GENERAL INFORMATION

SARS-CoV-2 is a new betacoronavirus that had been unknown until the outbreak of respiratory diseases—including atypical pneumonia—that started in late December 2019 in Wuhan, China.

The newly identified coronavirus is similar to some types of coronavirus previously found in bats, but it is different from SARS-CoV and MERS-CoV.

The genome of the newly discovered CoV consists of a positive-sense single-stranded RNA of approximately 30k nucleotides. Its genome organization is similar to that of other coronaviruses. It has been recently sequenced and contains the open reading frames (ORFs) common to all betacoronaviruses:

- The ORF1ab gene, which encodes most of the enzymatic proteins
- The spike glycoprotein gene (S)
- The small envelope protein gene (E)
- The matrix protein gene (M)
- The nucleocapsid protein gene (N)
- The gene that encodes non-structural proteins

Among the main priorities to ensure public health is the choice of the diagnostic *gold standard technique*. Detection by real-time RT-PCR has been proven before by public health laboratories during public health emergencies.

Imegen SARS-CoV-2 has been approved by Instituto de Salud Carlos III for the detection of the new coronavirus SARS-CoV-2 in respiratory samples

INTENDED USE

In accordance with technical guidelines developed by the World Health Organization for the detection of SARS-CoV-2, the **Imegen SARS-CoV-2** kit detects three specific targets in genes common to all betacoronaviruses:

- **The ORF1ab gene**, which encodes most of the enzymatic proteins
- **The S gene**, which encodes the spike glycoprotein
- **The N gene**, which encodes the nucleocapsid protein (N)

Likewise, the kit includes as an endogenous positive control a system that detects the human **RNase P** ribozyme.

This test enables reverse transcription (RT) of viral RNA and real-time detection via PCR (qPCR) of the target genes through a one-step RT-qPCR, using a combination of multiplexed oligonucleotides and fluorescent hydrolysis probes (FAM and VIC).

The results obtained from this test can be used to confirm the patient's diagnosis. This test is not optimal for the study of the SARS and MERS coronaviruses.

The **Imegen SARS-CoV-2** kit can be used for *in vitro* diagnosis, and it is aimed at professionals in the virology and molecular biology sectors.

TECHNICAL CHARACTERISTICS

The **Imegen SARS Cov-2** kit enables the detection of SARS-CoV-2 in previously purified RNA samples.

- **Sample type:** RNA extracted from nasopharyngeal cotton swabs, bronchoalveolar lavage, sputum, or any other respiratory sample.
- **Sample quantity:** 12 µL RNA
- **Sensitivity:** 100% for genomes reported in GISAID (29.04.2020)
- **Specificity:** 100% (all SARS and MERS cases will test negative)
- **Test time** (RT-qPCR): 1h 20 min
- Four specific targets detected in two amplification mixes:

Fluorophores	Mix 1	Mix 2
FAM	ORF1ab	N
VIC (HEX)	RNase P (Human)	S

SAMPLE PREPARATION

Below we highlight some of the most important requisites for the collection, preparation, and submission of the sample. For more information, review the procedure for action against cases of infection by the new SARS-CoV-2 coronavirus by the Spanish Ministry of Health (Ministerio de Salud) and Instituto de Salud Carlos III.

1. Sample type: Sputum, bronchoalveolar lavage of the lower respiratory tract, or nasopharyngeal and oropharyngeal cotton swabs taken simultaneously from the upper respiratory tract.

2. Sample collection: The sample collector must use an N96 or equivalent respirator and gloves. It is recommended to indicate sample type and the time the sample was taken.

3. Preparation for sample transport: Always use triple packaging, checking the tightness of each layer to prevent leakage during transport. Temperatures during transport must be maintained below 4ºC.

4. Sample storage prior to transport: If it is not possible to send the sample to the analysis laboratory within 72h of its collection, we recommend that the sample be stored at -80ºC and transported on dry ice whenever possible.

5. Extraction of viral RNA: Use an adequate viral RNA extraction method, be it manual or automated. It is recommended to thoroughly clean the surfaces and work equipment in order to eliminate nucleases (Rnase) before initiating the extraction protocol. Depending on the extraction method, the yield and pureness of the extracted RNA may differ. As an automated extraction method, the MagNA Pure Compact System with the corresponding MagNA Pure Compact Nucleic Acid Isolation Kit (Roche) has been used successfully.

CONTENT AND STORAGE CONDITIONS OF THE KIT

The kit contains the necessary reagents to carry out 96 RT-qPCR reactions with each specific Master Mix:

- **SARS-CoV-2 Master Mix I:** It contains the oligonucleotides and hydrolysis probes to carry out the amplification of the virus-specific ORF1ab system (FAM) and the endogenous human control, RNase P (VIC/HEX).
- **SARS-CoV-2 Master Mix II:** It contains the oligonucleotides and hydrolysis probes to carry out the amplification of the virus-specific N system (FAM) and the S gene (VIC/HEX).
- **RT-PCR Master Mix:** PCR Master Mix with the nucleotides, MgCl2, real-time PCR enzyme, and buffer necessary to carry out the real-time PCR.
- **RTase:** Reverse transcriptase enzyme to carry out RNA reverse transcription to complementary DNA (cDNA).
- **Positive control:** Positive control with the target sequences for the amplification of the S gene, the N gene, the ORF1ab gene, and the RNase P gene.

Reagents	Color	Quantity	Conservation
SARS-CoV-2 Master Mix I	Red cap	915 µL	-20º
SARS-CoV-2 Master Mix II	Blue cap	915 µL	
RT-PCR Master Mix	White cap	770 µL	
RTase	Yellow cap	96 µL	
Positive control	Green cap	60 µL	

Table 1. Components of the Imegen SARS-CoV-2 kit.

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NECESSARY EQUIPMENT AND MATERIALS NOT INCLUDED IN THE KIT

Equipment:

- Real-time PCR thermal cycler able to detect FAM and VIC fluorophores
- 10 µL, 20 µL, and 200 µL micropipettes
- Vortex mixer
- Centrifuge

Reagents:

- Viral RNA/total RNA extraction kit
- Nuclease-free water

Materials:

- Optical 96-well plates or 0.2 ml optical tubes
- Optical consumables compatible with the real-time PCR thermal cycler
- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5 ml tubes
- Latex gloves
- Surface decontaminant products such as "RNAse away"
- Material necessary for nucleic acid extraction

ASSAY PROTOCOL

Preparation for amplification reactions:

1. Thaw all kit reagents and RNA samples at room temperature and keep on ice once thawed.

2. Shake each reagent on a vortex mixer and keep cold.

3. Prepare the two PCR mixes as specified below, using two 1.5 ml tubes.

	Quantity per sample or control	
Reagents	MIX 1	MIX 2
SARS-CoV-2 Master Mix I	9.5 µL	-
SARS-CoV-2 Master Mix II	-	9.5 µL
RTase	0.5 µL	0.5 µL
RT-PCR Master Mix	4 µL	4 µL

NOTE: To estimate the necessary amount of reagents according to the number of samples and controls that will be simultaneously analyzed in each run, we recommend either including one extra reaction in the calculations or increasing the volume of each reagent by 10%.

4. Mix the reagents by pipetting several times, spin the PCR mixes, and dispense 14 µL into each well of the optical plate.

5. Once PCR mixes have been dispensed, add the following to the corresponding wells:

- 6 µL RNA samples
- 6 µL positive control
- 6 µL of nuclease-free water (negative control for PCR)

NOTE: It is recommended to add one negative PCR control per master mix to rule out reagent contamination, as well as one positive control per master mix to ensure the correct functioning of the PCR reaction.

6. Place the tubes or plates into the real-time PCR thermal cycler and configure settings for the amplification program as indicated in the next section.

Settings for the real-time PCR program

- Fluorophores of hydrolysis probes:

Probe	Emitter	Target	Quencher
N	FAM	N gene	BHQ1 (None)
ORF1ab	FAM	ORF1ab gene	BHQ1 (None)
S	VIC (HEX)	S gene	TAMRA
RNase P	VIC (HEX)	RNase P gene (human)	TAMRA

Table 2. Information about hydrolysis probes

- RT-PCR program:
  - **StepOne Plus Real-time PCR System:**
    - Type of experiment: Quantitation-Comparative Ct
    - Ramping speed: standard
    - ROX™ baseline reference: ROX
  - **CFX96 & CFX96 Touch Real-time PCR System (BioRad)**
    - Cq Determination mode: Single Threshold
    - Data Analysis: Quantification
  - **LightCycler 480 Real-time PCR System (Roche)**
    - Experiment: Dual Color Hydrolysis Probe/UPL Probe
    - Analysis: Abs Quant/Fit Points

Configure PCR settings as per the optimum program (1) indicated below:

Stage	No. of cycles	Temperature	Time
Reverse transcription	1	48ºC	20 minutes
Enzymatic activation	1	95ºC	10 minutes
PCR Denaturation, annealing, and extension	40	95ºC 59ºC 68ºC	5 seconds 15 seconds 15 seconds(2)

Table 3. Optimum PCR program for StepOne Plus, CFX96, CFX96 Touch and for Light Cycler 480 PCR Real-time PCR Systems

- **7500 FAST Real-time PCR System:**
  - Type of experiment: Quantitation-Comparative Ct
  - Ramping speed: standard
  - ROXTM baseline reference: ROX

  Configure PCR settings as per the optimum program (1) indicated below:

Stage	No. of cycles	Temperature	Time
Reverse transcription	1	48ºC	20 minutes
Enzymatic activation	1	95ºC	10 minutes
PCR Denaturation, annealing, and extension	40	95ºC 59ºC 68ºC	5 seconds 15 seconds 30 seconds(2)

Table 4. Optimum PCR program for 7500 FAST Real-time PCR System

(1) In the event that other thermal cycler models are available, please see chapter 11: Limitations.
(2) Fluorescence acquisition

ANALYSIS OF RESULTS

The following recommendations should be followed to ensure an accurate analysis of results:

- Make sure that no amplification occurred in negative PCR controls, either in the FAM or in the VIC (HEX) channels. If amplification is detected in a negative control, it is recommended to repeat the assay to rule out accidental contamination.
- Make sure that amplification occurred in positive controls for all FAM and VIC (HEX) targets.
- Make sure that amplification of the endogenous human RNase P gene occurred in all analyzed samples. A lack of amplification may indicate low RNA quality in the sample and will therefore invalidate any resulting conclusions.
- The specific software for the real-time PCR thermal cycler employed must be used to analyze samples. It is recommended to adjust the following settings:
  - Baseline: Ciblos 3 - 15
  - Threshold: StepOne Plus & 7500 FAST (Applied Biosystems): 0.05
    CFX96 & CFX96 Touch (BioRad): 100
    LightCycler 480 (Roche): Adjust *Noise band*

Below are the possible results obtained using the **Imegen SARS-CoV-2** kit.

1. The results from each assay must be classified individually according to the Ct values included in the following table:

N, S, ORF1ab assays	RNaseP assay (IPC)	Results of SARS-CoV-2
Ct < 37	Ct < 40	Positive
37 ≤ Ct < 40	Ct < 40	Inconclusive
Ct = Undetermined *or* Ct = 40	Ct < 37	Negative
Ct = Undetermined *or* Ct = 40	Ct ≥ 37	Invalid. Purify new RNA from the sample

Table 5. Classification of results according to Ct values

2. The results for each sample must be interpreted according to the following table:

Results of Imegen SARS-CoV-2	Interpretation of results
Two or more targets are positive	Presence of SARS-CoV-2
Only one target is positive	Inconclusive result
The three targets are negative	Absence of SARS-CoV-2

Table 6. Interpretation of results according to target amplification

Positive Controls:

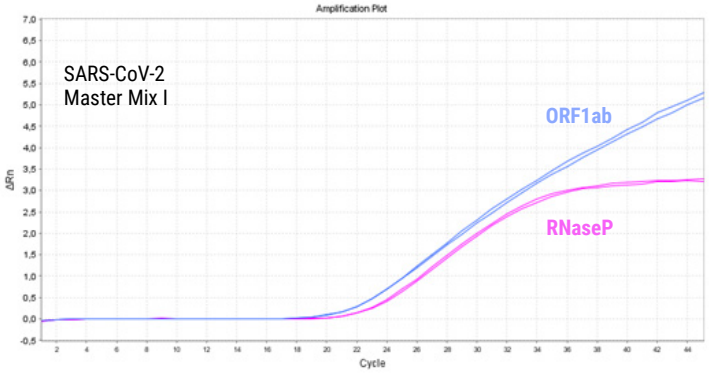

Figure 6. Result obtained from the positive control with SARS-CoV-2 premaster mix 1. Amplification of the virus-specific ORF1ab gene (FAM) is shown in blue, and amplification of the internal positive control RNaseP (VIC) is shown in pink

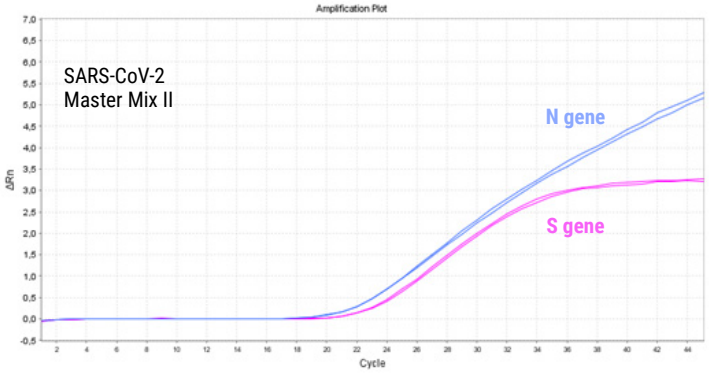

Figure 7. Result obtained from the positive control with SARS-CoV-2 premaster mix 2. Amplification of the virus-specific N gene (FAM) is shown in blue, and the virus-specific S gene (VIC) is shown in pink.

TROUBLESHOOTING

The following table shows results that may be obtained while using positive controls, negative controls, and viral RNA samples. If an unexpected result is obtained, the interpretation of the result and the most likely reason for the result are given in the following table:

Control	RNase P	S, N, ORF1ab targets	Result / Interpretation
Positive control	+	+	Expected result
	-	-	PCR failure [1]
RNA sample	+	+	Expected result
	+	-	
	-	-	RNA samples failed to amplify [2]
Negative control (NTC)	-	-	Expected result
	+	+	Contamination from positive samples or positive control material [3]

Table 7. Interpretation of possible results from Imegen-SARS-CoV-2

(1) PCR failure: An amplification error may occur due to a technical issue during PCR configuration.

Recommendation: Make sure the amplification program and fluorescence detection configuration are correct.

(2) Viral RNA sample amplification failure: Internal positive control (IPC) amplification failure in the RNA sample might suggest that the quantity or quality of the RNA sample is compromised.

Recommendation: Perform a second extraction and analysis before continuing with results interpretation.

(3) Contamination from positive samples or positive control material: PCR contamination could be caused by improper sample handling, the use of contaminated reagents, or environmental contamination, both from positive samples and positive control material.

Recommendation: Deep cleaning of the laboratory where PCRs are prepared, including equipment and material used. If necessary, use new aliquots from PCR reagents and finally prepare the PCR reactions containing the positive controls to avoid any cross-contamination