# COVID-19 Spike 1&2 IgG

## Enzyme Immunoassay for the determination of IgG antibodies to Spike antigens in human serum and plasma

- for “in vitro” diagnostic use only -


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REF COV19GSPIKE.CE
96, 192 Tests

# COVID-19 Spike 1&2 IgG

**A. INTENDED USE**

Enzyme ImmunoAssay (ELISA) for the determination of IgG antibodies to COVID-19 Spike antigens in human plasma and sera.

The IgG assay is intended for testing:

**(a) health-care workers** at risk of COVID-19 infection to verify whether or not they might be positive for potentially neutralizing anti Spike IgG;

**(b) normal population** for epidemiology studies on presence of potential "protective" IgG to COVID-19 Spike antigens;

**(c) PCR-negative infected individuals,** successfully recovered from infection, to assure presence of potentially neutralizing antibodies;

**(d) human donors,** recovered from COVID-19 infection**,** positive for anti-Spike IgG for the generation of hyperimmune plasma, as a possible immunotherapeutic approach to the disease;

**(e) vaccinated individuals** to assure a reliable positive immunization with development of anti Spike IgG antibodies.

For "in vitro" diagnostic use only.

**B. INTRODUCTION**

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was first identified amid an outbreak of respiratory illness cases in Wuhan City, Hubei Province, China and has since then caused a global pandemic. SARS-CoV-2 is a positive-sense single-stranded RNA virus and belongs to the Betacoronavirus Genus, which also includes SARS CoV (2003) and MERS CoV (2012). Same as all other coronaviruses, the genome of SARS-CoV-2 (2019-nCoV) encodes the spike protein, the envelope protein, the membrane protein, and the nucleocapsid protein. Those who are infected with COVID-19 may have little to no symptoms. Symptoms of COVID-19 are similar to a cold or flu and may take up to 14 days to appear after exposure to SARS-CoV-2. Symptoms have included: fever, cough, difficulty breathing, pneumonia in both lungs. In severe cases, infection can lead to death. Current tests for SARS-CoV-2 look for genetic material of the virus in oral swabs, using the polymerase chain reaction (PCR). PCR only give a positive result when the virus is still present. As antibodies to Spike glycoproteins, involved in the ACE2 receptor binding, are supposed to be neutralizing by the international Literature, the determination of such IgG is useful to monitor their presence.

**C. PRINCIPLE OF THE TEST**

Microplates are coated with recombinant Spike-1/RBD glycoprotein and Spike-2 antigens specific of COVID-19. The solid phase is first treated with the diluted sample and IgG antibodies are captured, if present, by the antigens.

The dispensation of specimens and components is monitored by the DiaPro's **S**ample **A**ddition **M**onitoring **S**ystem ("**SAMS**") where the correct addition of the samples and the reagents of the assay is followed up visually by a step-wise change of colors. After washing out all the other components of the sample, in the 2nd incubation bound antibodies are detected by the addition of polyclonal specific anti hIgG antibodies, labelled with peroxidase (HRP).

The enzyme captured on the solid phase, acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of anti COVID-19 IgG present in the sample.

An index (sample/cut-off) lets optical densities be interpreted into anti COVID-19 Spike specific IgG negative and positive results, and provide a quantification of IgG when present.

**D. COMPONENT**

The standard code COV19GSPIKE.CE contains reagents for 96 tests.

**Microplate** MICROPLATE

n° 1 microplate. 12 strips of 8 microwells coated with recombinant Spike-1/RBD glycoprotein and Spike-2 antigens. Plates are sealed into a bag with desiccant.

**Negative Control** CONTROL -

1x2ml/vial. Ready to use control. It contains 1% goat serum proteins, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.045% ProClin 300 as preservatives. The negative control is **yellow color coded**.

The Negative control contains 0 WHO IU/ml (1st WHO International Standard for anti-SARS-CoV-2 immunoglobulin NIBSC code 20/136) of anti SARS-CoV-2 antibodies.

**Positive Control** CONTROL +

1x2ml/vial. Ready to use control. It contains 1% goat serum proteins, human IgG positive to COVID-19, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.045% ProClin 300 as preservatives. The Positive Control is **green color coded**. The concentration value, expressed in WHO IU/ml (1st WHO International Standard for anti-SARS-CoV-2 immunoglobulin NIBSC code 20/136) is indicated on the vial label.

**Enzyme Conjugate CONJ**

1x16ml/vial. Ready to use and **pink/red color coded** reagent. It contains Horseradish Peroxidase conjugated goat polyclonal antibodies to human IgG, 5% BSA, 10 mM Tris buffer pH 6.8+/-0.1, 0.045% ProClin 300 and 0.02% gentamicin sulphate as preservatives.

**Chromogen/Substrate** SUBS TMB

1x16ml/vial. Ready-to-use component. It contains 50 mM citrate-phosphate buffer pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide or H2O2.

***Note: To be stored protected from light as sensitive to strong illumination.***

**Assay Diluent** DILAS

1x16 ml/vial. 10 mM tris buffered solution pH 8.0 +/-0.1 and bovine proteins containing 0.045% ProClin 300 for the pre-treatment of samples and controls in the plate, blocking interference.

***Note: The liquid changes color from light yellow to dark greenish blue when the diluted sample is added.***

**Sample Diluent:** DILSPE

1x50ml/bottle. It contains 1% goat serum proteins, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.045% ProClin 300 as preservatives. To be used to dilute the sample.

***Note: The diluent changes color from olive green to dark bluish green in the presence of sample.***

**Wash buffer concentrate** WASHBUF 20X

1x60ml/bottle. 20x concentrated solution. Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0+/-0.2, 0.05% Tween 20 and 0.045% ProClin 300.

**Sulphuric Acid** $H_2SO_4$ 0.3 M

1x15ml/bottle. It contains 0.3 M $H_2SO_4$ solution.

Attention: Irritant (H315; H319; P280; P302+P352; P332+P313; P305+P351+P338; P337+P313; P362+P363)

**Plate sealing foils n° 2**

**Package insert n° 1**

**Important note:** Upon request, Dia.Pro can supply reagents for 192 tests, as reported below**:**

| | |
|---|---|
| 1.Microplate | n°2 |
| 2.NegativeControl | 1x4.0ml/vial |
| 3.PositiveControl | 1x4.0ml/vial |
| 4.Enz. Conjugate | 2x16ml/vial |
| 5.Chromog/Subs | 2x16ml/vial |
| 6.Assay Diluent | 1x32ml/vial |
| 7.SampleDiluent | 2x50ml/bottle |
| 8.Wash buff conc | 2x60ml/bottle |
| 9.Sulphuric Acid | 1x32ml/bottle |
| 10.Plate seal foils | n°4 |
| 11.Pack. insert | n°1 |
| **Number of tests** | **192** |
| **Code** | **COV19GSPIKE.CE.192** |

**E. MATERIALS REQUIRED BUT NOT PROVIDED**

1. Calibrated Micropipettes (200ul and 10ul) and disposable plastic tips.
2. EIA grade water (bidistilled or deionized, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator capable to provide a temperature of +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

**F. WARNINGS AND PRECAUTIONS**

**1.** The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.

**2.** When the kit is used for the screening of blood units and blood components, it has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.

**3.** All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

**4.** All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.

**5.** The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen/Substrate from strong light and avoid vibration of the bench surface where the test is undertaken.

**6.** Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.

**7.** Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.

**8.** Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.

**9.** Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample. Do not reuse disposable tips.

**10.** Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one. Do not reuse disposable tips.

**11.** Do not use the kit after the expiration date stated on the external container and internal (vials) labels.

**12.** Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

**13.** The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.

**14.** Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min..

**15.** Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.

**16.** The Sulphuric Acid is an irritant. In case of spills, wash the surface with plenty of water

**17.** Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

**G. SPECIMEN: PREPARATION AND RECOMMANDATIONS**

**1.**Blood is drawn aseptically by venipuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.

**2.** Avoid any addition of preservatives to samples; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate, generating false negative results.

**3.** Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.

**4.** Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.

**5.**Sera and plasma can be stored at +2°…+8°C in primary collection tubes for up to five days after collection.
Do not freeze primary tubes of collection. For longer storage periods, sera and plasma samples, carefully removed from the primary collection tube, can be stored frozen at –20°C for at least 12 months. Any frozen samples should not be frozen/thawed more than once as this may generate particles that could affect the test result. See also Chapter S (Limitations).

**6.** If after thawing particles are present (as frequently happens with old samples in small volumes and to plasma), centrifuge at 2.000 rpm for 20 min or better filter using 0.2-0.8u filters to clean up the sample before testing.

**7.** As the sample diluent (DILSPE) contains a strong virus-inactivating substance, diluted samples may be duly stored at +2..8 °C only for 48 hrs.

**H. PREPARATION OF COMPONENTS AND WARNINGS**

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-use of the device and up to 6 months.

**Microplates**:

Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant is not turned to dark green, indicating a defect of manufacturing.
In this case call Dia.Pro's customer service.
Unused strips have to be placed back into the aluminum pouch, in presence of desiccant supplied, firmly zipped and stored at +2°..8°C.
When opened the first time, residual strips are stable till the indicator of humidity inside the desiccant bag turns from yellow to green.

**Negative Control**:

Ready to use. Mix well on vortex before use.

**Positive Control**:

Ready to use. Mix well on vortex before use. Handle this component as potentially infective even if the control by its chemical composition is inactivated.

**Wash buffer concentrate**:

The 20x concentrated solution has to be diluted with EIA grade water up to 1200 ml and mixed gently end-over-end before use. As some salt crystals may be present into the vial, take care to dissolve all the content when preparing the solution.
In the preparation avoid foaming as the presence of bubbles could give origin to a bad washing efficiency.
***Note:*** *Once diluted, the wash solution is stable for 1 week at +2..8° C.*

**Enzyme conjugate**:

Ready to use. Mix well on vortex before use.
Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.
If this component has to be transferred use only plastic, possibly sterile disposable containers.

**Chromogen/Substrate:**

Ready to use. Mix well on vortex before use.
Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.
Do not expose to strong illumination, oxidizing agents and metallic surfaces.
If this component has to be transferred use only plastic, possible sterile disposable container.

**Assay Diluent:**

Ready to use. Mix well on vortex before use**.**

**Sample Diluent:**

Ready to use. Mix well on vortex before use.

**Sulphuric Acid**:

Ready to use. Mix well on vortex before use.
Attention: Irritant (H315; H319; P280; P302+P352; P332+P313; P305+P351+P338; P337+P313; P362+P363).

Legenda:
Warning **H statements:**
**H315** – Causes skin irritation.
**H319** – Causes serious eye irritation.

Precautionary **P statements:**
**P280** – Wear protective gloves/protective clothing/eye protection/face protection.
**P302 + P352** – IF ON SKIN: Wash with plenty of soap and water.
**P332 + P313** – If skin irritation occurs: Get medical advice/attention.
**P305 + P351 + P338** – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
**P337 + P313** – If eye irritation persists: Get medical advice/attention.
**P362 + P363** – Take off contaminated clothing and wash it before reuse.

**I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT**

1. **Micropipettes** have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-2%. Decontamination of spills or residues of kit components should also be carried out regularly.
2. The **ELISA incubator** has to be set at +37°C (tolerance of +/-0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.
3. The **ELISA washer** is extremely important to the overall performances of the assay. The washer must be carefully validated in advance, checked for the delivery of the right dispensation volume and regularly submitted to maintenance according to the manufacturer's instructions for use. In particular the washer, at the end of the daily workload, has to be extensively cleaned out of salts with deionized water. Before use, the washer has to be extensively primed with the diluted Washing Solution.
   The instrument weekly has to be submitted to decontamination according to its manual (NaOH 0.1 M decontamination suggested).
   **5 washing cycles** (aspiration + dispensation of 350ul/well of washing solution + 20 sec soaking = 1 cycle) are sufficient to ensure the assay with the declared performances.
   If soaking is not possible add one more cycle of washing.
   An incorrect washing cycle or salt-blocked needles are the major cause of false positive reactions.
4. Incubation times have a tolerance of $\pm$5%.
5. The **ELISA microplate reader** has to be equipped with a reading filter of 450nm and with a second filter of 620-630nm, mandatory for blanking purposes. Its standard performances should be (a) bandwidth $\leq$ 10 nm; (b) absorbance range from 0 to $\geq$ 2.0; (c) linearity to $\geq$ 2.0; (d) repeatability $\geq$ 1%. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer 's instructions.
6. The use of **ELISA automated work stations** is recommended when screening a quite high number of samples (> 50 samples).
   When using an ELISA automated workstation, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section O "Internal Quality Control". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing.
   This must be studied and controlled to minimize the possibility of contamination of adjacent wells.
   When using automatic devices, in case the vial holder of the instrument does not fit with the vials supplied in the kit, transfer the solution into appropriate containers and label them with the same label peeled out from the original vial. This operation is important in order to avoid mismatching contents of vials, when transferring them. When the test is over, return the secondary labeled containers to 2..8°C, firmly capped.
   Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination

with the kit, in order to assure compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

**L. PRE ASSAY CONTROLS AND OPERATIONS**

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the Chromogen/Substrate is colorless or pale blue by aspirating a small volume of it with a sterile transparent plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Allow all the other components to reach room temperature (about 1 hr) and then mix as described.
5. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as reported in the specific section.
6. Turn on the ELISA reader at least 20 minutes before the reading operation.
7. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.
8. Check that the micropipettes are set to the required volume.
9. Check that all the other equipment is available and ready to use.
10. In case of problems, do not proceed further with the test and advise the supervisor.

**M. ASSAY PROCEDURE**

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

**Automated Assay:**

In case the test is carried out automatically with an ELISA system, follow the instructions reported for the Manual Assay to program the assay protocol. In case of a fixed needle, before the next sample is aspirated, needles have to be duly washed to avoid any cross-contamination among samples.

***Important Note:*** *Visually monitor that samples have been diluted and dispensed into appropriate wells. This is simply achieved by checking that the color of diluted samples has turned to dark bluish-green while the color of the negative control has remained yellow.*

It is strongly recommended to check that the time lap between the dispensation of the first and the last sample will be calculated by the instrument and taken into consideration by delaying the first washing operation accordingly.
For the next operations follow the operative instructions reported below for the Manual Assay.

**Manual assay:**

1. Place the required number of Microwells in the microwell holder. Leave the 1st well empty for the operation of blanking.
2. Dispense 200 ul of Negative Control (0 IU/ml) in duplicate and then 200 ul Positive Control (IU/ml reported on the label) in duplicate in proper wells. Do not dilute Controls as they are pre-diluted, ready to use!
3. Add 200 ul of Sample Diluent (DILSPE) to all the sample wells; then dispense 10 ul sample in each properly identified well. Mix gently the plate, avoiding overflowing and contaminating adjacent wells, in order to fully disperse the sample into its diluent.

***Important note:*** *Check that the color of the Sample Diluent, upon addition of the sample, changes from olive green to dark bluish green, assuring that the sample has been really added.*

4. Dispense 50 ul Assay Diluent (DILAS) into all the controls and sample wells. Check that the color of wells has turned to **dark blue**.
5. Mix gently the plate manually, avoiding overflowing and contaminating adjacent wells.
6. Incubate the microplate for **45 min at +37°C**.

***Important note:*** *Strips have to be sealed with the adhesive sealing foil, supplied, only when the test is carried out manually. Do not cover strips when using ELISA automatic instruments.*

7. Wash the microplate with an automatic washer by delivering and aspirating 350ul/well of diluted washing solution as reported previously (section I.3).
8. Pipette 100 µl Enzyme Conjugate into each well, except the 1st blanking well, and cover with the sealer. Check that this pink/red colored component has been dispensed in all the wells, except A1.

***Important note:*** *Be careful not to touch the plastic inner surface of the well with the tip filled with the Enzyme Conjugate. Contamination might occur.*

9. Incubate the microplate for **45 min at +37°C**.
10. Wash microwells as in step 7.
11. Pipette 100 µl Chromogen/Substrate mixture into each well, the blank well included. Then incubate the microplate at **room temperature (18-24°C) for 15 minutes**.

***Important note:*** *Do not expose to strong direct illumination. High background might be generated.*

12. Pipette 100 µl Sulphuric Acid into all the wells using the same pipetting sequence as in step 10 to stop the enzymatic reaction. Addition of acid will turn the positive control and positive samples from blue to yellow/brown.
13. Measure the color intensity of the solution in each well, as described in section I.5, at 450nm filter (reading) and at 620-630nm (background subtraction), blanking the instrument on A1 (mandatory).

***Important notes:***

1. *Ensure that no fingerprints are present on the bottom of the microwell before reading. Fingerprints could generate false positive results on reading.*
2. *Reading has to be carried out just after the addition of the Stop Solution and anyway not any longer than 20 minutes after its addition. Some self oxidation of the chromogen can occur leading to high background.*

**N. ASSAY SCHEME**

Scheme of the assay:

| **Method** | **Operations** |
|---|---|
| Controls<br>Sample Diluent (DILSPE)<br>Samples<br>Assay Diluent (DILAS) | 200 ul<br>200 ul<br>10 ul<br>50 ul |
| **1st incubation** | **45 min** |
| Temperature | +37°C |
| Wash step | n° 5 cycles with 20'' of soaking<br>OR<br>n° 6 cycles without soaking |
| Enzyme conjugate | 100 ul |
| **2nd incubation** | **45 min** |
| Temperature | +37°C |
| Wash step | n° 5 cycles with 20'' of soaking<br>OR<br>n° 6 cycles without soaking |
| TMB/H2O2 | 100 ul |
| **3rd incubation** | **15 min** |
| Temperature | r.t. |
| Sulphuric Acid | 100 ul |
| Reading OD | 450nm /620-630nm |

An example of dispensation scheme is reported below:

**Microplate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BLK | S4 | | | | | | | | | | |
| B | NC | S5 | | | | | | | | | | |
| C | NC | S6 | | | | | | | | | | |
| D | **PC** | S7 | | | | | | | | | | |
| E | **PC** | S8 | | | | | | | | | | |
| F | S1 | S9 | | | | | | | | | | |
| G | S2 | S10 | | | | | | | | | | |
| H | S3 | S11 | | | | | | | | | | |

Legenda: BLK = Blank NC = Negative Control
PC = Positive Control S = Sample

## O. INTERNAL QUALITY CONTROL

A check is carried out on the controls any time the kit is used in order to verify whether their OD450nm/620nm values are as expected and reported in the table below for the Screening method.

| Check | Requirements |
|---|---|
| Blank well | < 0.100 OD450nm value |
| Negative Control (NC) | < 0.150 mean OD450nm value after blanking |
| Positive Control | > 1.000 OD450nm value |

If the results of the test match the requirements stated above, proceed to the next section.
If they do not, do not proceed any further as data are invalid.

## P. CALCULATIONS

**Semi-quantitative determination**

The tests results are calculated by means of a cut-off value determined with the following formula on the mean OD450nm/620-630nm value of the Negative Control (NC):

**NC + 0.250 = Cut-Off (Co)**

The value found for the test is used for the interpretation of results as described in the next paragraph.

**Quantitative determination**

Calculate the mean OD450nm of the Negative (**N** = 0 IU/ml) and the Positive (**P** = IU/ml declared on its label) Controls.
For the next operations follow the manufacturer instructions reported in the Operator Manual of the reader (manual assay) or of the automated work station used for the Automated Assay.

***Important note**:*
*When the calculation of results is done by the operative system of an ELISA automated workstation be sure that the proper formulation is used to calculate the cut-off value and generate the right interpretations of results.*

## Q. INTERPRETATION OF RESULTS

**Semi-quantitative assay**

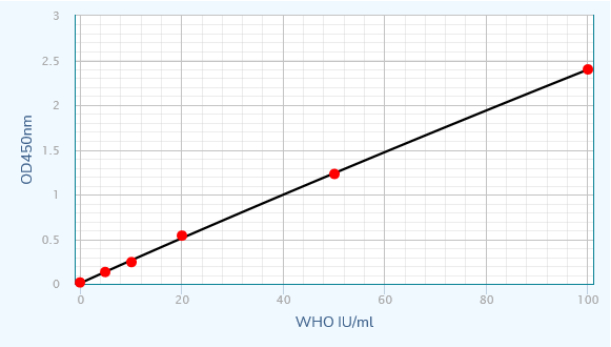
Results are interpreted as ratio of the sample OD450nm/620-630nm and the Cut-Off value (or S/Co) according to the following table:

| S/Co | Interpretation |
|---|---|
| < 0.9 | Negative |
| 0.9 ≤ S/Co < 1.1 | **equivocal** |
| 1.1 ≤ S/Co < 4 | Low positive |
| 4 ≤ S/Co < 8 | Medium positive |
| 8 ≤ S/Co < 12 | High positive |
| ≥ 12 | Very high positive |

A **negative** result (S/Co < 0.9) indicates that the subject has not IgG to COVID-19 Spike antigens.
An **equivocal** result (0.9 ≤ S/Co < 1.1) indicates the level of IgG to COVID-19 Spike antigens is closed to the cut-off of the assay (gray zone). It is recommended that a further sample is tested 7-14 days later.
A **positive** result (S/Co ≥ 1.1) is indicative of anti IgG to COVID-19 Spike antigens. The table above correlates S/Co values with a semi-quantitative interpretation.

**Quantitative assay**

Results are interpreted by turning OD450nm of samples into WHO IU/ml with a linear standard curve fitting system able to draw a straight line from the value **N** (0 IU/ml) to **P** (IU/ml declared on the label of the Positive Control).
Samples OD450nm values are then turned by the system in corresponding WHO IU/ml.
An example of standard curve is reported below:




Samples showing values > 100 WHO IU/ml have to be **diluted 1:20** (10 ul + 190 ul Sample Diluent), before retesting.
The diluted sample is then treated as reported in the table "Assay Scheme" described in chapter N, and briefly summarized as below in the first step of dispensation:

| | | |
|---|---|---|
| Negative Control (0 IU/ml) | 200 µl | A2+A3 |
| Positive Control (reported IU/ml) | 200 µl | A4+A5 |
| Sample Diluent (DILSPE) | 200 µl | In sample wells only |
| Sample prediluted 1:20 | 10 ul | In sample wells |
| Assay Diluent (DILAS) | 50 ul | In all the wells (except A1) |

The value of WHO IU/ml of such samples, calculated on the linear standard curve, has to be multiplied by 20 to ultimately obtain the right concentration of anti Spike/RBD IgG in the original undiluted sample.
WHO IU/ml values are interpreted as reported in the following table:

| WHO IU/ml | Interpretation |
|---|---|
| < 10 | Negative |
| 10 ≤ IU/ml < 12 | **equivocal** |
| 12 ≤ IU/ml < 50 | Low positive |
| 50 ≤ IU/ml < 250 | Medium positive |
| 250 ≤ IU/ml < 1000 | High positive |
| ≥ 1000 | Very high positive |

***Important Notes:***

1. *A conversion factor 1:1 between IU/ml and BAU/ml (1000 IU/ml corresponds to 1000 BAU/ml) is established in the official Instructions For Use issued by NIBSC, for First WHO International Standard for SARS-CoV-2, NIBSC code 20/136 (Version 2., Dated 17/12/2020)*
2. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
3. *Any positive result should be confirmed by an alternative method capable to detect IgG antibodies to SPIKE antigens*

*(example confirmation test Dia.Pro code COV19CONF.CE) before a diagnosis is formulated.*

4. *When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer.*
5. *The interpretation of results given in these Instructions has to be anyway taken as a presumptive diagnostic information to be further strengthened by clinical evidences and international directives.*
6. *No directive has been released so far by any international entity (WHO, CDC, NIH and national MOHs) on the titer of IgG antibodies such to make an individual be considered "protected" from a secondary infection. It is recommend anyway fulfilling the national regulations and directives in force in the single countries on this specific matter.*
7. *As recommended by FDA for anti covid-19 immunotherapy with hyperimmune plasma, in order to identify the right donor, the following procedure is reported:*
   - ***a)*** *Dilute the plasma 1:40 by dispensing 10 µl sample + 400 µl DILSPE. Mix and then dilute further to **1:160** by mixing 100 µl of dilution 1:40 + 300 µl DILSPE.*
   - ***b)*** *Dispense 50 ul of DILAS in each sample wells.*
   - ***c)*** *Dispense 200 ul of sample diluted 1:160, possibly in duplicate, and then proceed in testing as reported in the proper section M.*
   - ***d)*** *Samples showing a positive result (S/Co > 1.1) at the dilution of 1:160 are considered by FDA plasma suitable for immunotherapy.*

## R. PERFORMANCES

Whole performances are still under evaluation considering the difficulty to evaluate in particular specimens coming from infected hospitalized patients.

**Diagnostic specificity:**

The assay specificity, evaluated testing hundreds of samples collected before and after the outbreak of COVID-19, reached an overall value of > 98%.

Potential crossreactions with other respiratory infective agents were studied on samples positive for antibodies to: PIV1-3, Influenza A and B, H.influenzae, hCoV 229E, hCoV OC43, hCoV HKU1, hCoV NL63, Rhinovirus, RSV, Adenovirus, M.pneumoniae and C.pneumoniae.
No crossreactions were observed.

Antibodies, commonly present in human sera and plasma, to unrelated infective agents were also tested. Antibodies positive to CMV, EBV, HSV1&2, Toxoplasma, Rubella, H.pylori, Malaria sps, Coxsackie virus, Parvovirus B19 and HCV, HIV, Syphilis and HBsAg did not crossreacted.

No interferences were observed in pregnant women, abnormal levels of liver enzymes and other common organ-specific pathologies.

Well-known potentially interfering samples in EIA were studied. Results are reported in the table below:

| Substances | Concentrations | Score |
|---|---|---|
| Haemoglobin | Up to 500 mg/dl | negative |
| Bilirubin | Up to 20 mg/dl | negative |
| Triglyceride (milky samples) | Up to 3000 mg/dl | negative |
| Serum proteins | Up to 15g/dl | negative |
| RF+ | Up to 2500 U/ml **(*)** | negative |
| Anti-E-Coli Ab+ | Highly positive | negative |

***(*)*** *Cobas assay for RF determination.*

No false reactivity due to the method of analysis was found.

The same potential interfering samples were spiked with a specimen highly positive for IgG to COVID-19 Spike antigens. No false negative result was found assuring no interference of such substances in positive samples testing.Both plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and sera were used to determine the value of specificity. Frozen specimens were tested, as well, to check for interferences due to collection and storage. No interference was observed provided that the sample was clear and free of particles/aggregates.

Interferences were seen when fibrin aggregates, visible particles and lipid layers were present in the sample, giving usually a false positive result. These samples have to be cleared by filtration on a 0.22 u filter (lipid layers) or centrifuged for 30 min at 4000 rpm (aggregates) before testing, or discarded as not suitable for testing.

**Analytical and Diagnostic Sensitivity**

Recently NIBSC/WHO released the first WHO International Standard for anti-SARS-CoV-2 immunoglobulin NIBSC code 20/136 in IU/ml.
An analytical sensitivity better than 5 WHO IU/ml was observed.

NIBSC/WHO also released a panel - anti SARS-CoV-2 Verification Panel for Serology Assays code 20/B770 - aimed at providing data of performances in such IVD.
Results for the Screening assay are reported in the table below in comparison to the DiaPro's ELISA for anti RBD neutralizing antibodies code ACE2-RBDNEUTR.CE:

**NIBSC Verification Panel code 20/B770**

| Sample # | NEUTR result | SPIKE IgG S/Co | Sample # | NEUTR result | SPIKE IgG S/Co |
|---|---|---|---|---|---|
| 1 | pos | 3,4 | 20 | pos | 11,3 |
| 2 | pos | 3,7 | 21 | pos | 11,1 |
| 3 | pos | 13,5 | 22 | pos | 10,3 |
| 4 | pos | 12,7 | 23 | pos | 10,1 |
| 5 | pos | 13,7 | 24 | neg | 0,1 |
| 6 | pos | 9,9 | 25 | neg | 0,1 |
| 7 | pos | 11,1 | 26 | neg | 0,1 |
| 8 | pos | 11,8 | 27 | neg | 0,1 |
| 9 | pos | 14,6 | 28 | neg | 0,0 |
| 10 | pos | 15,0 | 29 | neg | 0,0 |
| 11 | pos | 8,5 | 30 | neg | 0,1 |
| 12 | pos | 9,8 | 31 | neg | 0,1 |
| 13 | pos | 10,1 | 32 | neg | 0,1 |
| 14 | pos | 8,4 | 33 | neg | 0,1 |
| 15 | pos | 9,4 | 34 | neg | 0,1 |
| 16 | pos | 7,7 | 35 | neg | 0,0 |
| 17 | pos | 10,2 | 36 | neg | 0,0 |
| 18 | pos | 12,3 | 37 | neg | 0,1 |
| 19 | pos | 10,5 | | | |

A multicentric international study was carried out in the context of the Public Health Emergency on more than 300 individuals.
With samples from: (***a***) a cohort of infected patients at end of infection with PCR negativity and clear clinical signs of full recovery, (***b***) normal population tested for epidemiologic studies and (***c***) healthcare operators in COVID-19 hospitals a sensitivity better than 98% was found.
When the samples above were tested on the DiaPro's Confirmatory Assay (code COV19CONF.CE), all the samples reacted to Spike 1 with a sensitivity value of 100% and some to Spike 2 as well.

When samples, collected during the course of the infection at sequential dates from outset of first symptoms and PCR positivity, were tested with the DiaPro's Confirmatory Assay, IgG to Spike antigens came out quite later than antibodies to Nucleocapsid (core) and kept on increasing in their S/Co values together with IgG to Nucleocapsid.

**Precision:**

Repeatability (intra-assay) was studied on 3 samples, one negative, one low positive and one high positive, examined in 16 replicates.
Results are summarized in the following table:

| | NEG | $_{high}$POS | $_{low}$POS |
|---|---|---|---|
| **average** | 0,059 | 2,191 | 0,450 |
| **STD** | 0,01 | 0,14 | 0,05 |
| **CV%** | 19,2 | 6,6 | 11,0 |

Reproducibility (inter-assay) was studied on the same 3 samples tested in 16 replicates for 3 times.
The variability found of 3-20 % depending on the sample (low-neg) did not lead in sample misclassification.

**S. LIMITATIONS**

In the first weeks of the onset of the infection with SARS-CoV-2 patients results may be negative for IgG to Spike antigens, that usually show up far later than IgG to anti Nucleocapsid antigen. In addition, patients with a low immunity status or other diseases that affect immune function, failure of important systemic organs and use of drugs that suppress immune function, might also lead to negative results of anti COVID-19 Spike IgG.
As reported in the proper section highly lipemic ("milky") and haemolized ("red) samples may generate false positive reactions.
When testing frozen samples, in particular those that:

(a) were submitted to several cycles of freezing&thawing;
(b) were already "dirty" in origin when aliquoting;
(c) were aliquoted in a small volume, due to tendency to get jellified by evaporation;
(d) are composed of plasma because of their tendency to form aggregates of fibrin upon thawing;
(e) IgM samples that by nature tends to aggregate upon freezing&thawing and become "sticky",

some false positive reaction it's quite likely to come out.

The device is able to detect antibodies generated by SARS-CoV-2, original Wuhan strain, infection or vaccines based on RNA/DNA specific for such strain.
Antibodies generated upon infection by SARS-CoV-2 mutants/variants might be detected with a different efficiency.

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All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

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