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Anti-SARS-Cov-2 *"Spike"* ELISA IgG

REF	Σ	IVD
IMS2907	96	CE

1. INTENDED USE

Qualitative immunoenzymatic determination of specific IgG antibodies against Covid-19 based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. The 96-well ELISA plates are coated with the specific recombinant antigen (Spike Protein or S protein) to bind the antibodies present in human serum and plasma samples (K2-EDTA, K3-EDTA, Heparin-Li, Sodium Citrate). The intended use of the assay is to aid in the identification of individuals with an adaptive immune response against SARS-CoV-2, indicating a previous or recent infection and contributing, in combination with other tests (PCR or antigen), to determining the stage of the infection. Additionally, this test can be used in research, seroprevalence studies, epidemiological surveillance and immune response of different vaccines against SARS-CoV-2.

2. FIELD OF APPLICATION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new virus that belongs to the family of coronaviruses, of the genus Betacoronavirus[1], which emerged in China in the city of Wuhan, Hubei province in December 2019 and is It has spread throughout the world until being declared by the WHO as a pandemic in March 2020. SARS-CoV-2, is a single-stranded, positive-sense RNA virus that presents similarities in the organization and expression of its genome with SARS-CoV, as well as with other human respiratory coronaviruses (NL63, 229E, OC43 and HKU1) and with bat coronaviruses, which is their zoonotic reservoir[2]. Thus, its genome codes for 28 proteins, 16 non-structural proteins and 4 structural proteins, protein S (spike protein), protein E (envelope), protein M (membrane) and protein N (nucleocapsid)[3].

This virus causes various clinical manifestations encompassed under the term COVID-19, which include respiratory conditions that vary from the common cold to severe pneumonia with respiratory distress syndrome, septic shock, and multiple organ failure. Most of the COVID-19 cases reported so far begin with mild symptoms[4].

The transmission routes of SARS-CoV-2 are similar to those described for other coronaviruses, highlighting transmission through the secretions of infected people by respiratory droplets of more than 5 microns, which are capable of being transmitted at distances of up to 2 meters, as well as through the hands or fomites contaminated with these secretions followed by contact with the mucosa of the mouth, nose or eyes. Similarly, the virus can be viable in the air, so airborne transmission by aerosols is also possible, although in a restricted way, not in open spaces and mainly in healthcare settings[5].

The host's immune system reacts to SARS-CoV-2 infection by producing specific antibodies that appear in serum or plasma. It has been described that infected individuals, after detection of viral RNA in swabs by reverse transcriptase polymerase chain reaction (RT-PCR), can develop antibodies in as little as 2 days to two weeks from the onset of symptoms.

There are five classes or isotypes of antibodies, also called immunoglobulins, in humans. Of these, three (IgG, IgM and IgA) are commonly used in serological tests for diagnosis. IgG is the most abundant immunoglobulin that is produced in response to an antigen and specifically SARS-CoV-2 and can be detectable in COVID-19 patients after initial exposure to the virus, conferring long-term immunity.

This assay is based on the detection of the viral spike (protein S), a glycoprotein that plays a key role in receptor recognition and the fusion process of the cell membrane, which is composed of two subunits, S1 and S2.

The S1 subunit contains the receptor-binding domain (RBD) that recognizes and binds to the host receptor's angiotensin converting enzyme 2 (ACE2), while the S2 subunit facilitates fusion between the viral envelope and the plasma membrane of its target cell6, as well as being the main target of most vaccines.

Finally, it should be noted that protein S presents the intrinsic instability typical of class I fusion proteins, and protein S tends to prematurely fold back to the post-fusion conformation, compromising the immunogenic properties, for this reason, for this test has used a stabilized trimer of protein S by introducing point mutations and disulfide bridges[7].

3. BASIS OF THE METHOD

The kit contains microtiter plates coated with recombinant protein S (stabilized trimer). Firstly, the diluted patient sample is incubated in the wells, allowing antigen-specific antibodies to bind to protein S. After washing the wells, to remove unbound antibodies, in a second incubation step, add an anti-human immunoglobulin (IgG) antibody conjugated to horseradish peroxidase (HRP). After a second washing step, tetramethylbenzidine (TMB) substrate is added causing the enzyme conjugated to the anti-IgG antibody to catalyze a colorimetric reaction.

The intensity of the color of the reaction is proportional to the number of antigen-specific antibodies present in the sample. The test can be carried out automatically or manually and It is for professional use only.

4. REAGENTS

4.1. Content of the kit

The reagents included in a kit are sufficient to perform 96 determinations. Each anti-SARS-CoV-2 "Spike" ELISA IgG kit contains:

MTP	**96-wells Microplate (12x8).** Multiwell strip microplates upholstered with SARS-CoV-2 recombinant antigen in a vacuum.sealed bag.
WASHBUF 20X	**50 ml wash buffer (20X).** HEPES, NaCl and detergents. Contains Proclin300 as a preservative (< 0.0014%).
DILUBU	**25 ml antibody and sample dilution buffer (1X - Ready to use).** Buffer that minimizes non-specific binding, cross-reactivity and matrix interference, with blue dye. Contains CMIT/MIT 3:1 as a preservative.
SUBS	**12 ml of tetramethylbenzidine (TMB)** chromogenic substrate (1X - Ready to use)
SOL	**12 ml of stopping solution** (1X - Ready to use) 0.5M sulphuric acid (H_2SO_4)
CNTRL + IgG	**1.7 ml of positive IgG control.** Contains CMIT/MIT 3:1 as a preservative. Ready to use.
CNTRL - IgG	**1.5 mL negative control.** Contains CMIT/MIT 3:1 as a preservative. Ready to use.
CNTRL ± IgG	**1.5 ml of calibrator.** Contains CMIT/MIT 3:1 as a preservative. Ready to use.
CONJ IgG	**120 µl of HRP-conjugated anti-human antibody (100X).** Contains CMIT/MIT 3:1 as a preservative.
INSTR	**1 Instructions for use.**
TAP	**2 units of protective film.**

4.2. Additional materials and equipment (not supplied in the kit).

- Calibrated spectrophotometer for Reading ELISA plates at 450 nm and 620 nm.
- Adjustable, calibrated micropipettes covering a range of 1-100 µL and corresponding disposable pipette tips.
- Automatic plate washer: recommended. Plate washing can also be performed manually.
- Incubator: for incubation of the microplate at +37° C.
- Distilled or deionized water.
- Timer.
- Disposable gloves.
- Waste container for biological substances

5. STORAGE AND HANDLING CONDITIONS

Store refrigerated between +2 and +8° C. DO NOT FREEZE.
Unopened, the kit is stable until the expiration date. Do not use it after this date. After opening, the reagents are stable if stored between +2 and +8° C and protected from contamination. Do not leave the reagents open and at a different temperature than the storage temperature for longer than necessary.

6. RECOMMENDATIONS AND WARNINGS.

⚠ DIAGNOSIS. For professional use only.

⚠ For trained laboratory personnel only.

⚠ Kit components contain Proclin300, sulphuric acid and CMIT/MIT. The compounds should be dissolved under running water before disposal. These conditions are recommended to avoid deposits in pipes. Safety Data Sheet (MSDS) available at www.immunostep.com

⚠ Before starting the analysis, read the instructions carefully. Deviations from recommended procedures could invalidate the test results. Do not replace or mix reagents from the Immunostep kit with reagents from other manufacturers.

⚠ Keep the kit components out of direct light exposure during the protocol. The substrate solution (TMB) is light sensitive.

⚠ Samples should be treated in the same way as those that could transmit infection. Appropriate methods must be available for handling them.

⚠ Reagents should not be used if the packaging shows clear evidence of deterioration.

⚠ Use personal protective equipment for sample handling. Wash your hands properly after handling the samples. All procedures must be carried out in accordance with approved safety standards.

⚠ The reagents in this kit include substances of animal and/or human origin. Although materials of human origin have been tested and found negative for hepatitis B surface antigen (HBsAg), hepatitis C and human immunodeficiency virus, all materials and patient samples must be handled and disposed of as potentially infectious using safe laboratory procedures.

7. SAMPLE COLLECTION

Sample collection (serum, EDTA plasma, heparin or citrate) must be done in suitable collection tubes, using the appropriate anticoagulant. Samples must remain at room temperature for no more than 8 hours. If the test is to be carried out after 8 hours, the samples must be cooled to between +2 and +8° C. If the test is not to be completed within 48 hours of sampling, then samples should be stored frozen at -20° C or below, avoiding unnecessary freezing and thawing cycles. Samples must be properly inactivated; an inactivation protocol may be to keep samples at 56° C for 30 minutes before use.

7.1. Sample preparation

Samples are diluted 100:1 in the antibody/sample dilution buffer (1X) included in the kit and mixed with a vortex shaker. The diluted samples should be tested within 8 hours. Except for the TMB solution and the stop solution.

8. PREPARATION OF THE REAGENTS

Temperature the reagents between +18° C and +24° C (room temperature) for 30 minutes.

Prepare a 1:100 dilution of HRP conjugate antibody in sample and antibody dilution buffer. The dilution should be prepared slightly before use, e.g. during the sample incubation step, as it is not advisable to leave the ready-to-use dilution of HRP conjugate antibody in storage, due to its poor stability.

The wash buffer included in the kit is a 20X concentrate. If crystallization is observed in the concentrated buffer during storage, heat to 37° C and shake well before making the dilution. To carry out the dilution, remove the required amount of concentrate from the bottle and dilute 1:20 with distilled water.

Microwell plate covered with the antigen. Remove the strips required to carry out the test and immediately after the removal of the strips, the remaining strips should be resealed in the aluminum foil bag together with the desiccant bag.

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9. TEST PROCEDURE

1.	Sample Incubation	Add 100 µl of the positive control, the negative control, the calibrator and the prepared sample (100:1 dilution) into the individual wells of the microplate. It is recommended to use two wells per sample, including the controls and in particular the calibrator. Incubate for 60 minutes at +37°C. When the process is manual, cover the microplate with one of the protective foils provided.
2.	Washing	If necessary, remove the protective film. Empty the wells and then wash 4 times using 300 µl of 1X wash buffer in each wash. Leave the wash buffer in each well for 30 to 60 seconds per wash cycle. After washing, completely remove all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.
3.	Incubation of the conjugate	Add 100 µl of the dilution (1:100) of the HRP-conjugated antibody to the wells and incubate for 30 minutes at +37°C. When the process is manual, cover the microplate with one of the protective films provided..
4.	Washing	If necessary, remove the protective film. Empty the wells and wash as described above (step 2).
5.	Substrate incubation	Add 100 µl of the chromogenic substrate solution (TMB) to each well of the microplate. Incubate for 10 minutes at room temperature (+18°C and +24°C) and protected from light.
6.	Stopping	Add 100 µl of the stop solution (1X - ready to use) to each well, trying to follow the same order in which the substrate solution was added.
7.	Absorbance measurement	Measure the optical densities (OD) of each well in a microplate spectrophotometer at 450 nm and a reference wavelength of between 620 and 650 nm within 30 minutes of adding the stopping solution. Before measurement, carefully shake the plate to ensure an even distribution of the solution.

10. RESULTS

10.1. Quality control

The controls and calibrator included in the kit must be used for each race. The controls serve as internal controls to validate the test results. Thus, the optical density (OD) values of the controls must be within the following ranges, otherwise, the test results are not valid, and the test should be repeated:

CONTROL	OD Value
Positive control	>1.5
Negative control	<0.5
Calibrator	≥1.5
	≤0.5

It is recommended that all tests include the laboratory's own controls in addition to those supplied with this kit whenever possible.

10.2. Interpretation of the results

The results can be evaluated by calculating the relation or ratio between the O.D. of the sample or the control, on the O.D. of the calibrator, according to the following formula.

$$Ratio = \frac{O.D\ control\ or\ sample}{O.D.mean\ of\ calibrator}$$

Ratio	Result	Interpretation
<0.8	No reactive	Negativo para anticuerpos IgG anti-SARS-CoV-2.
≥0.8 a <1.1	Borderline	No se puede valorar la muestra con seguridad. Es recomendable repetir el análisis y si vuelve a salir un valor limítrofe, se recomienda solicitar, en una o dos semanas, una nueva muestra del paciente, para volver a analizarlo.
≥1.1	Reactive	Positivo para anticuerpos IgG anti-SARS-CoV-2

10.2. Quantitative evaluation of the results

This ELISA kit against Protein S has been calibrated against the WHO First International Standard for anti-SARS-CoV-2 immunoglobulin (human), NIBSC Code 20/136.
In this way, it is possible to quantitatively report the concentration of IgG immunoglobulins in United Antibody Units (BAU / ml) against protein S. For this, the kit includes a positive control of known concentration (consult the certificate of analysis of each kit, concentration may vary between batches) with which to make a calibration line through serial dilutions (Fig. 1)

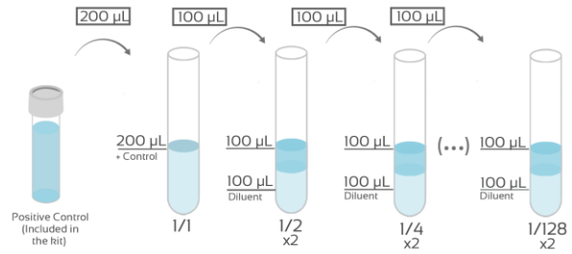

Figure 1: Serial dilution scheme (1: 2) of the positive control included in the kit for the construction of the calibration curve. Just as an example. A higher dilutional range may be required.

Each laboratory will have to determine its own dilutional range to obtain the regression model, which will preferably be linear, and for which it is recommended to use two replicates for each dilution and or concentration points.

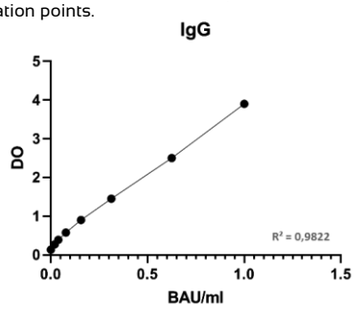

Figure 2: Representative calibration curve of a linear regression model. In some cases, it will be necessary to start by using higher dilutions (1: 2 or 1: 4) of the positive control as the first concentration point for the calibration curve.

Finally, it should be noted that the concentration of the positive control included in the kit is also reported in µg/ml, in addition to **BAU/ml** (consult the certificate of analysis for each kit, the concentration may vary between batches).

11. LIMITATIONS OF THE PROCEDURE

- The results of the samples should be assessed in combination with clinical symptomatology and other diagnostic procedures.
- A negative result does not exclude the possibility of SARS-CoV-2 infection. In an early stage of infection, the number of antibodies present in the sample may be below the detection limit of the test.
- A positive result demonstrates the presence of antibodies to SARS-CoV-2 and may therefore indicate either an ongoing or acute infection, or a past infection.
- Due to the high similarity between SARS-CoV-2 and other coronaviruses, cross-reactions of antibodies, especially against SARS-CoV, cannot be completely excluded.
- The results of the assay depend on whether the sample collection and processing procedures have been carried out correctly.

12. PERFORMANCE CHARACTERISTICS

12.1. Diagnostic sensitivity and specificity

For the assessment of diagnostic sensitivity, a couple of studies were carried out in 2 different clinical laboratories in Spain.

In the first one, 67 samples were analyzed and grouped according to the information available for each sample, in relation to the days that had passed since the confirmation of the PCR and the beginning of the symptoms.

Summary of results according to post-PCR days

Days after confirmation by PCR	N (Number of simples)	Negative	Borderline	Positive	%
0-7	10	4	1	5	50%
8-14	3	0	0	3	100%
15-21	5	0	0	5	100%
22-28	5	0	0	5	100%
29-35	2	0	0	2	100%
36-41	3	0	0	3	100%
42-48	3	0	0	3	100%
>48	2	0	0	2	100%
Total	34	4	1	29	

Counting borderline results as negative, the assay showed more than 50% positive agreement with PCR in the first days after PCR confirmation. Similarly, from day 15 after PCR, the agreement reached 100% (n = 21), while after 30 days the agreement remained at 100% (n = 10). Summary of the results according to the onset of symptoms:

Days from onset of symptoms	N (Number of simples)	Negative	Borderline	Positive	%
0-7	4	2	0	2	50%
8-14	6	2	1	3	50%
15-21	4	0	1	3	75%
22-28	6	0	0	6	100%
29-35	2	0	0	2	100%
36-48	5	0	0	5	100%
>48	6	0	0	6	100%
Total	33	4	2	27	

Counting borderline results as negative, the assay showed more than 50% positive agreement with CRP between days 0-7 after symptom onset. Similarly, from 15 to 21 days after the onset of symptoms, the concordance with CRP reaches 75% (n = 4), while from 22 days onwards the concordance reached 100% (n = 19).

To determine diagnostic specificity, the first study analyzed 76 samples from blood donors and sera from patients with rheumatoid arthritis, taken before the onset of the SARS-CoV-2 pandemic.
Descriptive summary of the results of O.D. obtained in the two series of samples:

Samples	N	Mean (O.D)	SD	Min	Max	95% of the confidence Interval for the mean value: Lowest limit	95% of the confidence Interval for the mean value: Highest limit
Blood donors (2018)	66	0,3606	0,1417	0,16	0,71	0,32	0,39
Serum Samples (2017)	10	0,2455	0,0487	0,21	0,35	0,21	0,28

Summary of the corresponding diagnostic specificity obtained for the two series of samples by counting the borderline values as negative:

Samples	N	Negative	Borderline	Positive	estimated
Blood donors (2018)	66	66	0	0	100%
Serum Samples (2017)	10	10	0	0	100%
Total	76	76	0	0	

In a second study, 45 donors divided into 3 groups were selected, a first group of patients vaccinated with the Pfizer-BioNTech vaccine (Comirnaty), 15 donors naturally infected by SARS-CoV-2 and 15 donors neither infected nor vaccinated. The objective of the study was to try to identify differences in the profile of those vaccinated and infected with respect to healthy non-vaccinated donors.

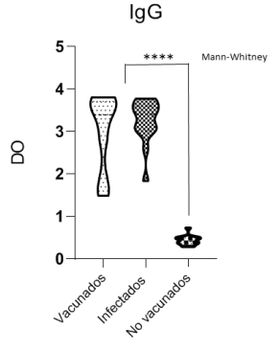

Figure 3: Violin diagram, comparing plasma samples from vaccinated donors and donors who have undergone the infection versus samples from donors neither exposed to the infection, nor vaccinated. Statistical significance was analyzed with a Mann-Whitney test. **** p, 0.0001.

The diagram shows a significant difference between samples from Comirnaty vaccinated donors and naturally infected donors relative to samples from non-infected and non-vaccinated donors. .

12.2. Precision

For the intra-laboratory precision study, three samples were selected (negative, borderline and positive) and following the CLSI and SEQC recommendations, a 20 x 2 x 2 experimental design was selected, consisting of a study that lasts at least 20 days. , with two races for each day that the test is carried out and with two replicates per sample tested in each race. The test was carried out on a single instrument. The results were the following:

	Sample 1		Sample 2		Sample 3	
Overall Result	Negative		Borderline		Positive	
N	78		78		78	
Mean value	0,263		0,892		3,135	
	SD	CV (%)	SD	CV (%)	SD	CV (%)
Repeatability	0,0276	11%	0,0367	4%	0.1919	6%
Between range	0,0384	15%	0.0761	9%	0,2840	9%
Within day	0,0345	13%	0,0592	7%	0,2577	8%
Within lab	0,0437	17%	0,0891	10%	0,3321	11%

12.3. Analytical specificity

To investigate the analytical specificity, the possible cross-reactivity of antibodies against other microorganisms that produce symptoms similar to SARS-CoV-2 infection was analyzed. In this way, 110 samples characterized as positive for IgG for the following microorganisms were selected: MERS-CoV (1), H. Influenzae (17), RSV (16), Influenza A (1), Influenza B (13), Parainfluenza (19), Adenovirus (7), Enterovirus (5) M. pneumoniae (13), Legionella (6), C. pneumoniae (12).

Due to the low homology of protein S between the coronavirus family, cross-reactivity with most human pathogenic representatives of this family of viruses is virtually excluded.

On the other hand, the homology of the S protein between SARS-CoV-2 and SARS-CoV, which emerged in China in 2013, is approximately 75%, which suggests that a cross-reactivity of antibodies against both viruses is possible.

12.4. Interference

The possible interference that high levels of hemoglobin, cholesterol and bilirubin could have on the performance of the assay was analyzed. For this, samples with different concentrations of IgG anti-SARS-CoV-2 antibodies were selected, which were enriched with potential interference and subsequently analyzed with the kit. The conclusion was that the performance of the assay is not affected by the use of hemolytic, lipemic, or jaundice samples, up to concentrations of 10 mg / ml of hemoglobin, 4 mg / ml of cholesterol and 0.4 mg / ml of bilirubin, respectively.

Furthermore, it has been proven that the presence of antinuclear antibodies (ANA) and rheumatoid factor (FA) in the sample can cause false positives. In this sense, it is recommended to use a dilution (1: 200) of patient samples with patients with rheumatoid arthritis and / or autoimmune diseases, to limit said positive interference.

On the other hand, the effects that the different matrices potentially would have on the trial results were also analyzed. For them, 20 donors with different concentrations of specific IgG antibodies against SARS-CoV-2 were selected.

Each of the donors was sampled in 4 types of tubes. 3 tubes of plasma each with a different anticoagulant: EDTA, heparin and citrate. And a tube with separator gel for the serum. The samples were analyzed following the kit instructions and in no case were significant differences observed between the different anticoagulants:

	N	Mean (O.D)	Desv	95% of the confidence Interval for the mean value: Lowest limit	95% of the confidence Interval for the mean value: Highest limit	Min	Max
Gel	20	1,4387	1,0163	0,7116	2,1657	0,19	2,73
EDTA	20	1,1590	0,9725	0,4633	1,8547	0,20	2,81
Heparin	20	1,1532	0,9785	0,4532	1,8531	0,17	2,86
Citrate	20	1,0698	0,9143	0,4157	1,7238	0,18	2,54
Total	80	1,2051	0,9704	0,5109	1,8993	0,18	2,73

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14. SYMBOL DESCRIPTION

	The content is sufficient for <n> analysis
REF	Reference of the producto
CE	CE Labelling
IVD	*in vitro* Diagnostic.
	Manufacturer
	Expiry date
LOT	Batch number
	Instructions of use
	Storage from x°C to y°C.
	Content per test
	Pay attention
	Biological Hazards
MTP	96-weel microplate (12x8)
WASHBUF	Wash Buffer 20X
DILUBU	Diluent Buffer
SUBS	Tetramethylobenzidine chromogenic substrate
SOL	0,5M of sulphuric acid (H2SO4).
CNTRL + IgG	IgG positive control.
CNTRL - IgG	IgG negative control.
CNTRL ± IgG	IgG calibrator.
CONJ IgG	Anti-human IgG conjugated.
INSTR	Instructions for use.
TAP	Protective film

15. MANUFACTURER INFO

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