Instructions of use

Imegen® SARS-CoV-2 Plus RNase P (Set B)

REF IMG-366-2

CE IVD

GESTIÓN PRODUCTO ISO 13485 SANITARIO
AEMPS [Nº 2019 09 0061 EN]

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Rev. 2 20/11/2020

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Imegen 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.** Imegen provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Imegen's sole obligation, in relation to the aforementioned 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. Imegen is not liable for any cost or expense, direct or indirect, or damage or harm incurred by the customer or user as a result of improper use of the product.

All Imegen products are subjected to rigorous quality control. The **Imegen® SARS-CoV-2 Plus RNaseP (Set B)** 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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Amendments to the Instructions for Use (IFU)	
Version 02	Amendment: Validation with CFX96 Real-time PCR Cycler (BioRad)

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1 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 reverse transcription real-time PCR (rRT-PCR) has been proven before by public health laboratories during public health emergencies.

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

2 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 Plus RNaseP (Set B)** kit detects 3 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 E gene**, which encodes the small envelope protein

Likewise, the kit includes as an endogenous positive control a system that detects the human **RNaseP** 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 Plus RNaseP (Set A)** kit can be used for *in vitro* diagnosis, and it is aimed at professionals in the virology and molecular biology sectors.

3 Technical characteristics

The **Imegen SARS Cov-2 Plus RNaseP (Set B)** 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**: 6 µL RNA
- **Inclusivity:** 100% for genomes reported in GISAID (31.07.2020)
- **Specificity (Cross-reactivity)**: 100% (Human SARS and MERS cases will test negative)
- **Test time** (rRT-PCR): 1h 15 min
- Four specific targets detected in 1 amplification mix:

Fluorophores	Mix 1
FAM	ORF1ab
VIC	S
Cy5	E
TexasRed	RNase P

In silico validation

The amplification systems have been designed using 4,115 SARS-CoV-2 genomes deposited in the database of viral sequences commissioned by the Global Initiative on Sharing All Influenza Data (GISAID).

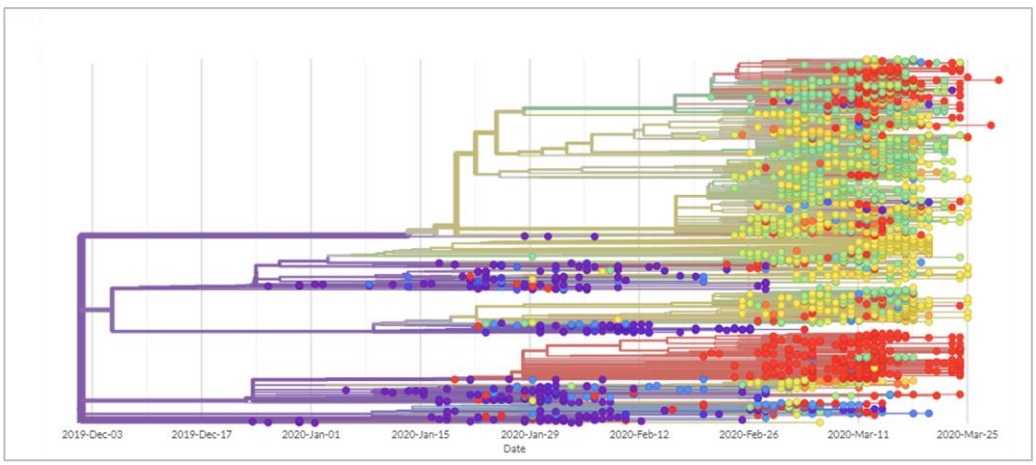

Figure 1. Graphic of 4,115 genomes analyzed between Feb 2020 and July 2020.

Sensitivity

The designs of the S, ORF1ab, and E 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 31/07/2020.

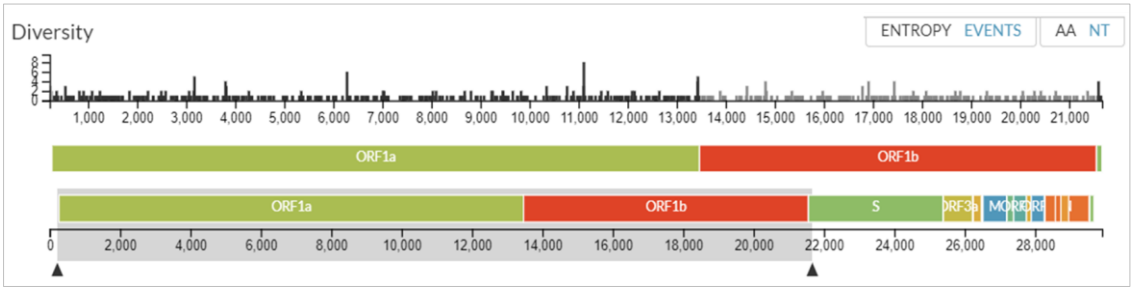

*Figure 2. Representation of the genetic variants in the ORF1ab gene (nucleotides 266-21555). The **Imegen SARS-CoV-2 Plus RNaseP (Set B)** system allows for the detection of the ORF1ab gene in all known betacoronavirus strains (SARS-CoV-2, 4,115 genomes). The position of the variants (EVENTS) in nucleotides (NT) is according to the reference genome of SARS-CoV-2 (Wuhan-Hu-1, GenBank MN908947).*

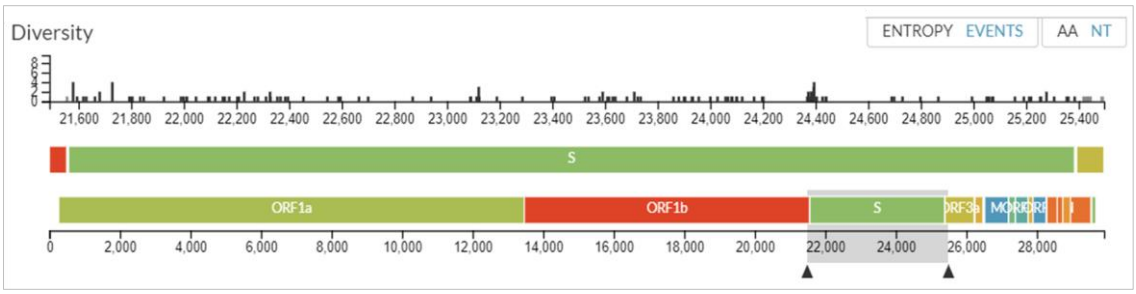

*Figure 3. Representation of the genetic variants in the S gene (nucleotides 21563-25384). The **Imegen SARS-CoV-2 Plus RNaseP (Set B)** system allows for the detection of the S gene in all known betacoronavirus strains (SARS-CoV-2, 4,115 genomes).*

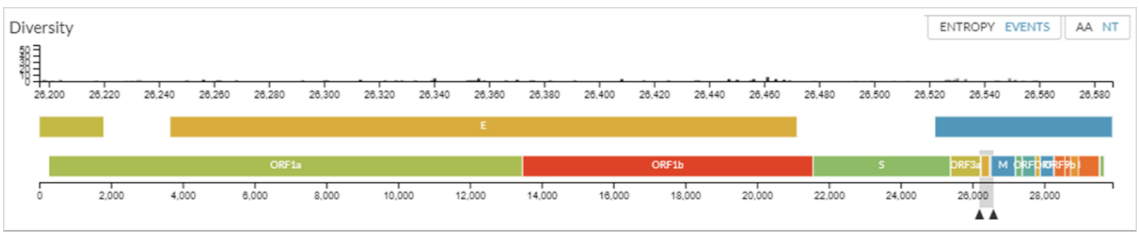

*Figure 4. Representation of the genetic variants in the E gene (nucleotides 26245-26472). The **Imegen SARS-CoV-2 Plus RNaseP (Set B)** system allows for the detection of the E gene in all known betacoronavirus strains (SARS-CoV-2, 4,115 genomes).*

The RNaseP system is used as a human endogenous control to confirm the integrity of the RNA sample.

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 Plus RNaseP (Set B)** 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.

Imegen is a biotechnology company certified to the **UNE EN ISO 13485:2018 Sanitary products** norm by AEMPS (Agencia Española del Medicamento y Producto Sanitario) for the design, development, fabrication, and commercialization of genetic analysis kits for *in vitro* diagnosis and for the development of software for bioinformatics analysis of genetic data.

4 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 4°C.

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 -80°C 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.

5 Warnings and precautions

1. It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
2. Do not mouth-pipette.
3. Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
4. Any cuts, abrasions, and other skin injuries must be properly protected.
5. 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.
6. 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.
7. Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
8. 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.
9. The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
10. 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.

6 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 Plus Master Mix** : It contains the oligonucleotides and hydrolysis probes to carry out the amplification of the virus-specific ORF1ab system (FAM), gene S (VIC), gene E (Cy5) and the human endogenous control RNase P (TexasRed).
- **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 E gene, the ORF1ab gene, and the RNase P gene.

Reagents	Color	Quantity	Conservation
SARS-CoV-2 Plus Master Mix	Red cap	915 µl	-20°C
RT-PCR Master Mix	White cap	385 µl	-20°C
RTase	Yellow cap	48 µl	-20°C
Positive control	Green cap	60 µl	-20°C

Table 1. Components of the Imegen SARS-CoV-2 Plus RNase P (Set B).

7 Necessary equipment and materials not included in the kit

Equipment:

- Real-time PCR thermal cycler able to detect FAM, VIC, Cy5 and TexasRed 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

8 Assay protocol

8.1 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 PCR mix as specified below using a 1.5 ml tube:

Reagents	Quantity per sample or control
SARS-CoV-2 Plus Master Mix	9.5 µL
RTase	0.5 µL
RT-PCR Master Mix	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.

8.2 Settings for the real-time PCR program

- Fluorophores of hydrolysis probes:

Probe	Emitter	Genotyping	Quencher
ORF1ab	FAM	Gene ORF1ab	MGB
E	Cy5	Gene E	BHQ2 (None)
S	VIC	Gene S	MGB
RNase P	TexasRed	Gene RNase P (Human)	BHQ2 (None)

Table 2. Information about hydrolysis probes

- RT-PCR program:

AriaMx (Agilent):

- Tipo de experimento: Quantitave PCR – Fluorescence Probe

CFX96 Touch Real-time PCR System (BioRad)

- Cq Determination mode: Single Threshold
- Data Analysis: Quantification

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

Stage	No. of cycles	Temperature	Time
Reverse transcription	1	48°C	15 minutes
Enzymatic activation	1	95°C	10 minutes
PCR Denaturation, annealing, and extension	40	95°C	5 seconds
		58°C	15 seconds
		68°C	15 seconds [1]

Table 3. Optimum PCR program for the AriaMx and CFX96 Real-time PCR Systems.

(1) Fluorescence acquisition

[2] In the event that other thermal cycler models are available, please see chapter 11: Limitations

9 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 fluorescence channels (FAM, VIC, Cy5, TexasRed). 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 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 use the **Auto Baseline** and the **Auto Threshold** in the analysis setting.

Analytical parameters are based on positive and negative controls. If an abnormal signal is observed, the value can be adjusted manually consulting the manufacturer's manual for real-time PCR system.

9.1 Interpretation of the results

Below are the possible results obtained using the **Imegen SARS-CoV-2 Plus RNaseP (Set B)**.

1. Verify the Ct values obtained for each sample.

Target E, S, ORF1ab	SARS-CoV-2 Results
Ct < 38	Positive (+)
38 ≤ Ct < 40	Inconclusive
Ct = Undetermined	Negative (–)

Table 5. Classification of results according to Ct values.

2. Interpret the results of each sample by following the following recommendations:

ORF1ab	S	E	RNase P	Status	Result	Action
–	–	–	Ct < 37	Valid	SARS-CoV-2 Negative	If the patient has symptoms, consider testing other respiratory virus.
Two or more SARS-CoV-2 positive targets			Any Ct value	Valid	SARS-CoV-2 Positive	Report the results to the healthcare provider.
–	–	+	Any Ct value	Valid	Betacoronavirus Positive	Report the results to the healthcare provider. Consider testing other respiratory virus.
Only one SARS-CoV-2 positive target			Any Ct value	Valid	SARS-CoV-2 Inconclusive	Repeat the test. If the repeated test remains inconclusive, additional confirmation testing should be conducted if clinically indicated.
Inconclusive SARS-CoV-2 targets in the absence of positive targets			Any Ct value	Valid		
–	–	–	Ct ≥ 37	Invalid	NA	The absence of RNase P could suggest that the quantity or quality of the RNA sample is compromised. Repeat the test. If the repeated test remains inconclusive, additional confirmation testing should be conducted if clinically indicated.

Table 6. Interpretation of results of SARS-CoV-2 Plus RNaseP (Set B).

Below are some examples of how some results obtained using the **Imegen® SARS-CoV-2 Plus RNaseP (Set B)** kit are displayed. **NEGATIVE CONTROL**

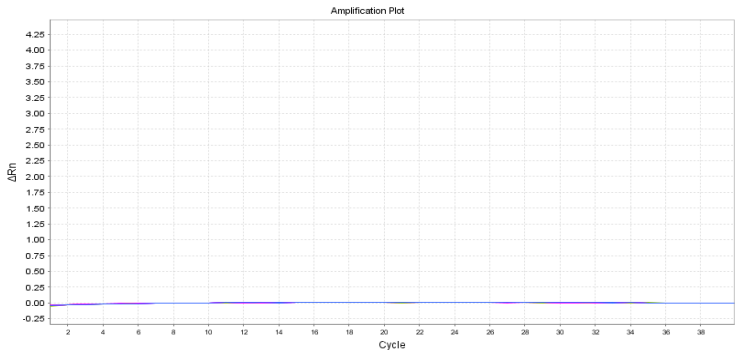

Figure 5. Expected result for negative PCR controls. No amplification signal is observed in any fluorescence channel.

POSITIVE CONTROL

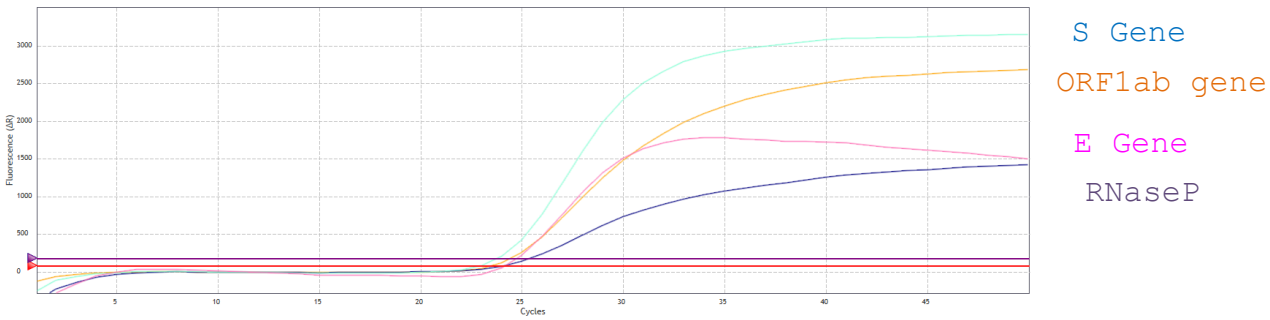

Figure 6. Result obtained from the positive control with SARS-CoV-2 Plus RNase P. Amplification of the virus-specific ORF1ab gene (FAM) is shown in orange, the S gene (VIC) shown in blue, the E gene (Cy5) shown in pink and the internal positive control RNase P gene (TexasRed) is shown in purple.

Example of COVID-19-negative sample:

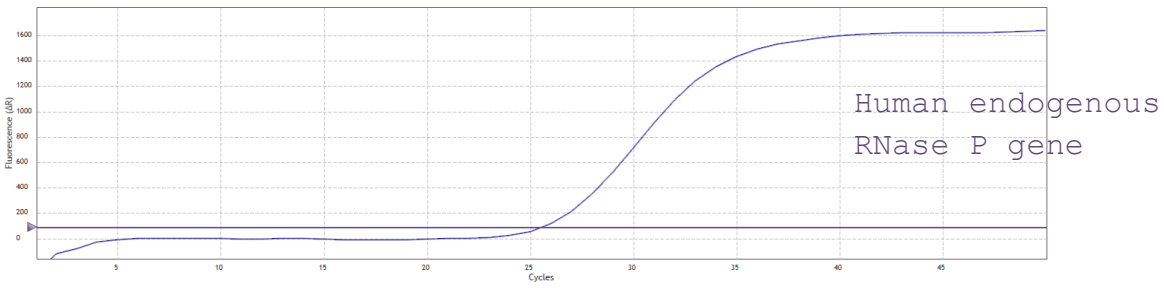

Figure 7. Result obtained from a negative SARS-CoV-2 sample. Amplification of the human endogenous RNase P gene (TexasRed) is shown in purple.

Example of COVID-19-positive sample:

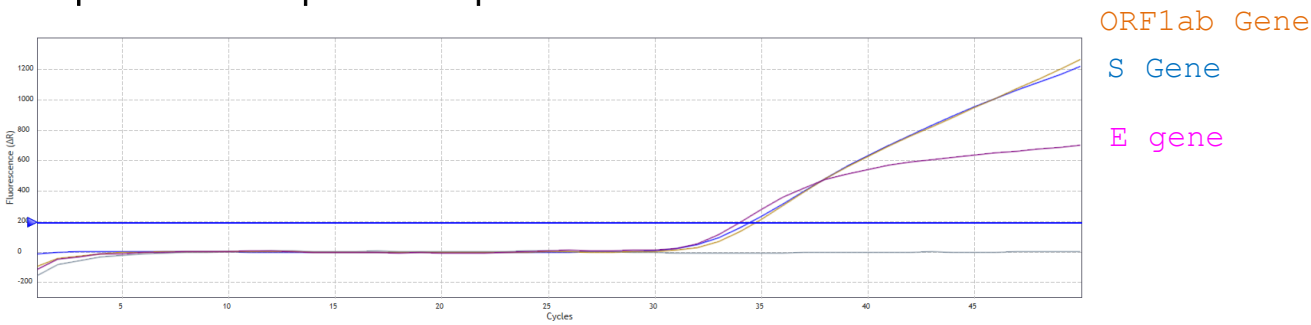

Figure 8. Result obtained from a positive sample. Amplification of the specific ORF1ab gene (FAM) is shown in orange, the S gene in blue (VIC), and the E gene (Cy5) in pink. The human endogenous gene is not detected when a high viral load is present in the sample.

Conclusion: Presence of COVID-19 due to the detection of two or more specific targets.

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, E, 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 Plus RNaseP (Set B).

[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: A failure to amplify the endogenous control (RNase P) in the RNA sample could suggest that the quantity or quality of the RNA sample is compromised. The RNA molecule is susceptible to degradation under suboptimal processing conditions (eg. lack of refrigeration, use of unsuitable transport medium, exposure to high temperatures).
Recommendation: Evaluate your preanalytical process and perform a second extraction and analysis before proceeding with the interpretation of the results.

[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

11.1 Equipment

Imegen SARS-CoV-2 Plus RNaseP (Set B) has been validated for use with the following PCR thermal cyclers:

- AriaMx Real-time PCR cycler (Agilent)
- CFX96 Real-time PCR cycler (BioRad)

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.

11.2 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 Imegen.

11.3 1Product stability

The Imegen SARS-CoV-2 Plus RNaseP (Set B) kit is stable for the entire shelf life, provided the storage conditions specified in this document are maintained.