Anti-SARS-Cov-2 ELISA IgG

REF	Σ	IVD
IMS2905	96	CE

1. 1. INTENDED USE

Qualitative enzyme-linked immunosorbent assay of specific IgG antibodies against Covid-19 based on the ELISA technique. The 96-well ELISA plates are coated with the recombinant specific antigen (Mpro or also called 3CLpro) 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 assist in the identification of individuals with an adaptive immune response to SARS-CoV-2, indicating a previous or recent infection and contributing in combination with other tests (PCR or antigen), to the determination of the stage of infection. Additionally, this assay can be used in research, seroprevalence studies and epidemiological surveillance.

1. FIELD OF APPLICATION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a new virus belonging 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 has spread worldwide until it was declared a pandemic by the WHO in March 2020. SARS-CoV-2 is a single-stranded, positive RNA virus that displays similarities in the organization and expression of its genome with SARS-CoV, in addition to other human respiratory coronaviruses (NL63, 229E, OC43 and HKU1) and with bat coronaviruses, which is its zoonotic reservoir[2]. Thus, its genome codes for 28 proteins, 16 non-structural proteins and 4 structural proteins, the S (spike protein), E (envelope), M (membrane) and N (nucleocapsid) proteins[3].

This virus causes various clinical manifestations that are covered by the term COVID-19, which include respiratory conditions ranging from the common cold to severe pneumonia with respiratory distress syndrome, septic shock and multiorgan failure. Most of the cases of COVID-19 reported so far debut with mild cases[4].

The routes of transmission of SARS-CoV-2 are similar to those described for other coronaviruses, highlighting transmission through the secretions of infected persons by respiratory droplets of more than 5 microns, which are capable of being transmitted at distances of up to 2 metres, as well as through 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 environments5.

The host 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, following the detection of viral RNA in swabs by reverse transcriptase polymerase chain reaction (RT-PCR), can develop antibodies from barely 2 days to 2 weeks from the onset of symptoms.

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

The vast majority of serological tests for specific antibodies to SARS-CoV-2 are based on serum detection of antibodies to either the S protein (the main target of neutralizing antibodies) or the N protein, which is known to be highly immunogenic protein in other coronaviruses and is the most widely used. In this sense, in an attempt to increase the diagnostic possibilities of COVID-19 patients, this assay is based on the detection of specific antibodies against one of the 16 non-structural proteins, specifically against the main viral protease (Mpro or 3CLpro), which plays a critical role in viral replication.

This is why it has been proposed as a target for specific inhibitors of virus replication7 and has also proved to be a powerful immunogen, allowing the detection of very low antibody titers.

Additionally, reactivity against Mpro protease in COVID-19 patients has been comparable to or stronger than reactivity against S or N protein, suggesting that humoral response against different virus antigens may be heterogeneous among different patients[8].

3. BASIS OF THE METHOD

The kit contains microtitre plates upholstered with the recombinant protein Mpro. In a first step, the patient's diluted sample is incubated in the wells, allowing specific antigen antibodies to bind to the Mpro protein. After washing the wells, to remove unbound antibodies, in a second incubation step, an anti-human immunoglobulin (IgG) antibody conjugated to horseradish peroxidase (HRP) is added. After a second washing step, the substrate tetramethylbenzidine (TMB) is added causing the enzyme-conjugated to the anti-IgG antibody catalyzes a colour reaction. The color intensity of the reaction is proportional to the amount of antigen-specific antibodies present in the sample.

The test can be performed automatically or manually and is for professional use.

4. REAGENTS

4.1. Contents of the Kit

The reagents included in a kit are sufficient to carry out 96 determinations. Each anti-SARS-CoV-2 IgG ELISA 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 (IX - 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 (IX - Ready to use)
SOL	**12 ml of stopping solution** (IX - Ready to use) 0.5M sulphuric acid (H_2SO_4)
CNTRL + IgG	**1.5 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.
INSTRU	**1 Instructions for use.**
TAP	**2 units of protective film.**

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

- Calibrated spectrophotometer for reading ELISA plates at 450 nm and 620 nm.
- Adjustable calibrated micropipettes covering a range of 1-1000 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.

6. 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.

7. RECOMMENDATIONS AND WARNINGS

⚠ FOR IN VITRO 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 1:100 in the antibody/sample dilution buffer (IX) included in the kit and mixed with a vortex shaker. The diluted samples should be tested within 8 hours.,

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.

Micro 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 aluminium foil bag together with the desiccant bag.

9. TEST PROCEDURE

1.	Sample Incubation	Add 100 µl of the positive control, the negative control, the calibrator and the prepared sample (1:100 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 IX 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 (IX - 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 gauge 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	O.D value
Positive control	>1.7
Negative control	<0.7
Calibrator	≥1.7
	≤0.7

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	Negative for IgG anti-SARS-CoV-2 antibodies.
≥0.8 a <1.1	Borderline	The sample cannot be assessed with certainty. It is advisable to repeat the analysis and if a borderline value is found again, it is recommended that a new sample be requested from the patient within one or two weeks, to be analysed again.
≥1.1	Reactive	Positive for anti-IgG anti-SARS-CoV-2 antibodies

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 amount 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 specifity

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

In the first one, 160 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 samples)	Negative	Doubtful	Positive	
0-1	23	7	3	13	57%
2-3	17	5	1	11	65%
4-5	21	7	0	14	67%
6-7	10	3	0	7	70%
8-14	16	2	1	13	81%
15-21	15	2	0	13	87%
22-28	14	1	0	13	93%
29-35	13	2	0	11	85%
36-41	9	0	0	9	100%
42-48	10	0	0	10	100%
Total	149	32	5	86	

Counting borderline results as negative, the trial showed more than 57% positive agreement with PCR in the first days after PCR confirmation. Similarly, from 15 to 21 days after PCR, concordance reached 87% (n=15), while from 36 to 41 days concordance reached 100% (n=9).

Summary of results according to the onset of symptoms:

Days from the onser of the symptoms	N (number of samples)	Negative	Doubtful	Positive	
0-7	32	12	3	17	53%
8-14	34	9	2	23	68%
15-21	18	3	0	15	83%
22-28	16	3	0	13	81%
29-35	12	2	0	10	83%
36-41	13	0	0	13	100%
Total	125	29	5	91	

Counting borderline results as negative, the trial showed an 53% positive agreement with PCR within days 0-7 after the onset of symptoms. Similarly, from 15 to 21 days after the onset of symptoms, concordance with PCR reached 83% (n=12), while from 36 to 41 days concordance reached 100% (n=13).

To determine diagnostic specificity, the first study analyzed 50 blood and serum donor samples from patients with rheumatoid arthritis, taken before the start of the SARS-CoV-2 pandemic.
Descriptive summary of the O.D. results obtained in the two series of samples:

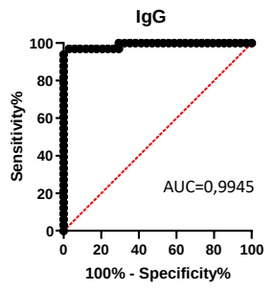
Samples	N	Mean (O.D)	SD	Minimum	Maximal	95% of the confidence interval for the mean	
						Lowest Limit	Highest Limit
Blood Donors (2018)	34	0,1576	0,1189	0,0455	0,7055	0,12	0,19
Serum Samples (2017)	16	0,1139	0,0533	0,0470	0,2830	0,11	0,13

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
Spanish Blood Donors (2018)	34	0	0	34	100%
Serum Samples (2017)	16	0	0	16	100%
Total	50	0	0	50	

In a second study, 36 patients diagnosed by PCR were selected, 9 of whom had active SARS-CoV-2 infection at the time of the study, while the rest had no detectable levels of virus. In parallel, 33 serum samples from patients with various pathologies (rheumatoid arthritis, allergy, monoclonal gammopathy) taken before June 2019 were selected to be used as negative controls.

Sensitivity and specificity were evaluated by analyzing a ROC (Receiver Operating Characteristics) curve



The graph represents the relationship between sensitivity and specificity, which for this trial was 97% and 100% respectively.

12.2. Precision

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

	Sample 1		Sample 2		Sample 3	
Overall Results	negative		Borderline		positive	
N	50		50		50	
Average value	0,067		0,895		2,851	
	SD	CV (%)	SD	CV (%)	SD	CV (%)
Repeatability	0,008	11,7%	0,0574	6%	0,0606	2%
Between Range	0,011	16,5%	0,1187	13%	0,1942	7%
Within day	0,010	14,2%	0,1001	11%	0,1791	6%
Within lab	0,012	18,4%	0,0857	10%	0,0963	3%

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. Thus, 110 samples were selected that were characterized as positive for IgG for the following microorganisms: 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).

Although the similarity between the Mpro of different coronaviruses SARS-CoV-2, HCovNL63, HCovOC43, and HCov229E is only about 40%, and although in principle it would not be necessary to carry out cross-reactivity studies, 70 pre-pandemic serum samples were analyzed, most of which contained antibodies to HCovOC43, a virus that causes mild illnesses similar to the common cold, while no antibodies to SARS-CoV-2 were detected, demonstrating that infections with this coronavirus do not generate cross-reactivity.

On the other hand, Mpro homology between SARS-CoV-2 and SARS-CoV, which emerged in China in 2013, reaches 96%, suggesting that cross-reactivity of antibodies to both viruses is possible.

12.4. Interference.

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

On the other hand, the effects that the different matrices would potentially have on the results of the trial 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 plasma tubes each with a different anticoagulant: EDTA, heparin and citrate. And a tube with separating gel for the serum. The samples were analyzed according to the instructions of the kit and in no case were significant differences observed between the different anticoagulants:

	N	Mean	Desv	95% confidence interval deviation for the mean		Minimum	Maximum
				Lowest Limit	Highest Limit		
Gel	20	1,1004	0,6976	0,7771	1,4327	0,26	2,23
EDTA	19	1,2245	0,64601	0,9132	1,5359	0,24	2,32
Heparin	20	1,0825	0,58197	0,8101	1,3548	0,27	1,94
Citrate	20	1,0347	0,58343	0,7616	1,3078	0,25	1,92
Total	79	1,1091	0,61873	0,9705	1,2477	0,24	2,32

13. REFERENCES

1. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS CoV-2. Nature Microbiology. 2020;5(4):536-544.
2. Zhou P, Yang X, Wang X, Hu B, Zhang L, Zhang W et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020;579(7798):270-273.
3. Wu A, Peng Y, Huang B, Ding X, Wang X, Niu P et al. Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China. Cell Host & Microbe. 2020;27(3):325-328.
4. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. New England Journal of Medicine. 2020;382(8):727- 733.
5. Van Doremalen N, Bushmaker T, Morris D, Holbrook M, Gamble A, Williamson B et al. Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. New England Journal of Medicine. 2020;382(16):1564-1567.
6. Zhang L, Lin D, Sun X, et al. Crystal structure of SARS-CoV- 2 main protease provides a basis for design of improved α-ketoamide inhibitors. Science 2020; 368: 409–412.
7. Martinez-Fleta P, Alfranca A, González-Álvaro I, Casasnovas J, Fernández Soto D, Esteso G et al. SARS-Cov-2 cysteine-like protease (Mpro) is immunogenic and can be detected in serum and saliva of COVID-19-seropositive individuals. 2020;.

14. WARRANTY

Warranted only to conform to the quantity and contents stated on the label or in the product labelling at the time of delivery to the customer. Immunostep disclaims hereby other warranties. Immunostep's sole liability is limited to either the replacement of the products or refund of the purchase Price.

15. EXPLICACIÓN DE LOS SÍMBOLOS

Σ	The content is sufficient for <n> analysis.
REF	Reference of the product
CE	CE labelling
IVD	In vitro diagnostic
	Manifacturer
	Expiry date.
LOT	Batch number
	Instructions for use
	Storage from x°C to °C.
	Content per test.
	Pay attention
	Biological hazards
MTP	96-well microplate (12x8).
WASHBUF 20X	Wash Buffer 20x.
DILUBUF	Diluent Buffer.
SUBS TMB	Tetramethylobenzidine chromogenic substrate..
SOL STOP	0,5M of sulphuric acid (H_2SO_4).
CNTRL + IgG	IgG positive control
CNTRL - IgG	IgG negative control.
CNTRL ± IgG	IgG calibrator.
CONJ IgG	Anti-human IgG conjugated.
INSTRU	Instructions for use
TAPE	Protective film.

16. MANUFACTURER

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