Serological SARS-CoV-2 Multiplex Assay

REF	Σ	IVD
IMS0509	96	CE

1. INTENDED USE

The SARS-CoV-2 multi-antigen IgG + IgA + IgM kit is a multiplex, bead-based immunoassay for the simultaneous and qualitative detection of specific IgG, IgM and IgA antibodies to 4 different antigens in SARS-CoV-2 array format by flow cytometry.

SARS-CoV-2 antigens included in the kit:

- Receptor-binding domain (RBD) of S-glycoprotein.
- Stable trimer of the spicule glycoprotein (S).
- Nucleocapsid protein (N).
- Main virus protease or 3C-type protease (3CLpro, Mpro)

Each array bead has a unique fluorescence intensity pattern and is coated with a recombinant virus-specific antigen and allows antibodies binding present in human serum and plasma samples (K2-EDTA, K3-EDTA, Heparin-Li, Sodium Citrate). After incubation and subsequent washing with anti-human IgG, IgA and IgM antibodies conjugated with different fluorochromes (FITC, PE and PE-Cyanine7), the samples are acquired in a flow cytometer.

The intended use of the assay is to assist in the identification of individuals with an adaptive immune response to SARS-CoV-2, indicating previous or recent infection and contributing, in combination with other tests (PCR or antigen), to the determination of the stage of infection. By testing several antigens and antibody classes at the same time, the assay can provide earlier and more sensitive results, contributing to its use in research, seroprevalence studies and epidemiological surveillance.

2. APPLICATION FIELD

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new virus belonging to the coronavirus family, of the genus Betacoronavirus1, which emerged in China in the city of Wuhan, Hubei province in December 2019 and has spread worldwide to be declared a pandemic by the WHO in March 2020. SARS-CoV-2 is a positive-sense, single-stranded RNA virus that has similarities in genome organisation and expression to SARS-CoV, as well as to other human respiratory coronaviruses (NL63, 229E, OC43 and HKU1) and bat coronaviruses, which is its zoonotic reservoir2. From 5' onwards, two thirds of the SARS-CoV-2 genome encodes for two polyproteins, ppla and pplab, called replicase. These polyproteins are in turn cleaved into 16 non-structural proteins, including RNA-dependent RNA polymerase (RdRp), by the action of two viral proteases essential in virus replication: 3C-type protease (3CLpro) and papain-like protease (PLpro). The other third of the virus genome codes for 8 accessory proteins, which are not essential for replication, and 4 structural proteins, protein S (spicule glycoprotein), protein E (envelope), protein M (membrane) and protein N (nucleocapsid)3 Thus, its genome codes for a total of 28 proteins.

In relation to the viral proteins included in this assay, protein S, which plays a key role in receptor recognition and the cell membrane fusion process, 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 (ECA2), while the S2 subunit facilitates fusion between the viral envelope and the plasma membrane of its target cell. On the other hand, the SARS-CoV-2 viral membrane is surrounded by a helical nucleocapsid in which the viral genome is encapsulated by the nucleocapsid protein (N), this viral protein is produced at high levels within infected cells, improving the efficiency of viral RNA transcription and is essential for viral replication. Lastly, the main viral protease (Mpro or 3CLpro) also plays a critical role in viral replication, which is why it has been proposed as a target for specific inhibitors of virus replication4 and has also been found to be a potent immunogen.

This virus causes various clinical manifestations grouped under the term of COVID-19, including respiratory symptoms ranging 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[5].

The routes of transmission of SARS-CoV-2 are similar to those described for other coronaviruses, notably through the secretions of people infected with respiratory droplets larger than 5 microns, which are capable of being transmitted over distances of up to 2 metres, as well as through hands or fomites contaminated with these secretions followed by contact with mucous membranes of the mouth, nose or eyes. Similarly, the virus can be viable in the air, so airborne transmission by aerosols is also possible, albeit in a restricted way[5].

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

There are five different classes of human antibodies, also called immunoglobulins. Three of them (IgG, IgM and IgA) are commonly used in serological assays 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 it may be detectable in COVID-19 patients after initial exposure to the virus, conferring long-term immunity.

IgA is present in high concentrations in mucous membranes, particularly in the inner walls of the respiratory tract and gastrointestinal tract, as well as in saliva and tears, and in fact, it is the most important immunoglobulin to combat infectious pathogens in the respiratory and digestive system at the point of pathogen entry. The detection of specific IgA in the serum of SARS-CoV-2 infected patients has been widely described, making its appearance earlier, stronger and more persistent than IgM. Overall, the median time to IgA, IgM and IgG seroconversion has been reported to be 4-6, 4-6 and 5-10 days after the onset of the symptoms, respectively[6]. These data suggest that IgA detection may improve the diagnostic outcome in the early stages of infection and make them good potential markers to identify patients with COVID-19.

IgM is mainly found in the blood and lymphatic fluid; This is the first antibody the body makes to fight a new infection. It is also called macroglobulin due to its size: it is the largest immunoglobulin (950,000 Daltons), although the size is not exclusively due to the actual molecular weight of the molecule, but rather it has the ability, through its Fc region, to interact with four other IgM molecules, forming a high molecular weight complex of five IgM molecules.

The ability of IgM to form these complexes - which makes it very easy to bind the complement - is what gives it the power to opsonize certain antigens, causing the lysis of bacteria, enveloped viruses as in the case of SARS-CoV- 2 and other pathogens. Although it is widely described that IgM is the first type of immunoglobulin synthesized in response to infection, this is not entirely clear in COVID-19 patients, where IgG and especially IgA can be present at the same time or even prior to IgM in some cases.

The vast majority of serological tests for the detection of SARS-CoV-2 specific antibodies are based on the detection in serum of antibodies against protein S (the main target of neutralising antibodies) or protein N, known to be highly immunogenic in other coronaviruses, and which is the most widely used. However, it seems reasonable to think that exposure to other antigens released from infected cells, such as the major viral protease (Mpro or 3CLpro), which plays a critical role in viral replication, might stimulate an antibody response that could correlate with tissue damage and, therefore, may have some value as a prognostic indicator. Additionally, it seems clear that the humoral response against different viral antigens may be heterogeneous between different patients[7]. It has been described how the decrease in antibody titre is more pronounced for some antigens[8], or how the humoral response against some antigens may correlate with the severity of the infection. The differential profile between adults and children in relation to humoral response has also been reported[9] and how this may be related to possible cross-reactivities to other human coronaviruses (HCoV)[10].

Therefore, it is important that the detection of SARS-CoV-2 specific antibodies in any sample, positive or negative, be tested against several viral antigens, improving the sensitivity of the serological assay, thus avoiding incomplete or erroneous interpretation and contributing to improve the diagnosis of COVID-19 patients.

3. METHOD

The kit allows the detection of IgG, IgA and IgM antibody classes against four (4) magnetic beads with a unique and differential fluorescence intensity pattern for each of them and which are each coated with one of the SARS-CoV-2 antigens (N, Mpro, S, RBD) included in the kit. In a first step, the diluted patient sample is incubated with the beads, allowing allowing the antigen-specific antibodies to bind to the different proteins that are coating the beads. After washing the beads, to remove unbound antibodies, in a second incubation step, Fluorescein-conjugated anti-human immunoglobulin IgG, IgA and IgM antibodies (FITC), Phycoerythrin (PE) and Phycoerythrin Cyanine 7 (PE-Cyanine 7) respectively are added. After a second washing step, the beads are resuspended and analyzed in the cytometer. The fluorescence intensity is proportional to the number of antigen-specific antibodies present in the sample. The assay can be performed on any conventional flow cytometer, and it is for professional use.

4. REAGENTS

4.1. Content of the kit

The reagents included in one kit are sufficient to carry out 96 determinations. Each SARS-CoV-2 Multi-Antigen IgG + IgA + IgM kit contains:

BEADS	Magnetic polystyrene microspheres with a diameter (µm) 5.5 ±0.2 (CV<5%), coated with the following SARS-CoV-2 antigens: • RBD - bead population no. 1. • S - bead population no. 2. • N - bead population no. 3. • Mpro - bead population no. 4. The microspheres are supplied in 4 separate vials at the following concentration: 1000 microspheres/test (10µl/test) - 1ml/vial and in a buffered aqueous solution containing protein stabiliser and 0.09% sodium azide (NaN3) as anti-microbial agent..
WASHBUF 10X	**20 ml wash buffer (10X).** PBS 10% BSA, pH 7.4 - 10X. Contains 10% albumin in 10mM sodium phosphate, 150mM NaCl, pH 7.4, contains KATHON™ anti-microbial agent. Dilute the contents of the 10X assay buffer to 1X (PBS 1% BSA) in PBS, for use in this assay.
DILUBU	**10 ml of sample diluent buffer (1X - Ready to use).** Buffer that minimises non-specific binding, cross-reactivity and matrix interference. Contains CMIT/MIT 3:1 as preservative.
CNTRL +	**0.16 ml positive control.** Contains CMIT/MIT 3:1 as preservative. Ready to use.
CNTRL -	**0.16 ml negative control.** Contains CMIT/MIT 3:1 as preservative. Ready to use.
CAL	**80 µl calibrator.** Contains CMIT/MIT 3:1 as preservative. Ready to use. It is advisable to give it a spin before each use.
CONJ	• 500 µl FITC-conjugated anti-human IgG antibody (5 µl/test). • 500 µl PE-conjugated anti-human IgA antibody (5 µl/test). • 500 µl of PE-conjugated anti-human IgM antibody - Cyanine 7 - (5 µl/test). The antibodies are supplied in 3 different vials, use concentration and buffered aqueous solution containing protein stabiliser and 0.09% sodium azide (NaN3) as anti-microbial agent.
PLATE	**12×8 well (12X8) black microtiter plate with cap.** For protocol with plate.
INSTR	1 **Instructions for use.**

4.2. Required materials, reagents and equipment not supplied.

- Flow cytometer equipped with at least one blue laser, 488 nm, and fluorescent channels for FITC (Ex-Max 494 nm/Em-Max 520 nm), PE (Ex-Max 496 nm/Em-Max 578 nm) (Ex max: and PE-Cyanine 7 (Ex-Max 496 nm/Em-Max 785 nm).
- Adjustable calibrated micropipettes covering a range of 1-1000 µL and corresponding disposable pipette tips.
- Magnetic rack; MagneSphere(R) Mag. Sep. Stand 12- hole, 12x75mm (PROMEGA, Ref Z5343). For tube protocol.
- 12x75 mm Polystyrene round bottom tubes (Cytometer tubes). For tube protocol.
- Magnetic separation block for 96-well flat or conical bottom (Merck, Ref.40-285). For plate protocol.
- Timer.
- Disposable gloves.
- Waste container for biological substances.

5. STORAGE AND HANDLING CONDITIONS

Store refrigerated between +2 and +8° C. DO NOT FREEZE.

The unopened kit is stable until the expiry date. Do not use after this date. After opening, reagents are stable if stored at +2 to +8° C and protected from contamination. Do not leave the reagents open and at a different temperature from the storage temperature for longer than strictly necessary.

6. RECOMENDATIONS AND WARNINGS.

⚠ FOR IN VITRO DIAGNOSTIC USE. For professional use only.

⚠ For qualified laboratory personnel only.

⚠ The kit components contain KATHON™, sodium azide (NaN3) and CMIT/MIT. The compounds should be dissolved with tap water before disposal. These conditions are recommended to avoid deposits in pipes. Material Safety Data Sheet (MSDS) available on the web. www.immunostep.com

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

⚠ Keep the kit components away from direct light exposure during the protocol. Fluorescently conjugated antibodies are sensitive to light.

⚠ Specimens should be treated in the same way as those that could transmit infections. Appropriate methods of handling should be available..

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

⚠ Wear personal protective equipment for sample handling. Wash hands properly after handling samples. All procedures should 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 should be handled and discarded as potentially infectious using safe laboratory procedures.

7. SAMPLING COLLECTION

Collection of samples (serum, EDTA plasma, heparin or citrate) should be done in suitable collection tubes, using the appropriate anticoagulant. Samples should remain at room temperature for no more than 8 hours. If the assay is to be performed beyond 8 hours, samples should be refrigerated at +2 to +8° C. If the assay is not to be completed within 48 hours of sample extraction, then samples should be stored frozen at - 20° C or below, avoiding unnecessary freeze-thaw cycles. Samples must be properly inactivated. An inactivation protocol may be to hold samples at 56° C for 30 minutes before use.

7.1. Sampling preparation.

Samples are diluted 1:20 in the antibody and sample diluent buffer (1X) included in the kit and mixed with a vortex agitator. Diluted samples should be assayed within 8 hours.

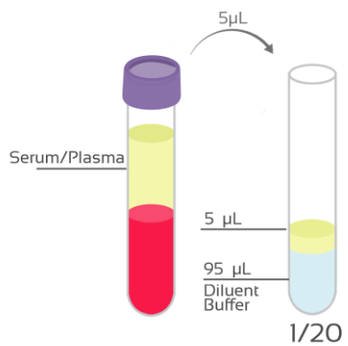

Fig. 1: graphical representation of a 1:20 sample preparation. It is recommended to prepare two replicates per sample. It is advisable to work with sample volumes of at least 5 µL to minimize pipetting errors.

8. PREPARATION OF REAGENTS

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

The wash buffer included in the kit is a 10X concentrate. If crystallization is observed in the concentrated buffer during storage, warm to 37°C and properly shake before diluting. To carry out the dilution, the amount required for the assays is removed from the concentrate bottle and diluted 1:10 in PBS.

If the protocol is to be carried in a tube, it will be necessary to prepare and label as many 12x75 mm polystyrene round bottom tubes (cytometer tube) as samples and controls are to be tested.

9. ASSAY PROCEDURE

The protocol can be carried out in a cytometer tube (12x75 mm) or in a 96-well microplate (ANNEX I and II).

1.	Preparation of microspheres	Resuspend each of the magnetic bead vials capped with the viral antigens by vortexing for approximately 20 seconds. Add 10 µl of each of the four antigen-capped beads (RBD, S, N and Mpro) to the tubes listed above. Then add 10 µl of the sample (see 7.1 Sample preparation) to the corresponding tube. Shake with a vortex for about 20 seconds.
3.	Preparation of controls	Add 10µl of each control to each of the tubes identified as such above. Shake with a vortex for about 20 seconds.
4.	Incubation of the sample	Incubate for 60 minutes at room temperature, in the dark and shaking, either tube or plate. When the process is microplate, cover it with the lid.
5.	Washing	After incubation, wash the sample (antigen-specific antibodies bound to the beads) twice using 1 ml (tube) or 300 µl (microplate) of 1X wash buffer (see point 8. Preparation of reagents) for each wash. Leave the wash buffer in each tube or well for 30 to 60 seconds per wash cycle. Subsequently, collect the magnetic beads by placing the tubes or the microplate in a magnetic rack or plate respectively and incubate for 5 minutes. Collection of the beads can also be performed by centrifugation at 2500xg for 5 minutes. Remove the supernatant from the tubes or the microplate by manual decanting if using the magnetic plate or rack, or by aspiration if centrifugation is used. Be careful not to disturb the beads and make sure to leave a minimum volume of 50 µl and a maximum of 85µl of supernatant in the tube or well.
6.	Incubation of the conjugate	Add 5 µl of each of the 3 fluorescently conjugated anti-Ig-human antibodies (IgG, IgA and IgM) to the cytometer tubes or microplate wells. Vortex for about 20 seconds and incubate for 30 minutes at room temperature, in the dark and shaking. When the process is microplate, cover the microplate with the lid.
7.	Washing	After incubation wash 2 times as described above (step 5).
8.	Acquisition	Resuspend the sample in 200µl in PBS and acquire in a flow cytometer or store protected from light for a maximum of 30 min at 2-8°C, until analysis is carried out.

10. ACQUISITION AND ANALYSIS OF THE ASSAY IN THE CYTOMETER.

A proper bead population selection strategy will allow the removal of doublets and dirt traces, contributing to the correct identification of the 4 bead populations (RBD, S, N and Mpro),
For this purpose, the first step is the selection the bead population on the FSC-H/FSC-A dot plot to remove doublets (A), followed by a selection of the bead population on the SSC-A/FSC-A dot plot to remove dirt traces and reduce background marking (B) is recommended, allowing the correct identification of the 4 bead populations on a dot plot for any of the following channels PerCP/APC, PerCP-Cy5/APC or PerCP-Cy5.5/APC (C).

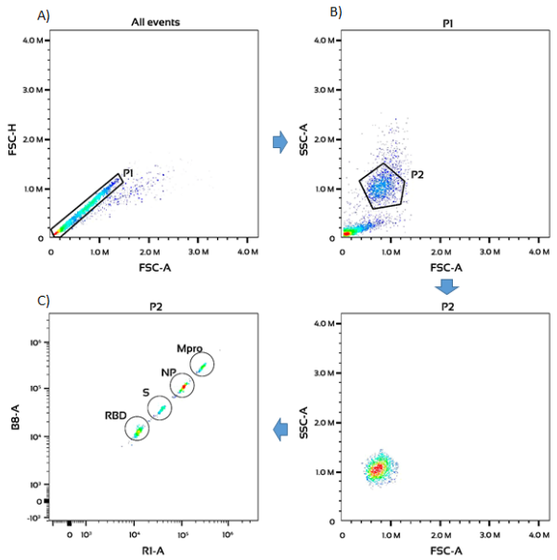

Fig. 2: Analysis strategy for bead population selection in the FSC-H/FSC-A (A); SSC/FSC (C) and PerCP/APC (C) dot plots.

11. RESULTS

11.1. Interpretation of results

The threshold or borderline value is equal to the mean value from the median fluorescence intensity values of the replicates corresponding to the negative control times 3 times the standard deviation. Threshold (limiting value) = µ (median) ± 3σ (of the negative control).

Whenever possible, it is recommended that all assays include the laboratory's own negative controls in addition to the one supplied with this kit for the calculation of the threshold.

11.1.1. Qualitative assessment

Result	Interpretation
Negative	Samples with fluorescence intensity values below the lower limit of the limiting value.
Borderline or Thershold	Lower limit = threshold Upper limit = threshold + 20% (threshold x 1.2)
Positive	Samples with fluorescence intensity values above the upper limit of the limiting value

11.1.1. Quantitative assessment.

The results can be evaluated by calculating the corresponding antibody index in units per ml, using the ratio of the MFI of the sample to the MFI of the negative control, according to the following formula:

$$Antibody\ Index = \frac{control\ or\ sample\ IMF}{Threshold} \text{x10}$$

The unit of measurement AU/ml is an arbitrary unit that is directly related to the concentration of antibody in the sample but does not allow to know its direct concentration (mg/ml).

Índice Abs (AU/ml)	Results	Interpretation
<10	Negative	Negative for anti-SARS-CoV-2 antibodies.
≥10 a ≤12	Borderline	The sample cannot be assessed with certainty. It is advisable to repeat the analysis and if a borderline value is found again, is recommended to request a new sample from the patient in one or two weeks for retesting.
>12	Positivo	Positive for anti-SARS-CoV-2 antibodies.

The range corresponding to the antibody index is variable, between samples, antibodies (IgG, IgA and IgM) and between the different viral antigens used in the test (RBD, S, N and Mpro). The following is an example of the range corresponding to the antibody index, in a standard assay (n=87), with 28 negative samples and 59 positive samples by PCR:

IgG

	Statistician	SD	95% confidence interval for the mean Lower limit	95% confidence interval for the mean Upper limit	Minimum	Maximum	Range
RBD	35,3793	4,4422	26,5486	44,2100	6,00	208,00	202,00
S	66,2759	12,3684	41,6883	90,8634	4,00	635,00	631,00
N	33,2069	5,1322	23,0044	43,4094	2,00	211,00	209,00
Mpro	36,2069	5,3984	25,4753	46,9385	2,00	219,00	217,00

IgA

	Statistician	SD	95% confidence interval for the mean Lower limit	95% confidence interval for the mean Upper limit	Minimum	Maximum	Range
RBD	14,0230	1,29275	11,4531	16,5929	6,00	87,00	81,00
S	62,3793	11,74747	39,0261	85,7325	5,00	619,00	614,00
N	41,0920	9,54217	22,1227	60,0612	2,00	581,00	579,00
Mpro	49,2644	11,39184	26,6181	71,9106	3,00	693,00	690,00

IgM

	Statistician	SD	95% confidence interval for the mean Lower limit	95% confidence interval for the mean Upper limit	Minimum	Maximum	Range
RBD	28,2299	4,48669	19,3106	37,1491	2,00	236,00	234,00
S	91,7931	23,94170	44,1986	139,3876	0,00	1046,00	1046,00
N	17,5517	4,96130	7,6890	27,4145	0,00	231,00	231,00
Mpro	19,7701	5,52227	8,7922	30,7480	0,00	276,00	276,00

In another assay with a PCR positive sample, linearity of the assay was demonstrated during evaluation within the following measurement ranges (AU/ml) for each viral antigen and antibody:

	RBD Abs Index (AU/ml)	RBD R^2	S Abs Index (AU/ml)	S R^2	N Abs Index (AU/ml)	N R^2	Mpro Abs Index (AU/ml)	Mpro R^2
IgG	160 - 17	0,99	1942 - 6	0,97	780 - 4	0,99	267 - 1	0,99
IgA	44 - 6	0,99	354 - 6	0,98	94 - 2	0,98	84 - 2	0,98
IgM	64 - 1	0,99	989 - 2	0,99	11 - 0,1	0,99	25 - 0,2	0,99

11.1.3. Results Report in International Units (IU/ml) for IgG.

The SARS-CoV-2 multiplex assay has been calibrated against the WHO First International Standard for Anti-SARS-CoV-2 Immunoglobulin (Human), NIBSC Code 20/136.

In this sense, it is possible to quantitatively report the concentration of IgG immunoglobulins in International Units (IU / ml) against RBD or in United Antibody Units (BAU / ml) against S, N and Mpro for IgG. For this reason, the kit includes a calibrator or standard of known concentration (consult the certificate of analysis of each kit, since the concentration may vary between batches) with which to make a calibration line through serial dilutions (Fig. 2) to each of the viral antigens used in the test (RBD, S, N, Mpro) in which to interpolate the fluorescence values resulting from the assay of the samples, thus obtaining the concentration in IU / ml or BAU / ml corresponding to each sample.

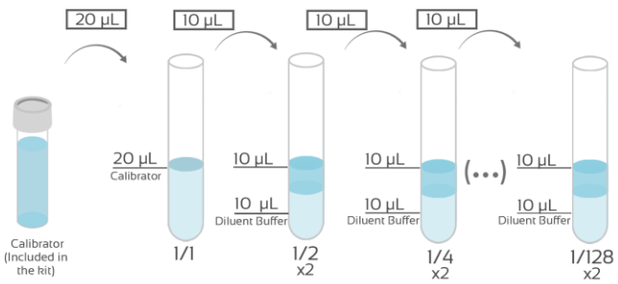

Fig. 3: Graphical Representation of the serial dilution (I: 2) of the calibrator included in the kit for the construction of the calibration curve. This is an example; you may require a higher dilution range.

Each laboratory will have to determine its own dilutional range to obtain the regression model, which will preferably be linear. It is recommended to use two replicates for each dilution and 8 concentration points.

The calibrator preparation is added to the beads in a 1:5 dilution ratio (10 µl of the calibrator dilution and 40 µl corresponding to the 4 types of beads, 10 µl of each), and therefore, the concentration of the first serial dilution tube corresponds to a 1:5 dilution of the calibrator concentration.

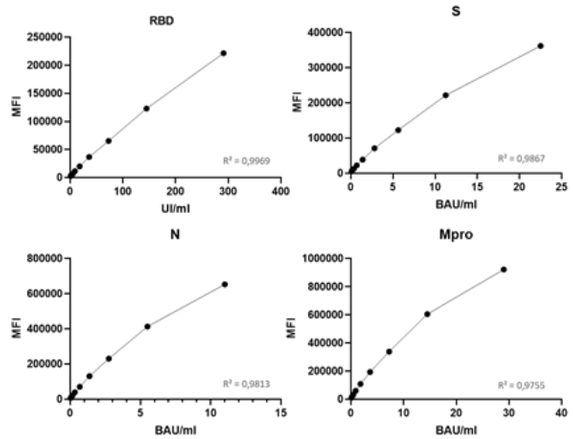

Fig. 4: Representative calibration curve of a linear regression model, generated using the SARS-CoV-2 multiplex serological assay for IgG. This is an example.

It is possible that lower dilutions (1:1 to 1:8 or 1:16) contain an excess of antibody that saturates the assay and produces a broad effect and therefore could not be used as concentration points for the construction of the linear regression model.

13. QUALITY CONTROL

The controls included in the kit must be used in each race. Controls serve as internal controls to validate assay results. Thus, the antibody index values (AU / ml) of the controls must be within certain ranges (consult the certificate of analysis of each kit, since the concentration may vary when changing the lot), otherwise the test results are invalid, and the test should be repeated.

It must be considered that the dynamic range of the assay is different for each antigen and immunoglobulin class and therefore it is recommended, whenever possible, that all the assays include the laboratory's own controls in addition to those supplied with this kit.

14. LIMITATIONS OF THE PROCEDURE

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

15. PERFORMANCE CHARACTERISTICS

15.1. Diagnostic sensitivity and specificity

For the evaluation of diagnostic sensitivity, a study was carried out in 2 different clinical laboratories in Spain, where samples were analysed and grouped according to the information available for each sample, in relation to the days that had elapsed since PCR confirmation and symptom onset.

Summary of results according to days post-PCR for each of the immunoglobulin classes

IgG						
Days	VP	FN	Total	PPA	95% (C.I)	
<7	14	2	16	87,5%	72,1%	100,0%
7-21	10	0	10	100,0%	38,0%	100,0%
>21	14	1	15	93,3%	80,6%	100,0%

IgA						
Days	VP	FN	Total	PPA	95% (C.I)	
<7	14	3	17	82,4%	64,3%	100,0%
7-21	10	0	10	100,0%	38,0%	100,0%
>21	19	1	20	95,0%	85,4%	100,0%

IgM						
Days	VP	FN	Total	PPA	95% (C.I)	
<7	11	4	15	73,3%	50,9%	95,7%
7-21	9	0	9	100,0%	38,0%	100,0%
>21	13	5	18	72,2%	49,5%	94,9%

(TP, true positive; FN, false negative)

Counting borderline results as negative, the assay showed 72%, 82% and 73% positive concordance (PPA) with PCR in the first days after PCR confirmation for immunoglobulins IgG, IgA and IgM respectively. Similarly, from 7 to 21 days after PCR, concordance reached 100% for all the three immunoglobulins. Summary of results according to the onset of symptoms:

IgG						
Days	VP	FN	Total	PPA	95% (C.I)	
<7	30	5	35	85,7%	75,0%	100,0%
7-15	47	3	50	94,0%	86,4%	99,3%
>15	25	1	26	96,2%	86,1%	100,0%

IgA						
Days	VN	FP	Total	PPA	95% (C.I)	
<7	33	5	38	83,3%	74,5%	98,7%
7-15	46	10	56	80,0%	71,5%	92,7%
>15	28	1	29	96,6%	86,7%	99,9%

IgM						
Days	VN	FP	Total	PPA	95% (C.I)	
<7	29	7	36	80,6%	64,3%	96,9%
7-15	47	9	56	90,0%	65,3%	100,0%
>15	20	5	25	80,0%	57,3%	100,0%

(TP, true positive; FN, false negative)

Counting borderline results as negative, the assay showed more than 83% positive concordance with CRP between days 0-7 after symptom onset for immunoglobulins IgG and IgA, while for IgM the concordance in the first days was 80%. Similarly, from 7 to 15 days after symptom onset, concordance with PCR reached 94%, 80% and 90% for IgG, IgA and IgM respectively, and these percentages were maintained beyond day 15, except for IgA and IgM, which showed an increase and slight decrease respectively.

To determine the diagnostic specificity, 77 samples from blood donors collected before the onset of the SARS-CoV-2 pandemic were tested. Descriptive summary of the results of the Abs index obtained in the sample series for each antigen and immunoglobulin

IgG						95% confidence interval for the mean	
Antígen	N	Mean (Abs Ratio)	DS	Mínimum	Maximum	Lower limit	Upper limit
RBD	77	7,0	1,6	3,0	12,0	6,7	7,4
S	77	6,9	3,2	1,0	20,0	6,2	7,7
N	77	5,7	3,3	2,0	20,0	5,0	6,5
Mpro	77	5,7	3,3	2,0	18,0	5,0	6,5

IgA						95% confidence interval for the mean	
Antígen	N	Mean (Abs Ratio	DS	Mínimum	Maximum	Lower limit	Upper limit
RBD	79	8,0	2,4	4,0	14,0	7,4	8,5
S	79	7,8	3,4	1,0	21,0	7,1	8,6
N	79	6,3	3,0	2,0	20,0	5,6	6,9
Mpro	79	6,9	6,4	2,0	52,0	5,5	8,4

IgM						95% confidence interval for the mean	
Antígen	N	Mean (Abs Ratio	DS	Mínimum	Maximum	Lower limit	Upper limit
RBD	78	7,4	3,6	1	25	5,0	6,8
S	78	5,8	3,8	1	19	4,9	6,6
N	78	5,8	4,8	0	29	4,7	6,9
Mpro	78	5,7	5,2	1	25	4,5	6,9

Summary of the corresponding diagnostic specificity obtained for each antigen and immunoglobulin counting borderline values as negative:

Igs	VN	FP	Total	NPA	95% (C.I)	
IgG	293	5	298	98,3%	93,7%	100,0%
IgA	296	12	308	95,9%	91,3%	100,0%
IgM	271	21	292	92,3%	87,7%	96,9%

(TN, true negative; FP, false positive; NPA, negative percentage agreement).

15.2. Precision

ParFor 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 lasting at least 20 days, with two runs for each day the assay is performed and with two replicates per sample tested in each run. The trial was conducted on a single instrument. The results were as follows:

IgA

			Whitin-run	Between-run	Between-day	Whitin-lab
Positive	RBD	N	70			
		Mean value	8649,50			
		SD	124,79	184,79	124,79	184,79
		CV (%)	6,59%	9,76%	6,59%	9,76%
	S	Mean value	19627,03			
		SD	2679,68	4975,39	2679,68	4975,39
		CV (%)	13,65%	25,35%	13,65%	25,35%
	N	Mean value	61045,46			
		SD	9268,69	15209,23	9268,69	15209,23
		CV (%)	15,18%	24,91%	15,18%	24,91%
	Mpro	Mean value	45462,93			
		SD	7747,71	12759,67	7747,71	12759,67
		CV (%)	17,04%	28,07%	17,04%	28,07%
intermediate	RBD	N	63			
		Mean value	1893,37			
		SD	133,58	192,00	133,58	192,00
		CV (%)	6,97%	10,20%	6,97%	10,20%
	S	Mean value	17699,11			
		SD	3199,75	4748,41	3199,75	4748,41
		CV (%)	18,08%	27,56%	18,08%	27,56%
	N	Mean value	7177,06			
		SD	902,23	1596,62	902,23	1596,62
		CV (%)	12,57%	22,92%	12,57%	22,92%
	Mpro	Mean value	4989,23			
		SD	597,75	1013,26	597,75	1013,26
		CV (%)	11,98%	20,31%	11,98%	20,31%
Negative	RBD	N	70			
		Mean value	1599,00			
		SD	68,27	127,77	68,27	127,77
		CV (%)	4,27%	7,99%	4,27%	7,99%
	S	Mean value	1415,00			
		SD	63,43	150,04	63,43	150,04
		CV (%)	4,48%	10,60%	4,48%	10,60%
	N	Mean value	3179,03			
		SD	226,55	424,22	226,55	424,22
		CV (%)	7,13%	13,34%	7,13%	13,34%
	Mpro	Mean value	3076,16			
		SD	253,23	473,85	253,23	473,85
		CV (%)	8,23%	15,40%	8,23%	15,40%

IgG

			Whitin-run	Between-run	Between-day	Whitin-lab
Positive	Positive	N	69			
		Mean value	11247,86			
		SD	1536,93	2470,39	1536,93	2470,39
		CV (%)	13,66%	21,96%	13,66%	21,96%
	S	Mean value	55729,29			
		SD	6339,42	10488,48	6339,42	10488,48
		CV (%)	11,38%	18,82%	11,38%	18,82%
	N	Mean value	84236,10			
		SD	10214,74	16270,19	10214,74	16270,19
		CV (%)	12,13%	19,31%	12,13%	19,31%
	Mpro	Mean value	65252,78			
		SD	7416,06	12429,36	7416,06	12429,36
		CV (%)	11,37%	19,05%	11,37%	19,05%
intermediate	RBD	N	69			
		Mean value	1098,70			
		SD	62,04	98,34	62,04	98,34
		CV (%)	5,65%	8,95%	5,65%	8,95%
	S	Mean value	1383,46			
		SD	108,16	166,27	108,16	166,27
		CV (%)	7,82%	12,02%	7,82%	12,02%
	N	Mean value	2235,76			
		SD	245,55	260,43	245,55	260,43
		CV (%)	10,49%	11,12%	10,49%	11,12%
	Mpro	Mean value	1213,05			
		SD	190,19	224,68	190,19	224,68
		CV (%)	15,68%	18,52%	15,68%	18,52%

IgG

			Whitin-run	Between-run	Between-day	Whitin-lab
Negative	RBD	N	70			
		Mean value	1116,51			
		SD	59,32	97,57	59,32	97,57
		CV (%)	5,31%	8,74%	5,31%	8,74%
	S	Mean value	899,62			
		SD	53,85	102,82	53,85	102,82
		CV (%)	5,99%	11,43%	5,99%	11,43%
	N	Mean value	1661,09			
		SD	230,10	283,60	230,10	283,60
		CV (%)	10,29%	12,68%	10,29%	12,68%
	Mpro	Mean value	1287,49			
		SD	88,33	139,45	88,33	139,45
		CV (%)	6,86%	10,83%	6,86%	10,83%

IgM

			Whitin-run	Between-run	Between-day	Whitin-lab
Positive	RBD	N	70			
		Mean value	18781,80			
		SD	21526,18	41638,15	21526,18	41638,15
		CV (%)	11,50%	22,24%	11,50%	22,24%
	S	Mean value	342904,16			
		SD	39057,11	72529,30	39057,11	72529,30
		CV (%)	11,39%	21,15%	11,39%	21,15%
	N	Mean value	38721,60			
		SD	4850,56	9213,06	4850,56	9213,06
		CV (%)	12,53%	23,79%	12,53%	23,79%
	Mpro	Mean value	24396,91			
		SD	2593,71	5059,69	2593,71	5059,69
		CV (%)	10,63%	20,74%	10,63%	20,74%
intermediate	RBD	N	63			
		Mean value	6191,84			
		SD	747,66	1137,07	747,66	1137,07
		CV (%)	12,07%	18,36%	12,07%	18,36%
	S	Mean value	8271,79			
		SD	1010,30	2334,59	1010,30	2334,59
		CV (%)	12,21%	28,22%	12,21%	28,22%
	N	Mean value	17589,10			
		SD	2296,28	4534,86	2296,28	4534,86
		CV (%)	12,98%	25,64%	12,98%	25,64%
	Mpro	Mean value	7613,56			
		SD	939,81	1352,94	1268,73	1050,72
		CV (%)	12,34%	17,77%	16,66%	13,80%
Negative	RBD	N	70			
		Mean value	3470,81			
		SD	257,61	474,11	257,61	474,11
		CV (%)	7,42%	13,66%	7,42%	13,66%
	S	Mean value	1457,13			
		SD	225,08	236,43	225,08	236,43
		CV (%)	15,45%	16,23%	15,45%	16,23%
	N	Mean value	16396,60			
		SD	1466,56	3479,33	1466,56	3479,33
		CV (%)	8,94%	21,22%	8,94%	21,22%
	Mpro	Mean value	10314,03			
		SD	815,77	2265,74	815,77	2265,74
		CV (%)	7,91%	21,97%	7,91%	21,97%

15.3. Analytical Sppecificity

To investigate analytical specificity, the possible cross-reactivity of antibodies against other micro-organisms producing symptoms similar to SARS-CoV-2 infection was analysed. Thus, 110 samples characterised as IgG positive 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).

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 tested, most of which contained anti-HCoVOC43 antibodies, 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 antibody cross-reactivity to both viruses is possible.

15.4. Interference

The possible interference that high levels of haemoglobin, cholesterol and bilirubin could have on the performance of the assay was analysed. For this purpose, samples with different concentrations of anti-SARS-CoV-2 antibodies were selected, spiked with potential interferents and subsequently analysed with the kit. The conclusion was that the performance of the assay is not affected by the use of haemolytic, lipaemic and icteric samples, up to concentrations of 10 mg/ml haemoglobin, 4 mg/ml cholesterol and 0.4 mg/ml bilirubin, respectively.

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On the other hand, the effects that the different matrices would potentially have on the test results were also analysed. Ten donors with different concentrations of SARS-CoV-2 specific antibodies were selected. Each donor was sampled in 4 types of tubes, 3 tubes of plasma each with a different anticoagulant: EDTA, heparin and citrate, and one tube with separator gel for serum. The samples were analysed according to the kit instructions and in no case were significant differences observed between the different anticoagulants.

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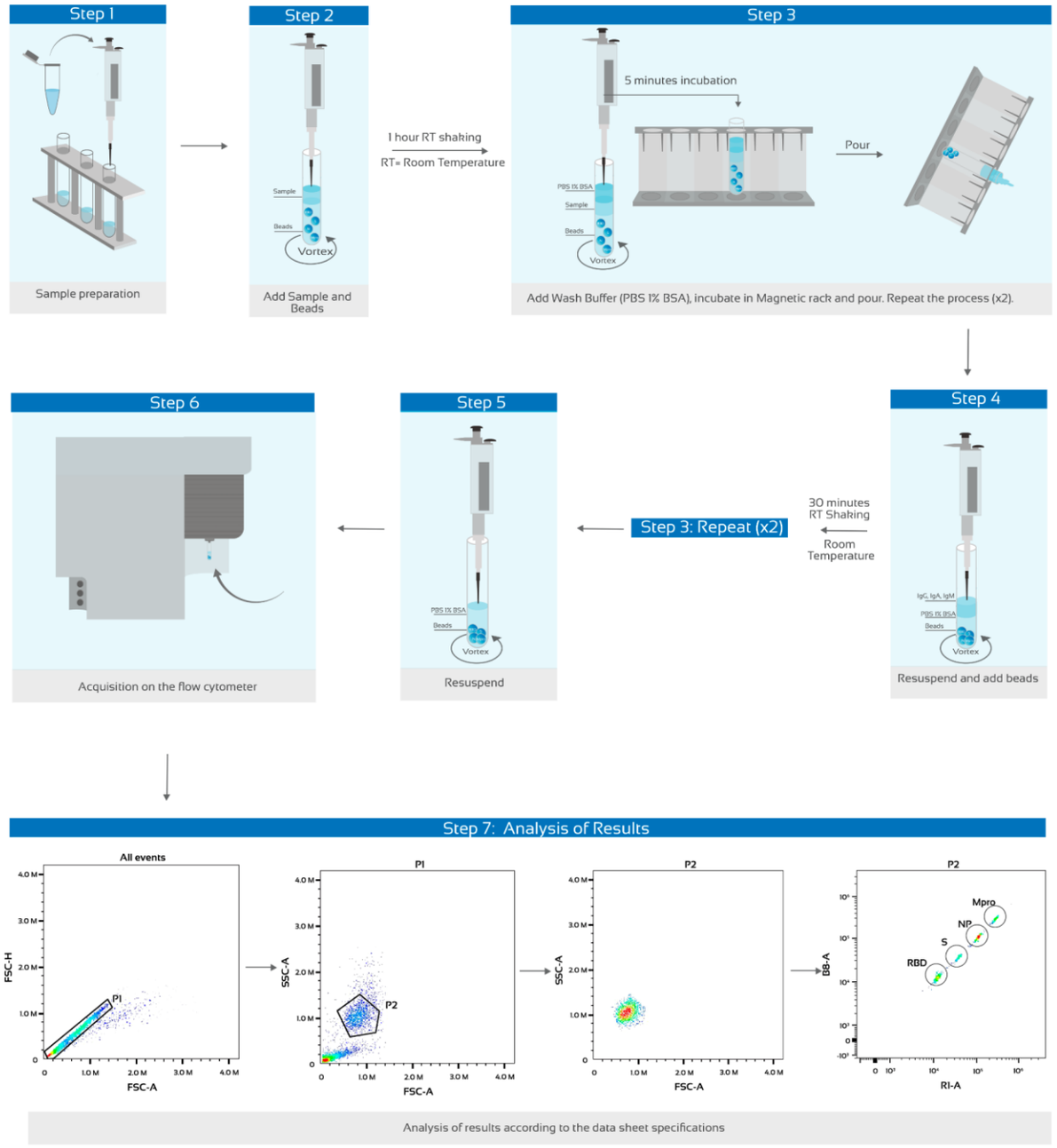
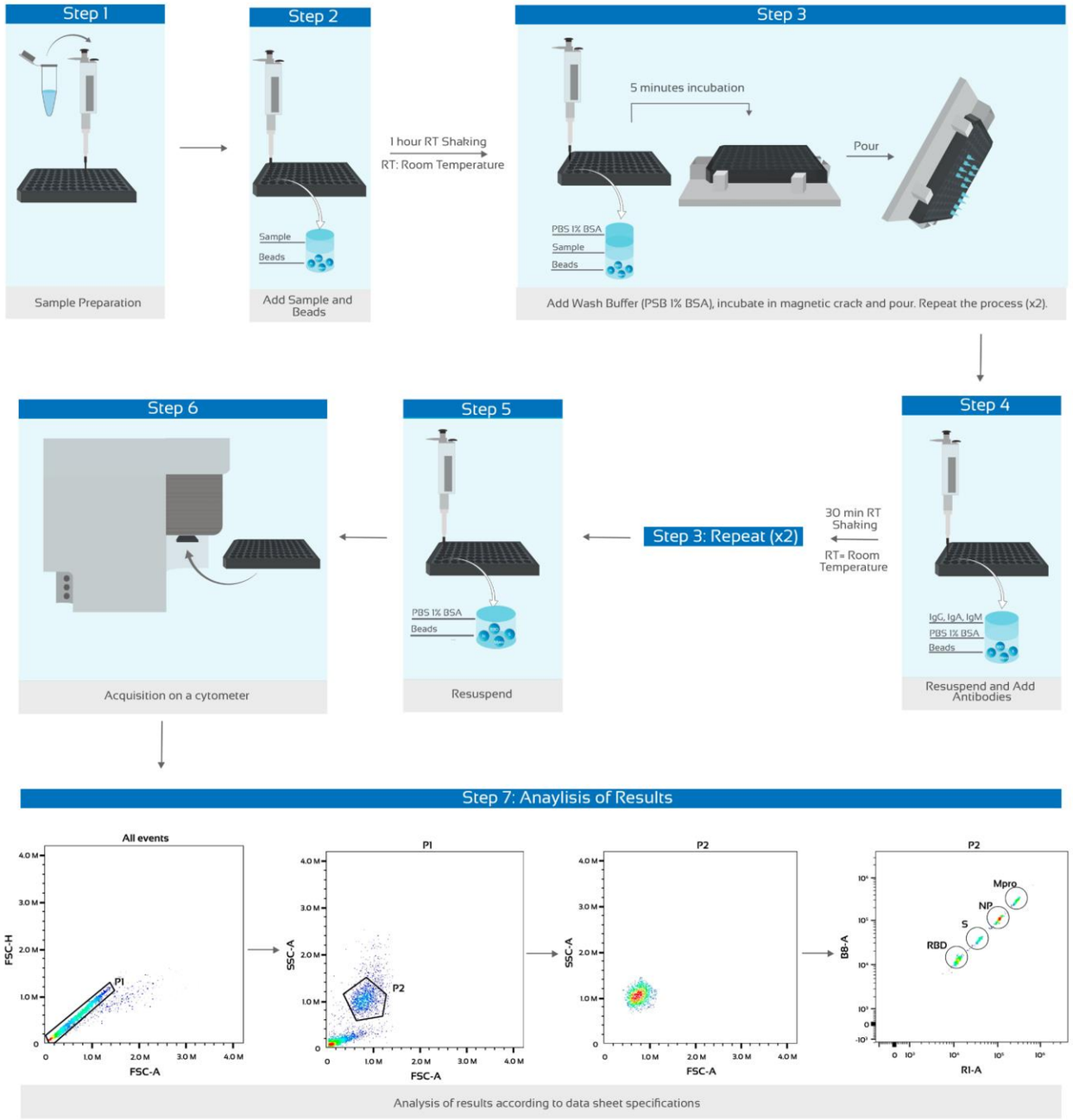
17. EXPLANATION OF SYMBOLS

n	The content is sufficient for <n> analysis.
REF	Reference of the product
CE	CE Regulatory Status
IVD	*in vitro Diagnosis kit*.
	Manufacturer
	Expiration Date
LOT	Lot Number
	Observe the instructions for use.
	Conservation from x°C to y°C.
	Content of each test.
	Pay attention.
	Biological hazards.
WASHBUF	10x wash buffer.
DILUBU	Buffer diluent.
CNTRL +	Igs positive control.
CNTRL -	Negative control Igs.
CAL	Antigen-specific recombinant human Igs
CONJ IgG	Conjugate of anti-human Igs.
PLATE	96-well microplate with lid
INSTRU	Instructions for use.

ANNEX I. Cytometer tube workflow



ANNEX II. 96-well microplate workflow.



18. MANUFACTURER INFO

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