# Instructions for use

## Imegen® SARS-CoV-2

Ref. IMG-356

CE IVD

Manufactured by:
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Code: HIC-PT-KIT 03-F-03 V.01

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Health in Code S.L guarantees that its products are free of defects, both in materials and workmanship. This guarantee is valid until the expiration date, provided the storage conditions specified in this manual are maintained.

**Our products are intended for *in vitro* diagnostic use.** Health in Code S.L. provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Health in Code S.L sole obligation, in relation to the guarantees, shall be to either replace the product or reimburse the cost of it, per the client's preference, provided that materials or workmanship are found to be defective. Health in Code S.L is not liable for any cost or expense, direct or indirect, or damage or harm incurred by the customer or user because of improper use of the product.

All Health in Code S.L products are subjected to rigorous quality control. The Imegen® SARS-CoV-2 kit has passed all internal validation tests, thus guaranteeing the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, feel free to contact our Technical Department:

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| Instructions for Use (IFU) modifications | | |
|---|---|---|
| Version 15 | FEB 2023 | Change in manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain. |
| Version 14 | SEP 2022 | Change of manufacturer's identification from Imegen to Health in Code. |
| Version 13 | JUL 2020 | Modification of the analysis threshold (page 16). Applies from lot 35620C012. |
| Version 12 | MAY 2020 | PCR program modification (pages 14-15). |
| Version 11 | MAY 2020 | Review of the genomic inclusivity in GISAID (page 6). |
| Version 10 | APR 2020 | Addition PCR program for 7500 FAST Rea-time PCR cycler (page 14). |
| Version 09 | APR 2020 | Addition of PCR conditions for CFX96 Real-time PCR cycler (page 14). |
| Version 08 | APR 2020 | Modification of the PCR program (table 3, page 14). |

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# 01 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.

**NOTE**

*Imegen® SARS-CoV-2* is approved by Instituto de Salud Carlos III for the detection of the new coronavirus SARS-CoV-2 in respiratory samples.

References


- *Shu, Y., McCauley, J. (2017) GISAID: Global initiative on sharing all influenza data – from vision to reality EuroSurveillance, 22(13) doi:10.2807/1560-7917.ES.2017.22.13.30494 PMCID: PMC5388101 Web: www.gisaid.org*
- *Corman VM, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveillance 2020; 25: 2000045. Web: www.eurosurveillance.org*
- *Procedure for action against cases of infection with the new coronavirus (SARS-CoV-2). Web: www.mscbs.gob.es*

# 02 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.

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

# 03 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
- **Inclusivity**: 100% for genomes reported in GISAID (29.04.2020)
- **Specificity (cross-reactivity)**: 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 1 |
|---|---|---|
| FAM | ORF1ab | N |
| VIC (HEX) | RNase P (human) | S |

## ↘ *In silico* validation

The amplification systems have been designed using 3,123 SARS-CoV-2 genomes deposited in the database of viral sequences commissioned by the *Global Initiative on Sharing All Influenza Data* (GISAID).

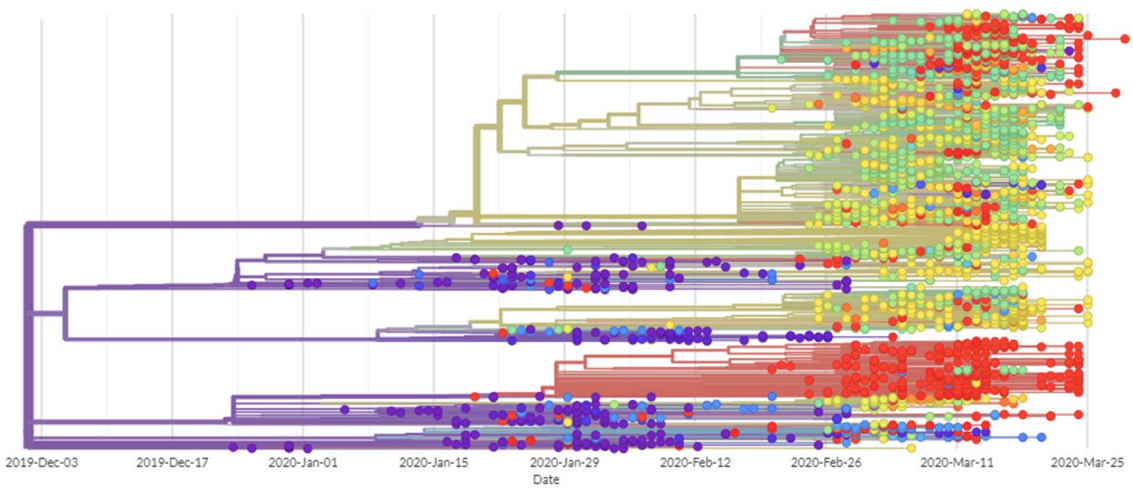


*Figure 1. Graphic of 3,123 genomes analyzed between Feb 2020 and April 2020*

## Sensitivity

The designs of the S, ORF1ab, and N genes were done using bioinformatics tools and existing genome information in the GISAID database, where all genetic variants are shown and classified according to the country, region, and host they were detected in. The design of oligonucleotides and hydrolysis probes allows for the detection of all genomic variants identified until 29/04/2020.

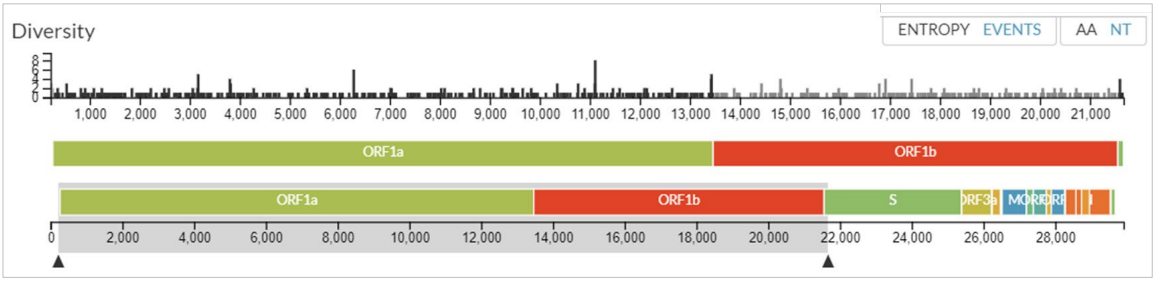


*Figure 2. Representation of the genetic variants in the ORF1ab gene (nucleotides 266-21555). The Imegen® SARS-CoV-2 system allows for the detection of the ORF1ab gene in all known betacoronavirus strains (SARS-CoV-2, 2,499 genomes). The position of the variants (EVENTS) in nucleotides (NT) is according to the reference genome of SARS-CoV-2 (Wuhan-Hu-1, GenBank MN908947).*

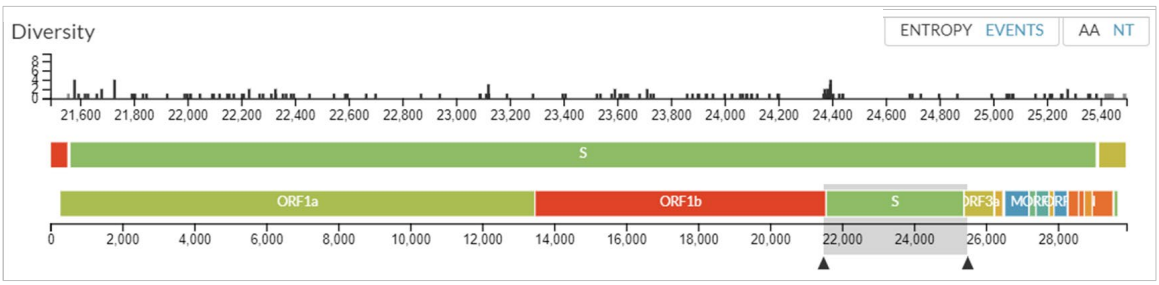


*Figure 3. Representation of the genetic variants in the S gene (nucleotides 21563-25384). The Imegen® SARS-CoV-2 system allows for the detection of the S gene in all known betacoronavirus strains (SARS-CoV-2, 2,499 genomes).*

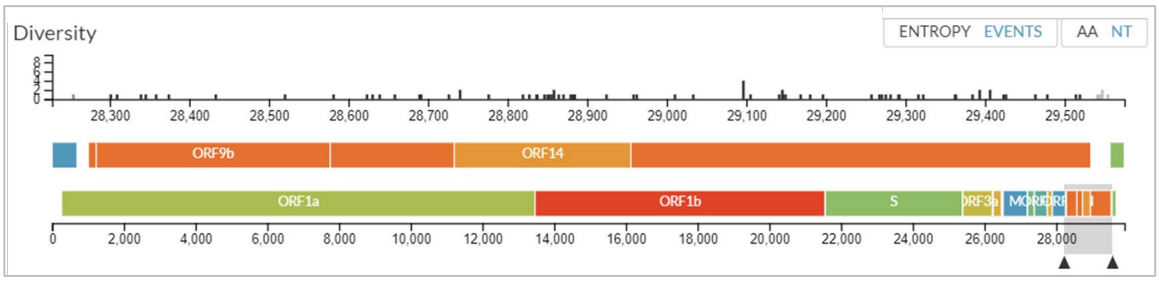


*Figure 4. Representation of the genetic variants in the N gene (nucleotides 28274-29533). The Imegen® SARS-CoV-2 system allows for the detection of the N gene in all known betacoronavirus strains (SARS-CoV-2, 2,499 genomes).*

The RNase P ribozyme system, used as a human endogenous control to confirm the integrity of the RNA sample, is based on the method published by the *Centers for Disease Control and Prevention* (CDC).

## Specificity

The genome sequences suggest the presence of a virus associated with severe acute respiratory syndrome (SARS) that is closely related to the members of a viral species called CoV, a species defined by the agent of the 2002/03 outbreak of SARS in humans. Because of

this, each system's specificity has been evaluated to confirm its analytical specificity via BLAST in the NCBI and GISAID public databases.

The Imegen-SARS-CoV-2 detection systems show partial similarity with bat betacoronavirus, but not with human SARS or MERS, which confirms the specificity of the systems for the new betacoronavirus, SARS-CoV-2.

## ↘ Analytical validation

The kit has been validated for samples from nasopharyngeal cotton swabs, bronchoalveolar lavages, and sputum from patients diagnosed via a commercial diagnosis kit. In addition, a control of the complete SARS-CoV-2 genome (Twist) has been included, together with certified synthetic vectors (GenScript) that contain the targets of interest. This vector is included in the kit and its use as a positive control to verify the correct functioning of the PCR is recommended. A complete validation process assures a sensitive and specific diagnostic method.

Health in Code S.L. is a biotechnology company certified to the **UNE-EN ISO 13485:2018 Sanitary products** norm by the SPANISH AGENCY OF MEDICINES AND MEDICAL DEVICES (AEMPS) for the Design, development, and production of medical devices for *in vitro* diagnostic use:

- Genetic testing kits
- Software for the bioinformatics analysis of genetic data

# 04 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*.

- **Sample type**: Sputum, bronchoalveolar lavage of the lower respiratory tract, or nasopharyngeal and oropharyngeal cotton swabs taken simultaneously from the upper respiratory tract.
- **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.
- **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 4°C.
- **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 -80°C and transported on dry ice whenever possible.
- **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.

# 05 Safety warnings and precautions

- It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- Do not mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the case of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucose mebranes and rinse with a large amount of water.
- Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
- This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and to manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
- This kit has been validated with specific equipment and under specific conditions that could noticeably vary among laboratories. Therefore, it is recommended that each laboratory verify compliance with the technical specifications of the manufacturer when the kit is to be used for the first time.

# 06 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, $MgCl_2$, 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 indicator | Quantity | Conservation |
|---|---|---|---|
| **SARS-CoV-2 Master Mix I** | Red cap | 915 µl | -20°C |
| **SARS-CoV-2 Master Mix II** | White cap | 915 µl | -20°C |
| **RT-PCR Master Mix** | White cap | 770 µl | -20°C |
| **RTase** | Yellow cap | 96 µl | -20°C |
| **Positive control** | Green cap | 60 µl | -20°C |

*Table 1. Components of the Imegen® SARS-CoV-2 Kit*

# 07 Equipment, reagents and material 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

# 08 Assay protocol

## 08.1 | Preparation of amplification reactions

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

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

03 Prepare the two PCR mixes as specified below, using two 1.5 ml tubes:

| | Quantity per sample or control | |
|---|---|---|
| **Reagent** | **Mix I** | **Mix II** |
| 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%.

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

05 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.

06 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.

# 08.2 | Settings for the real-time PCR program

- Fluorophores of hydrolysis probes:

| Probe | Emitter | Genotyping | 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 | 48°C | 20 minutes |
| **Enzymatic activation** | 1 | 95°C | 10 minutes |
| **PCR**<br>**Denaturation, annealing, and extension** | 40 | 95°C | 5 seconds |
| | | 59°C | 15 seconds |
| | | 68°C | 15 seconds [2] |

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

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

*(2) Fluorescence acquisition.*

## 7500 FAST Real-time PCR System

- **Type of experiment**: Quantitation-Comparative Ct
- **Ramping speed**: Standard
- **ROX™ baseline reference**: ROX

Configure PCR settings as per the optimum program[1] indicated below:

| Stage | No. of cycles | Temperature | Time |
|---|---|---|---|
| **Reverse transcription** | 1 | 48°C | 20 minutes |
| **Enzymatic activation** | 1 | 95°C | 10 minutes |
| **PCR Denaturation, annealing, and extension** | 40 | 95°C | 5 seconds |
| | | 59°C | 15 seconds |
| | | 68°C | 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.*

# 09 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:

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

| N, S, ORF1ab assays | RNase P 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*

02 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*

Below are some examples of how some results obtained using the Imegen® SARS-CoV-2 kit are displayed:

## ↘ NEGATIVE CONTROL

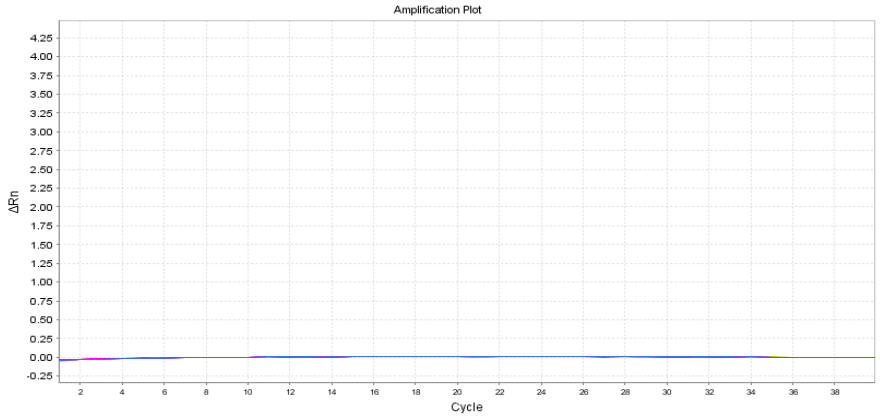


*Figure 5. Expected result for negative PCR controls. No amplification signal is observed in any fluorescence channel.*

## ↘ POSITIVE CONTROL

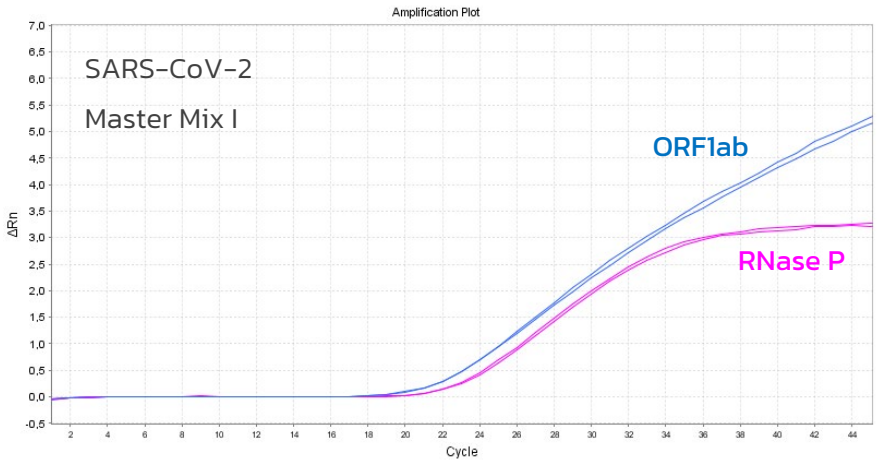


*Figure 6. Result obtained from the positive control with SARS-CoV-2 premaster mix 1. Amplification of the virus-specific ORF1ab gene (FAM) is shown in blue, and amplification of the internal positive control RNaseP (VIC) is shown in pink.*

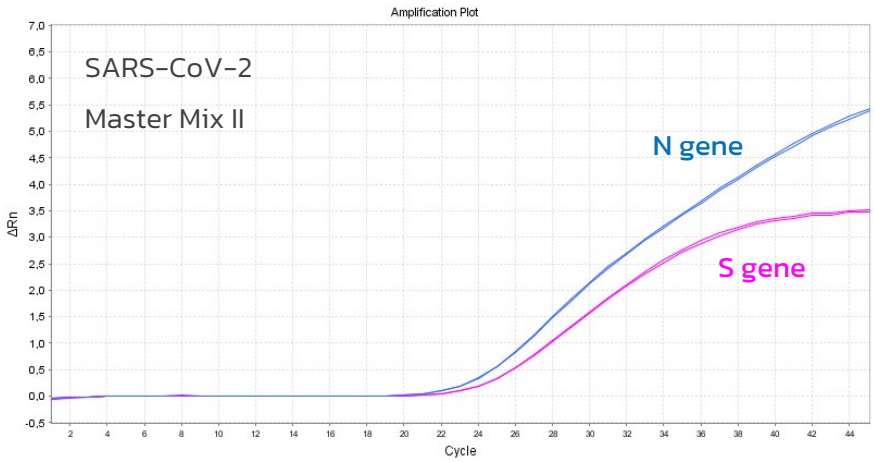


*Figure 7. Result obtained from the positive control with SARS-CoV-2 premaster mix 2. Amplification of the virus-specific N gene (FAM) is shown in blue, and the virus-specific S gene (VIC) is shown in pink.*

## Example of COVID-19-positive sample

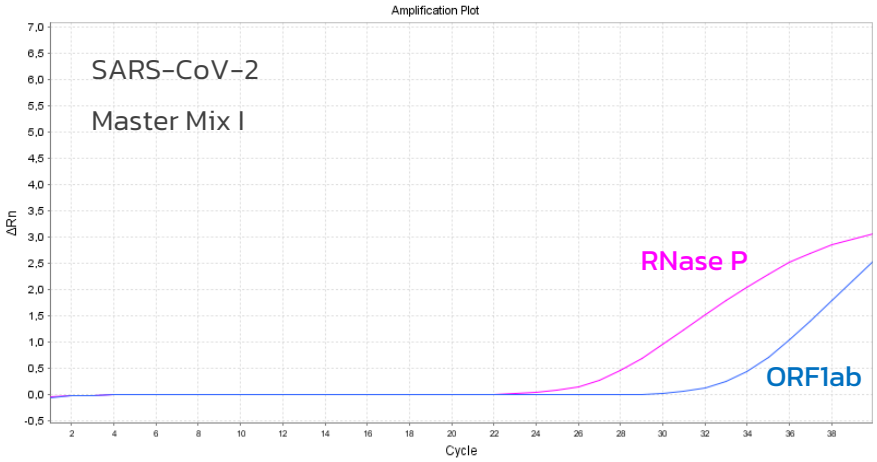


*Figure 8. Result obtained from the positive control with SARS-CoV-2 premaster mix 1. Amplification of the virus-specific ORF1ab gene (FAM) is shown in blue, and amplification of the internal positive control RNase P (VIC) is shown in pink.*

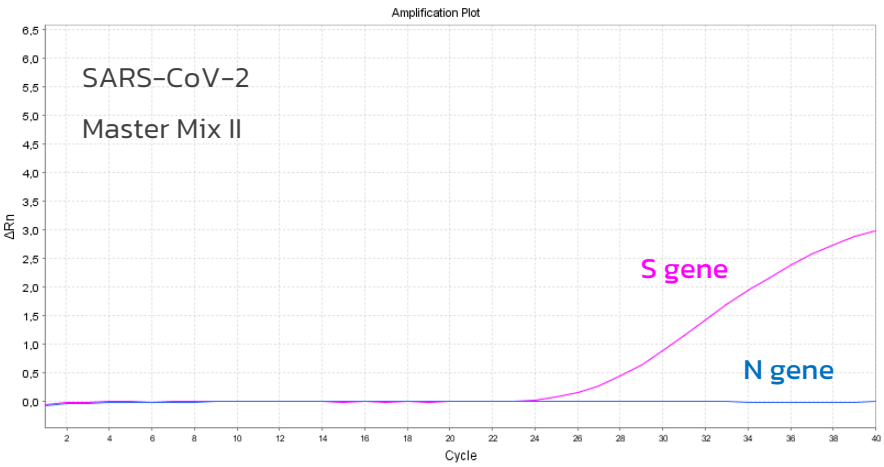


*Figure 9. Result obtained from the positive control with SARS-CoV-2 premaster mix 2. Amplification of the virus-specific S gene (VIC; shown in pink) is detected. No amplification of the virus-specific N gene (FAM; shown in blue) is detected.*

## CONCLUSION

Presence of COVID-19 due to the detection of two COVID-19-specific genes with Ct < 37


*Figure 10. View of the Imegen® SARS-CoV-2 Kit.*

# 10 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.

# 11 Limitations

## 10.1 | Equipment

Imegen® SARS-CoV-2 has been validated for use with the following PCR thermal cyclers:

- *7500 FAST Real-Time PCR System* (ThermoFisher Scientific)
- *StepOne Real-Time PCR System* (ThermoFisher Scientific)
- *CFX96 Touch Real-Time PCR System* (BioRad)
- *CFX96 Real-Time PCR System* (BioRad)
- *Light Cycler 480 PCR System* (Roche)

If a different brand or model is used, the amplification program may need to be adjusted. Should you need further information or advice, please contact our technical service.

## 10.2 | Reagents

The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code S.L.

## 10.3 | Product stability

The Imegen® SARS-CoV-2 kit is stable for the entire shelf life, provided the storage conditions specified in this document are maintained.

For any questions about the applications of this product or its protocols, please contact our Technical Department:

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