, the internal control (HEX/VIC/JOE channel, GAPDH) should show a fluorescence growth curve that crosses the threshold with a Ct value of under 35 cycles.
• The result is interpreted as **INVALID** if a fluorescence growth curve is not generated in any of the measurement channels, or if the aforementioned positive or negative criteria are not met.
In the case of **invalid** samples, repeating the test is recommended. Should the sample again produce an invalid result, repeating the sample collection is recommended (for direct sample testing) or the extraction process for nucleic acids, in case an inhibitor is present in the sample. In the case of nucleic acids, and if the result repeats after the new extraction, it could be repeated by eluting a smaller volume in order to rule out a low nucleic acid concentration problem.
* It is important to check the amplification profiles of each sample to confirm that the result obtained is correct and cannot be attributed to background or device noise generated during the reaction. All samples should show the typical sigmoidal amplification profile.

Orf1ab	E	N	GADPH	RESULT
+	+	+	+ or -	POSITIVE
+	-	+	+ or -	POSITIVE
-	+	+	+ or -	POSITIVE
+	+	-	+ or -	POSITIVE
-	-	-	+	NEGATIVE
+	-	-	+	NEGATIVE
-	+	-	+	NEGATIVE
-	-	+	+	NEGATIVE
-	-	-	-	INVALID

QUALITY CONTROL

It is necessary to confirm the correct functioning of the kit in every assay by amplifying a negative control (DEPC-treated water) and the positive controls included in the kit.
A blank that gives a positive test for either gene (E gene, orf1ab gene, N gene or GAPDH) in the test indicates a contamination problem. In this case, the process should be repeated, ensuring that the work area and equipment are adequately decontaminated and that extreme precautions are taken during the PCR.
A positive control that returns a negative result indicates a failure of the PCR reaction, either due to inadequate reaction preparation, failure of the reagents, or failure of the positive control. Before repeating the amplification process, make sure that all reagents have been stored correctly and have not expired.

TEST LIMITATIONS

• As with any other diagnostic test, results of Real SARS-CoV-2 PLUS kit must be interpreted by a healthcare professional, taking into account the patient's medical history, clinical symptoms and any other diagnostic tests available.
* The results obtained with this product depend on the proper collection, transportation, storage and processing of samples. To avoid erroneous results, it is necessary to pay particular attention to these stages and carefully follow the instructions provided with the products for the extraction and storage of the nucleic acids.
* This assay could be used with other types of samples, although it has only been validated with samples of nasopharyngeal, oropharyngeal or nasal swabs and saliva.
* There is the possibility of false negatives or of variable results when working with samples with a low number of copies of the target template, below the detection limit of the test (see sensitivity heading).
* Due to the high analytical sensitivity of the technique, there is a possibility of false positives due to cross-contamination with SARS-CoV-2 from positive samples with high viral load, with the positive control or with the same products of the amplification reaction. These phenomena can only be avoided with good laboratory practice and carefully following product instructions.
* A positive result does not necessarily indicate the presence of viable viruses and does not imply that these viruses are infectious or that they are the causative agents of clinical symptoms. However, a positive result may be indicative of the presence of the target viral sequences (E gene, orf1ab gene, N gene).
* Negative results do not exclude suffering from SARS-CoV-2 infection and should not be used as the sole basis for treatment or other patient management decisions. Optimal sample types and when maximum viral load levels are reached during SARS-CoV-2 infections have not been determined. Collecting multiple samples (sample types and at various points over time) from the same patient may be necessary to detect the virus. If diagnostic tests for other respiratory diseases are negative and the patient's clinical presentation and epidemiological information suggest a possible SARS-CoV-2 infection, then the result should be considered a false negative and further testing of the patient should be discussed.

FEATURES / EXTERNAL AND INTERNAL EVALUATIONS

1. Diagnostic sensitivity and specificity agreement with the Multiple Real Time PCR kit for detection of 2019-CoV (XABT)
Tests were performed for the presence of SARS-CoV-2 in respiratory samples (nasopharyngeal swabs, nasal swabs, oropharyngeal swabs and saliva) and nucleic acids extracted from those samples with the Real SARS-CoV-2-PLUS kit (respiratory sample and nucleic acid) and the Multiple Real Time PCR Kit for detection of 2019-CoV (XABT, Beijing Applied Biological Technologies Co, Ltd; only nucleic acid). This evaluation was performed in Operon's facilities (Cuarte de Huerva, Spain).
When testing **direct respiratory samples**, 302 samples positive for SARS-CoV-2 with the reference test (123 nasal swab samples, 61 oropharyngeal swab samples, 10 nasopharyngeal swab samples, and 108 saliva samples) were tested, and 340 samples negative for SARS-CoV-2 with the reference test (117 nasal swab samples, 99 oropharyngeal swab samples, 69 nasopharyngeal swab samples, and 56 saliva samples).
When testing **nucleic acids**, 309 samples from patients positive for SARS-CoV-2 with the reference test (123 nasal swab samples, 68 oropharyngeal swab samples, 10 nasopharyngeal swab samples, and 108 saliva samples) were tested, and 322 samples from patients negative for SARS-CoV-2 with the reference test (114 nasal swab samples, 101 oropharyngeal swab samples, 51 nasopharyngeal swab samples, and 56 saliva samples).
The following tables contain the results:

DIRECT SAMPLE		Multiple Real Time PCR Kit for detection of 2019-CoV		
		+	-	Total
Real SARS-CoV-2 PLUS	+	295	0	295
	-	7	340	347
	Total	302	340	642
Concordance Sensitivity: 97.7% (IC95%: 95.3–98.9%) Concordance Specificity: 100% (IC95%: 98.9%-100%) Prevalence: 47% PPV: 100% NPV: 98.0% Efficiency: 98.9%				

NUCLEIC ACID		Multiple Real Time PCR Kit for detection of 2019-CoV		
		+	-	Total
Real SARS-CoV-2 PLUS	+	307	0	307
	-	2	322	324
	Total	309	322	631
Concordance Sensitivity: 99.4% (IC95%: 97.7–99.8%) Concordance Specificity: 100% (IC95%: 98.9%-100%) Prevalence: 49.0% PPV: 100% NPV: 99.4% Efficiency: 99.7%				

2. Diagnostic sensitivity and specificity agreement with the "Allplex 2019-nCoV assay" (Seegene) kit
Tests were performed for the presence of SARS-CoV-2 in respiratory samples (oropharyngeal swabs, nasopharyngeal swabs, nasal swabs and saliva) and nucleic acids extracted from those samples with the Real SARS-CoV-2-PLUS kit (respiratory sample and nucleic acid) and the "Allplex 2019-nCoV assay" (Seegene, only nucleic acid). This evaluation was performed in Operon's facilities (Cuarte de Huerva, Spain).
When testing **direct respiratory samples**, 67 samples positive for SARS-CoV-2 with the reference test (2 nasal swab samples, 39 oropharyngeal swab samples, 26 nasopharyngeal swab samples, and 108 saliva samples) were tested, and 62 samples negative for SARS-CoV-2 with the reference test (40 oropharyngeal swab samples and 22 nasopharyngeal swab samples).
When testing **nucleic acids**, 74 samples from patients positive for SARS-CoV-2 with the reference test (2 nasal swab samples, 39 oropharyngeal swab samples, 26 nasopharyngeal swab samples, and 7 saliva samples) were tested, and 62 samples from patients negative for SARS-CoV-2 with the reference test (40 oropharyngeal swab samples and 22 nasopharyngeal swab samples).
The following tables contain the results:

DIRECT SAMPLE		Allplex 2019-nCoV assay		
		+	-	Total
Real SARS-CoV-2 PLUS	+	65	0	65
	-	2	62	64
	Total	67	62	129
Concordance Sensitivity: 97.0% (IC95%: 89.8–99.1%) Concordance Specificity: 100% (IC95%: 94.3%-99.9%) Prevalence: 51.9% PPV: 100% NPV: 96.9% Efficiency: 98.5%				

NUCLEIC ACID		Allplex 2019-nCoV assay		
		+	-	Total
Real SARS-CoV-2 PLUS	+	74	2	76
	-	0	60	60
	Total	74	62	136
Concordance Sensitivity: 100% (IC95%: 95.2–99.7%) Concordance Specificity: 96.8% (IC95%: 89.1%-99%) Prevalence: 54.4% PPV: 97.4% NPV: 100% Efficiency: 98.5%				

The two false positives detected using the Real SARS CoV-2 PLUS kit were confirmed as weak positives (Cts >36) using the kit from XABT

3. Diagnostic sensitivity and specificity agreement with the kit "GSD NovaPrime SARS-CoV-2" (NovaTec Immundiagnostica GmBH)
Tests were performed for the presence of SARS-CoV-2 in 200 nasopharyngeal swab samples (99 positive and 101 negative), comparing the results from using the kits "GSD NovaPrime SARS-CoV-2" (NovaTec Immundiagnostica GmBH; only nucleic acid) and "Real SARS-CoV-2 PLUS" (Operon; respiratory sample and nucleic acid). The evaluation was performed in the facilities at Eurofins Megalab, SAU (Madrid, Spain).
The following tables contain the results:

DIRECT SAMPLE		GSD NovaPrime SARSCoV2		
		+	-	Total
Real SARS-CoV-2 PLUS	+	91	0	91
	-	8	101	109
	Total	99	101	200
Concordance Sensitivity: 91.9% (IC95%: 84.9–95.8%) Concordance Specificity: 100% (IC95%: 96.4%-99.9%) Prevalence: 49.5% PPV: 100% NPV: 92.7% Efficiency: 96.0%				

NUCLEIC ACID		GSD NovaPrime SARSCoV2		
		+	-	Total
Real SARS-CoV-2 PLUS	+	98	1	99
	-	1	100	101
	Total	99	101	200
Concordance Sensitivity: 99% (IC95%: 94.6–99.8%) Concordance Specificity: 99% (IC95%: 94.7%-99.8%) Prevalence: 49.5% PPV: 99,0% NPV: 99.0% Efficiency: 99,0 %				

ANALYTICAL SENSITIVITY

The analytical sensitivity of the Real SARS CoV-2-PLUS test was evaluated by making positive mixes of negative samples of the different specimens compatible with the test (oropharyngeal swabs, nasal swabs, nasopharyngeal swabs and saliva) with different concentrations of the WHO international standard for SARS-CoV-2 RNA (NIBSC code 20/146, inactivated virus), establishing the test's limit (LoD) as the concentration providing 19 positive results from 20 tests. The following table shows the results for the different specimens, based on whether a direct respiratory sample or extracted nucleic acids are tested:

Specimen	Nucleic acid	Direct sample
	LoD (IU/ml)	LoD (IU/ml)
Oropharyngeal swab	750	8,000
Nasopharyngeal swab	1,000	8,000
Nasal swab	750	8,000
Saliva	3,000	30,000

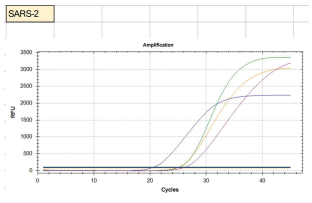
On the other hand, and for swab samples, there is evidence that the analytical sensitivity is not significantly affected by the transport medium in which it is stored (tested transport mediums: Operon S.A. virus transport medium, based on the CDC recipe; Vircell S.L. virus transport medium; Universal Transport Medium from Copan Diagnostics Inc; Transport Medium One from CPM Scientifica).
Testing a series of dilutions with a positive RNA control (Twist Bioscience, sequence MN908947.3) provided an analytical sensitivity, defined as the minimum amount of SARS-CoV-2 RNA in which two or three genes are detected as positive (orf1ab gene, gene N, gene E) from 6.25 copies of RNA by PCR (1.25 copies/µl in the nucleic acid extract).

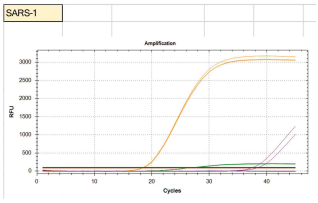
SPECIFICITY

The presence of cross reaction with the following micro-organisms was ruled out by adding quantified cultures of the different micro-organisms to make mixes of negative respiratory samples positive:
* Bacteria/yeasts (10^7 CFU/ml): *Bordetella holmesii, Bordetella parapertussis, Bordetella pertussis, Candida albicans, Haemophylus influenzae, Haemophylus parahaemolyticus, Haemophylus parainfluenzae, Klebsiella pneumoniae, Legionella pneumophila, Moraxella catarrhalis, Moraxella lacunata, Mycoplasma tuberculosis, Neisseria gonorrhoeae, Neisseria meningitidis, Neisseria subflava, Proteus vulgaris, Serratia marcescens, Staphylococcus haemolyticus, Staphylococcus saprophyticus, Staphylococcus aureus, Streptococcus A, Streptococcus B, Streptococcus C, Streptococcus D, Streptococcus F, Streptococcus G, Streptococcus mitis, Streptococcus mutans, Streptococcus oranlis, Streptococcus pneumoniae, Streptococcus salivarius, Streptococcus sanguinis, Pseudomonas aeruginosa, Chlamydophila pneumoniae* and *Mycoplasma pneumoniae.*
* Virus (5.33 x 10^3- 8.9 x 10^6 $TCID_{50}$/ml): coronavirus *OC43, NL63, 229E* and *MERS*, the *parainfluenza viruses* (HPIV) 1, 2, 3, 4A, and 4B, with *Adenovirus, Enterovirus, Metapneumovirus, RSV A* and *B, Rhinovirus, and Influenza A and B.*
Furthermore, cross-reactivity with other possible common respiratory tract micro-organisms has been ruled out by analysis of a pool of human nasopharyngeal washings.
The lack of cross reaction was confirmed for all cases by "in silico" analysis of every available sequence in GenBank.
Given the high level of homology with the SARS-CoV-1 coronavirus and that one of the genes amplified with the kit is the specific gene for the sarcovirus present in both SARS-1 and SARS-2 (gene E), samples positive for SARS-1 will show amplification curves with Real SARS-CoV-2-PLUS. However, the amplification pattern is clearly different, so in the presence of SARS-CoV-1, the product will be able to differentiate it. The following is detected rather than similar amplification curves for all three genes:

* orf1ab: a practically flat amplification curve due to unsuitable hybridisation of the probe (very low RFUs).
* Gene E: a normal amplification curve, equivalent to that of SARS-CoV-2.
* Gene N: amplification is only detected with high Cts values.

Orange line: gene E. Green line: orf1ab. Purple line: gene N.





HOOK EFFECT

The presence of a Hook effect was ruled out by adding increasing amounts of a RNA control positive for SARS-CoV-2 (Twist Bioscience, sequence MN908947.3) to the RT-PCR reaction, up to a maximum of 5 x 10^6 copies/PCR.

INTRA-ASSAY PRECISION

Analysing 5 replicates of 7 series of dilutions of an RNA control positive for SARS-CoV-2, 4 respiratory samples and their corresponding nucleic acids, a positive control, a negative control, and a blank with three different product batches, evidence was provided for a high inter-assay precision for the Real SARS-CoV-2-PLUS test, obtaining RSDs between the Ct values obtained in the different replicates under 4 %.

INTER-DAY PRECISION

Analysing 2 replicates of 7 series of dilutions of an RNA control positive for SARS-CoV-2, 4 respiratory samples and their corresponding nucleic acids, a positive control, a negative control, and a blank over 5 consecutive days, evidence was provided for a high inter-day precision for the Real SARS-CoV-2-PLUS test, obtaining RSDs between the Ct values obtained in the different replicates under 3 %.

INTER-LABORATORY PRECISION

Analysing 2 replicates of 7 series of dilutions of an RNA control positive for SARS-CoV-2, 4 respiratory samples and their corresponding nucleic acids, a positive control, a negative control, and a blank by 3 different operators and different equipment (micropipettes, cabinet, Real Time PCR thermal cyclers), evidence was provided for a high inter-laboratory precision for the Real SARS-CoV-2-PLUS test, obtaining RSDs between the Ct values obtained in the different replicates under 8 %.

INTER-LOT PRECISION

Analysing 2 replicates of 7 series of dilutions of an RNA control positive for SARS-CoV-2, 13 respiratory samples and their corresponding nucleic acids, and a blank with three different product batches, evidence was provided for a high inter-batch precision for the Real SARS-CoV-2-PLUS test, obtaining RSDs between the Ct values obtained in the different replicates under 3 %.

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