Instructions for use

Imegen® Neumo ViralDx

Ref. IMG-386

CE IVD

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All the products marketed by Health in Code S.L. undergo rigorous quality control. The Imegen® Neumo ViralDx kit has passed all internal validation tests, which guarantee the reliability and reproducibility of each manufactured batch.

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

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Modifications to the instructions for use (IUF)		
Version 05	DEC 2022	Modification of section 4 Warnings and precautions.
Version 04	NOV 2022	Change of manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 03	SEP 2022	Change of manufacturer's identification: from Imegen to HEALTH IN CODE, S.L.
Version 02	OCT 2020	Modification of section 9. Analysis of results.

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01 General information

SARS-CoV-2 is a novel betacoronavirus previously unknown in humans, which was detected during an outbreak of respiratory disease, including atypical pneumonia, that began in mid-December 2019 in the city of Wuhan in China. The genome of the newly emerging CoV consists of a single positive-stranded RNA that is approximately 30k in length. The overall genome organization of the emerging CoV is similar to other coronaviruses, encoding the open reading frames common to all betacoronaviruses, including the ORF1ab gene encoding most enzymatic proteins, the spike surface glycoprotein gene (S), the gene encoding the small envelope protein (E) and the nucleocapsid protein gene (N), among others.

Given that all forecasts indicate that SARS-CoV-2 will be present in the coming months, the arrival of other seasonal respiratory pathologies such as influenza and respiratory syncytial virus (RSV), which have a high incidence in the pediatric population, pose an added difficulty in identifying and isolating positive SARS-CoV-2 cases.

Among the main priorities to ensure public health is using gold-standard technology for its diagnosis. The Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC) recommends multiplex PCR for the diagnosis of viral infections in the respiratory field, since the symptoms or clinical debut (fever, odynophagia, cough, etc.) is identical for all viral pathogens.

In this sense, the comprehensive diagnosis of respiratory viruses in a single multiplex PCR assay allows for the rapid and cost-effective management of a patient with symptoms compatible with different respiratory viruses. Compared to current systems for PCR diagnosis of COVID-19 patients, this assay will reduce the number of uncertain and false negative diagnoses, with SARS-COV-2 being immediately ruled out if the patient tests positive for another respiratory virus.

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 Website: www.gisaid.org.*
- *Corman VM, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveillance 2020; 25: 2000045. Website: www.eurosurveillance.org.*
- *Procedimiento de actuación frente a casos de infección por el nuevo coronavirus (SARS-CoV-2). Website: www.mscbs.gob.es.*

02 Intended use

The Imegen® Neumo ViralDx kit is designed to detect the presence of genomic RNA of the following respiratory viruses: SARS-CoV-2, influenza A and B and respiratory syncytial virus (RSV).

This assay allows reverse transcription (RT) of viral RNA and real-time PCR (qPCR) detection of target genes to be carried out by single-step RT-qPCR using a combination of oligonucleotides and multiplexed fluorescent hydrolysis probes (FAM, VIC, Cy5 and Texas Red). The targets of the assay include the detection of the following specific genes for each of the respiratory viruses:

- **SARS-CoV-2 coronavirus:**
  - *ORF1ab* gene encoding most enzymatic proteins
  - *S* gene encoding the glycoprotein of the spike surface
- **Influenza (influenza A and influenza B):**
  - *MP* gene encoding the matrix protein
- **Respiratory syncytial virus (RSV-A and RSV-B):**
  - *M2-1* gene encoding the matrix protein

In addition, the kit includes a detection system as an endogenous positive control for the *RNase P* gene encoding human ribonuclease P.

The results obtained from this assay confirm the patient's diagnosis.

Imegen® Neumo ViralDx can be used for *in vitro* diagnostic use and is intended for professionals in the virology and molecular biology sector.

03 Technical characteristics

The Imegen® Neumo ViralDx kit allows SARS-CoV-2, influenza A and B, and respiratory syncytial virus (RSV-A and RSV-B) to be detected in previously purified RNA samples.

Technical specifications:

- Type of sample: RNA extracted from human respiratory samples
- Sample quantity: 10 µL RNA
- Inclusivity: Detection of SARS-CoV-2 (study of 4,115 genomes), influenza and RSV viral sequences described between 2016 and 2020.
- Specificity: 100% against other human viruses and bacteria
- Assay time (RT-qPCR): 1 h 20 min
- 4 specific targets detected in 1 amplification mix:

Fluorophores	Mix 1
FAM	SARS-CoV-2
VIC	Influenza A & B
Cy5	RSV-A & RSV-B
Texas Red (ROX)	Human RNase P

Genomic inclusivity

The **SARS-CoV-2 coronavirus** detection system includes simultaneous detection of the *S* gene and the *ORF1ab* (RdRP) gene. The design was based on the existing genomic information in the GISAID database, which shows all genetic variants classified by the country where they were detected, the region and host. Specific amplification systems have been designed on the 4,115 SAR2-CoV-2 genomes deposited in the viral sequence database commissioned by the Global Initiative on Sharing Avian Influenza Data (GISAID).

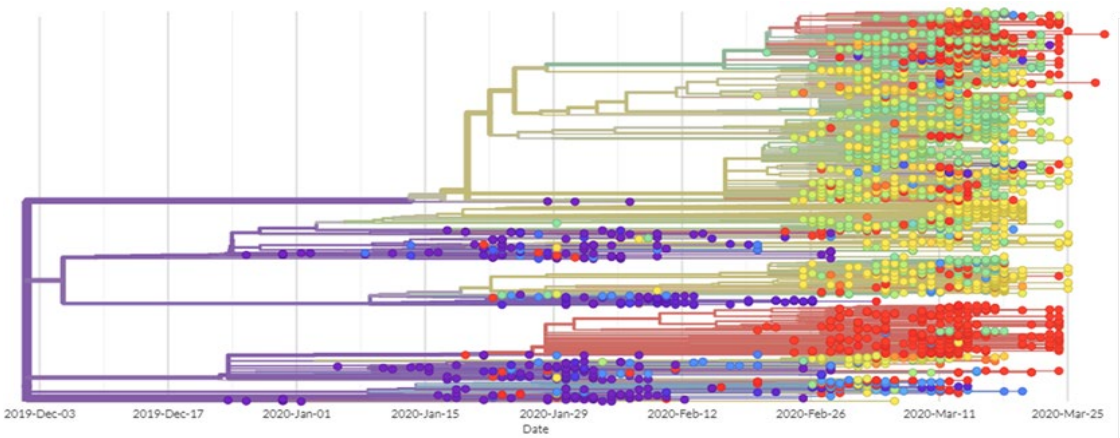

Figure 1. Graph of the 4,115 genomes analyzed between Feb 2020 and July 2020.

Influenza virus detection systems have been carried out by analyzing genomes extracted from the EpiFlu database (GISAID), the Flu Database and NCBI. The study included a total of 5,433 influenza A viral sequences and a total of 1,998 for influenza B, including the 2020-2021 cell-type vaccine strains:

- A/Hawaii/70/2019 (H1N1) pdm09
- A/Hong Kong/45/2019 (H3N2)
- B/Phuket/3073/2013 (strain B/Yamagata)
- B/Washington/02/2019 (strain B/Victoria)

The **respiratory syncytial virus (RSV)** detection system has been designed including all genomes identified since 2016, including a total of 441 viral sequences extracted from specific Virus Pathogen Resources (ViPR) and NCBI databases.

Genomic exclusivity

The genome sequences of SARS-Cov-2 suggest the presence of a virus closely related to members of a viral species called severe acute respiratory syndrome (SARS)-related CoV, a species defined by the agent of the 2002/03 outbreak of SARS in humans. Therefore, the specificity of each detection system was evaluated to confirm its analytical specificity by BLAST in the NCBI and GISAID public databases.

Clinical validation

The kit has been validated on nasopharyngeal swabs, oropharyngeal swabs and sputum samples from clinically diagnosed patients using a scientifically approved diagnostic method. In addition, whole genome samples (ATCC, United States) of influenza A (H1N1, H3N2), Influenza B (Yamagata, Victoria), and respiratory syncytial virus (RSV-A2, RSV-B), as well as synthetic samples of the SARS-CoV-2 genome (Twist) and certified synthetic vectors (GenScript) containing the targets of interest have been included in the validation. This vector is included in the kit and is recommended for use as a positive control to verify the correct operation of the PCR. Full validation provides a robust and specific diagnostic method. Diagnostic specificity tests have been carried out including samples from healthy patients previously diagnosed as such by molecular diagnostic methods.

Health in Code S.L. is a biotechnology company which has been certified according to **the standard UNE-EN ISO 13485:2018 Medical Devices** by AGENCIA ESPAÑOLA DE MEDICAMENTOS Y PRODUCTOS SANITARIOS (AEMPS, Spanish Agency for Medicinal Products and Medical Devices) for the design, development, manufacture and marketing of genetic analysis kits for *in vitro* diagnosis, as well as for the development of software for the bioinformatics analysis of genetic data.

04 Sample preparation

Below, we highlight some of the most important requirements for sample collection, preparation and shipment. For more information, please review the procedure for dealing with cases of infection by the new SARS-CoV-2 coronavirus from the Spanish Ministry of Health and the Carlos III Health Institute of Spain.

- **Type of sample**: sputum, bronchoalveolar lavage from the lower respiratory tract or nasopharyngeal and oropharyngeal swabs taken simultaneously from the upper respiratory tract.
- **Sampling**: the sampler should wear an N96 respirator or equivalent and gloves. It is recommended to record the type of sample and time of collection.
- **Preparation of the sample for transport**: a triple container should always be used, checking the seal of each layer to avoid spills during transport of the samples. Transport at 4°C.
- **Storage of the sample before transport**: if it is not possible to send the sample to the analytical laboratory within 72 h of collection, we recommend that you store the sample at -80°C and transport it on dry ice whenever possible.
- **Viral RNA extraction**: use an appropriate viral RNA extraction method, either manual or automated. It is recommended that surfaces and equipment be thoroughly cleaned to remove nucleases (RNase) before starting the extraction protocol. The yield and purity of extracted RNA may differ depending on the extraction method.

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 pipette by mouth.
- Do not smoke, eat, drink or apply cosmetics in the areas where kits and samples are handled.
- Any skin conditions, as well as cuts, abrasions and other skin lesions should be properly protected.
- Do not pour reagent residues into the drinking water system. It is recommended to use the waste containers set out by the legal regulations and to manage them via an authorized waste manager.
- In the case of accidental spillage of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with plenty of water.
- Material safety data sheets (MSDS) for all hazardous components contained in this kit are available upon request.
- This product requires the handling of samples and materials of human origin. It is recommended that all human-sourced materials be considered potentially infectious and handled in accordance with the OSHA Biosafety Level 2 standard for bloodborne pathogens or other relevant biosafety practices should be used for materials that contain or are suspected of containing infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive and do not cause biological environmental contamination.
- This kit has been validated with specific equipment and under specific conditions that may vary among laboratories. It is therefore recommended that each laboratory verify the correct operation of the assay when using the kit for the first time.
- The manufacturer is not responsible for the assay not working properly when the reagents included in the kit are replaced by other reagents not supplied by Health in Code S.L.
- The manufacturer does not guarantee the reproducibility of the assay when the user includes reagents not validated by Health in Code S.L., considering them equivalent to those supplied in the kit.

06 Content and storage conditions of the kit

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

- ***Neumo Master Mix:*** contains the oligonucleotides and hydrolysis probes to perform system-specific amplification of SARS-CoV-2 (FAM), influenza (VIC), RSV (Cy5) and human endogenous control, RNase P (Texas Red).
- ***RT-PCR Master Mix:*** PCR Master Mix with the nucleotides, $MgCl_2$, real-time PCR enzyme and buffer required to perform real-time PCR.
- ***RTase:*** retrotranscriptase enzyme to carry out the reverse transcription of RNA to complementary DNA (cDNA).
- ***Positive Control:*** positive control with the target sequences for the amplification of the SARS-CoV-2, influenza, RSV and RNase P endogenous control targets.

Reagents	Color	Quantity	Storage
Neumo Master Mix	Red cap	530 µL	-20°C
RT-PCR Master Mix	White cap	385 µL	-20°C
RTase	Yellow cap	48 µL	-20°C
Positive control	Green cap	100 µL	-20°C

Table 1. Imegen® Neumo ViralDx kit components

07 Equipment, reagents and materials not included in the kit

Equipment:

- Real-time PCR thermal cycler that detects FAM, VIC, Cy5 and Texas Red fluorophores.
- 10 µL, 20 µL and 200 µL micropipettes
- Vortex
- Centrifuge

Reagents:

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

Materials:

- 96-well optical plates or 0.2 mL optical tubes
- Optical consumables compatible with the real-time PCR thermal cycler
- Pipette tips with filter (10 µL, 20 µL and 200 µL)
- 1.5 mL sterile tubes
- Latex gloves
- Surface cleaning material such as RNAse AWAY
- Material required for nucleic acid extraction

08 Assay protocol

08.1 | Preparation of amplification reactions

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

02 Vortex each of the reagents and keep cold.

03 Prepare PCR premaster as specified below using 1 x 1.5 mL tube:

Reagents	Volume per reaction
Neumo Master Mix	5.5 µL
RTase	0.5 µL
RT-PCR Master Mix	4 µL

NOTE: To estimate the quantity of reagents needed depending on the number of samples and controls to be analyzed simultaneously per run, we recommend adding one more reaction or increasing the volume of each reagent by 10% when making the calculations.

04 Mix the reagents by pipetting several times, spin the PCR mixes and dispense 10 µL into the corresponding wells of the optical consumables.

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

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

NOTE: It is recommended to add a negative PCR control per master mix to rule out contamination of the reagents and also a positive control per master mix to ensure the correct operation of the PCR reaction.

06 Place the tubes or plates in the real-time PCR thermal cycler and set up the amplification program as indicated in the following section.

08.2 | Real-time PCR program setup

- Fluorophores of hydrolysis probes:

Probe	Issuer	Genotyping	Quencher
SARS-CoV-2	FAM	SARS-CoV-2	MGB
RSV	Cy5	RSV-A & B	BHQ2 (None)
Influenza	VIC	Influenza A & B	MGB
RNase P	Texas Red	RNase P gene (Human)	BHQ2 (None)

Table 2. Hydrolysis probe information

- RT-PCR program:

  - *QuantStudio 5 Real-time PCR System* (Applied Biosystems)

    - **Type of experiment**: Quantitation-Comparative Ct
    - **Ramp speed**: Standard
    - **ROX™ baseline reference**: NONE

  - *CFX96 Touch Real-time PCR System* (BioRad)

    - **Cq Determination mode**: Single Threshold
    - **Data analysis**: Quantitation

    Configure the PCR program according to the optimal program [1] shown 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	30 seconds [2]

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

(1) If other models of thermal cyclers are available, see chapter 11: Limitations.
(2) Fluorescence acquisition

09 Analysis of results

It is recommended to follow the indications below for the results to be analyzed properly:

- Check that there is no amplification in any of the fluorescence channels (FAM, VIC, Cy5, Texas Red) in the negative PCR controls. If amplification is detected in a negative control, it is recommended to repeat the assay to rule out accidental contamination.
- Check that there is amplification of all the targets in the positive controls: SARS-CoV-2, RSV, influenza and RNase P endogenous control.
- Check for amplification of the endogenous human *RNase P* gene in all samples tested. The absence of amplification may indicate low RNA quality in the sample and will invalidate conclusions.
- In order to analyze the samples, the specific software for the real-time PCR thermal cycler employed must be used. It is recommended to use Auto Baseline and Auto Threshold in the analysis settings.

09.1 | Interpretation of results

The possible results obtained with the Imegen® Neumo ViralDx kit are shown below:

01 Verify the Ct value of the result obtained for each sample.

Viral target assay	SARS-CoV-2, influenza and RSV results
Ct ≤ 35	Positive (+)
35 > Ct	Negative (-)
Ct = Undetermined	

Table 5. Cut-off values for SARS-CoV-2, influenza and RSV

02 Interpret the results of each sample according to the following recommendations:

SARS-CoV-2	Influenza	RSV	RNase P	Status	Result	Share
-	-	-	Ct < 37	Valid	Analyzes viruses not detected	Consider other infectious causes associated with clinical symptoms
+	-	-	Any value	Valid	Presence of SARS-CoV-2 RNA	Report results to the health system
-	+	-	Any value	Valid	Presence of influenza A and/or B RNA	Follow the recommendations of the health system
-	-	+	Any value	Valid	Presence of RSV-A and/or B RNA	Follow the recommendations of the health system
Simultaneous detection of several viral targets			Any value	Valid	Presence of co-infection	Inform the health system if SARS-CoV-2 is detected
-	-	-	Ct ≥ 37	Invalid	NA	Repeat the test. If the repeated result is invalid, an additional test to confirm is recommended

Table 6. Interpretation of Imegen® Neumo ViralDx results.

Below are some examples of how some results are visualized using the Imegen® Neumo ViralDx kit:

↘ NEGATIVE CONTROL

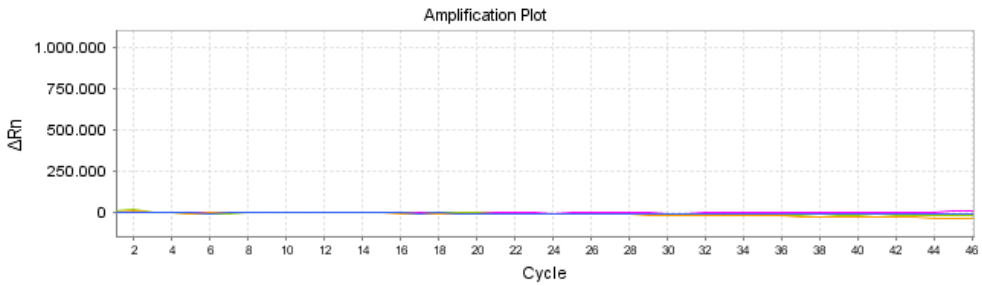

Figure 2. No amplification signal is observed in any fluorescence channel.

↘ POSITIVE CONTROL

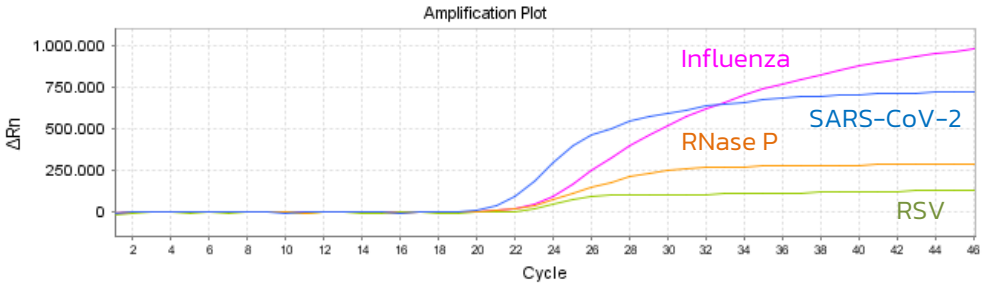

Figure 3. Result obtained from the positive control. Amplification of the ORF1ab and/or S targets of SARS-Cov-2 (FAM) is shown in blue, the amplification of influenza A and/or B (VIC) targets in pink, the amplification of the respiratory syncytial virus specific gene (Cy5) in green, and the amplification of the internal positive control RNase P (Texas Red) in orange.

Example of negative sample

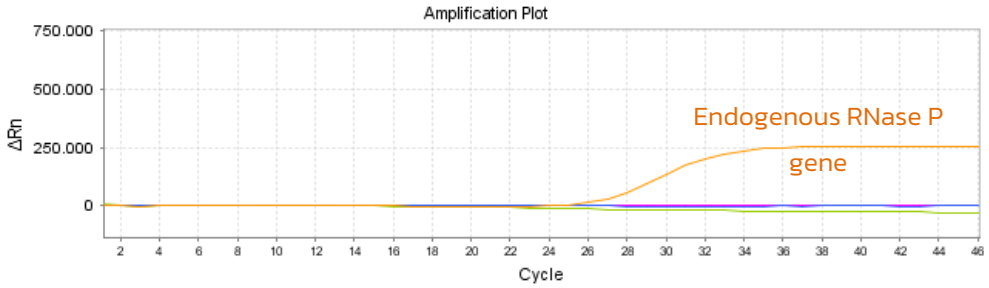

Figure 4. Result obtained from a negative SARS-CoV-2 sample. Amplification of the human endogenous RNase P gene (Texas Red) is shown in orange

10 Troubleshooting

The following table shows the results that could be obtained using the positive and negative controls, and viral RNA samples. In the case that an unexpected result is obtained, the interpretation of the result and the most probable reason for such a result are given in the following table:

Control	RNase P	Viral targets	Results/interpretation
Positive control	+	+	Expected result
	–	–	Failed PCR setup[1]
Patient sample	+	+	Expected result
	+	–	
	–	–	Failed amplification of the DNA samples[2]
Negative control (NTC)	–	–	Expected result
	+	+	Contamination with positive samples or the positive control[3]

Table 7. Interpretation of possible results obtained using Imegen® Neumo ViralDx.

(1) Failed PCR configuration: An error in amplification may be due to a technical problem during PCR setup.

↘ Recommendation: verify that the amplification program and fluorescence detection settings are correct.

(2) Failed amplification of the DNA sample: A failure to amplify the human endogenous control in the RNA sample could suggest that the quantity or quality of the RNA sample is compromised.

↘ Recommendation: perform a second extraction and analysis before proceeding to the interpretation of the results.

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

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

11 Limitations

11.1 | Equipment

Imegen® Neumo ViralDx has been validated using the following PCR thermal cyclers:

- *7500 FAST Real-Time PCR System* (ThermoFisher Scientific)
- *QuantStudio 5 Real-Time PCR System* (ThermoFisher Scientific)
- *CFX96 Touch Real-Time PCR System* (BioRad)

If you use another make or model of thermal cycler, you may need to adjust the amplification program. Please contact our technical support for any questions or clarifications.

11.2 | Reagents

The manufacturer is not responsible for the assay not working properly if the reagents included in the kit are replaced by other reagents not supplied by Health in Code S.L.

11.3 | Product stability

Imegen® Neumo ViralDx is stable during its shelf life, provided that the storage conditions set out in these instructions are followed.

Contact our Technical Department for any questions about the applications of this product or its protocols:

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