ACE2-RBD Neutralization Assay

ELISA for the determination of the neutralizing activity of anti SARS-CoV-2 antibodies by ACE2-RBD binding inhibition in human serum and plasma

- for “in vitro” diagnostic use only -

DIA.PRO
Diagnostic Bioprobes Srl
Via G. Carducci n° 27
20099 Sesto San Giovanni
(Milano) - Italy
Phone +39 02 27007161
Fax +39 02 44386771
e-mail: info@diapro.it

REF ACE2-RBDNEUTR.CE
96 Tests

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A. INTENDED USE

Enzyme ImmunoAssay (ELISA) for the determination of inhibition activity of RBD-ACE2 binding induced by antibodies to SARS-CoV-2 in human plasma and sera.

The assay is intended for:

(a) Confirmation of anti RBD antibodies neutralizing activity in patients positively recovered from COVID-19 showing antibodies to RBD/Spike;

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

(c) Screening of vaccinated individuals to assure a reliable positive and efficient immunization with development of high titer neutralizing anti RBD/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 **RBD/Spike protein**, the envelope protein, the membrane protein, and the nucleocapsid protein or NCP.

While antibodies to NCP are the first to appear in seroconversion, antibodies to RBD/Spike, involved in the ACE2 receptor binding, are produced later during the infection, are considered a marker of recovery and are known to have a neutralizing effect on SARS-CoV-2 by blocking the binding of the virus to ACE2 receptor.

The product code ACE2-RBDNEUTR.CE, in addition to the determination of the presence of total antibodies to RBD (the "molecule"), provides an important additional information on the real biological activity of such molecule in inhibiting the binding of RBD of **SARS-CoV-2** to its receptor ACE2, thus preventing the virus from entering into the target cells.

C. PRINCIPLE OF THE TEST

The inhibition of the binding between ACE2 and RBD is determined by means of an ELISA carried out on plasma/sera whose antibodies neutralizing action wants to be measured.

Microplates are coated with SARS-CoV-2 specific recombinant glycosylated RBD.

The sample is incubated allowing anti RBD/Spike antibodies, if present, to bind to such antigen.

After washing free RBD/Spike are determined by the addition of recombinant ACE2 biotinylated antigen and then in sequence of SAV-HRP.

A color will be generated by TMB/H2O2 if no antibodies have bound to RBD while a strong inhibition on the color development will be observed in case antibodies to RBD have blocked the binding of the biotin-labelled ACE2 to it.

The presence of such antigen on the solid phase is finally determined by the addition of SAV-HRP, which will bind to ACE2 if no neutralizing antibodies are present or not in case antibodies have blocked the coated RBD.

In case the sample is diluted serially in the assay a titer can be calculated by the system providing a value of neutralization.

The content in WHO IU/ml of the Positive Control can be used for a quantification of neutralizing antibodies to RBD.

D. COMPONENTS

The product contains reagents for 96 tests in screening and 12 tests of neutralization titration.

Microplate MICROPLATE

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

Negative Control CONTROL -

1x12ml/vial. Ready to use control. It contains human serum negative for antibodies to RBD/Spike, 0.09% Na-azide and 0.045% ProClin 300 as preservatives. The control is used also as diluent of the sample in case of titration of the neutralizing activity of its anti RBD/Spike antibodies. The Negative Control (or **NC**) contains 0 WHO IU/ml and is **bluish color coded**.

Positive Control CONTROL +

1x2ml/vial. Ready to use control. It contains neutralizing antibodies to SARS-CoV-2, 0.09% Na-azide and 0.045% ProClin 300 as preservatives. The Positive Control (or **PC)** can be used for quantification and contains 1000 IU/ml ± 100**. It is pink color coded**.

ACE2-biotin CONJ 1

4 lyophilized vials. It contains recombinant biotin-labelled ACE2, 5% BSA, 10 mM Tris buffer pH 6.8+/-0.1, 0.045% ProClin 300 and 0.02% gentamicin sulphate as preservatives. To be dissolved with 4 ml CONJ 2.

Streptavidin-HRP CONJ 2

1x18ml/vial. Ready to use and **red color-coded** reagent. It contains Streptavidin conjugated to HRP, 5% BSA, 10 mM Tris buffer pH 6.8+/-0.1, 0.045% ProClin 300 and 0.02% gentamicin sulphate as preservatives.

Assay Diluent DILAS

1x 16ml/vial. 10 mM tris buffered solution pH 8.0 +/-0.1 containing 0.045% ProClin 300 for the in-well treatment of samples and controls.

Note: After dispensing the liquid into the control and sample wells, the respective color turns to dark blue.

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.

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 O.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

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.

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. The Positive Control contains 1000 WHO IU/ml ± 100.

Assay Diluent:

Ready to use. Mix on vortex before use

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.*

ACE2/SAV conjugate complex:

Mix Conjugate 2 on vortex.

Before the test is started dissolve the content of the lyophilized ACE2 (CONJ # 1) with 4 ml of Streptavidin-HRP **(**CONJ # 2). Mix gently on vortex; this operation is essential.

The complex is stable **for 5 days at +2..8°C. In this case take care to return the liquid complex to the fridge as soon after use.** The complex is also stable for 1 month frozen at -20°C but, once thawed, has to be used only in the run of the day**.**
Anyway, possibly dissolve only the number of vials necessary for daily routine.

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.

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 workstations** 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 manufacturer' 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.

The assay can be easily automated on most of the best ELISA workstation available in many diagnostic laboratories for both screening and titration methods.

M1. SCREENING ASSAY:
The screening assay is recommended as a first test for antibodies to RBD/Spike in COVID-19 recovered people and vaccinated individuals.

1. Prepare the conjugate complex ACE2-SAV as reported in section H. Place the required number of wells in the microwell holder.
2. Leave A1 well empty for blanking operations.
3. Dispense 50 ul of Assay Diluent (DILAS) in all the wells with the exclusion of the blank well A1.
4. Dispense 100 ul of Negative Control (**NC**) in triplicate and then 100 ul Positive Control (**PC**) in single in proper wells.
5. Then dispense 100 ul of samples in proper sample wells.
6. Incubate the microplate for **60 min at +37°C**.

Important notes:

- *The A1 well is used for Blanking operations so only the Chromogen/Substrate mixture and the Sulphuric Acid are pipetted in A1 well. The other reagents are not delivered in the A1 well*
- *In the Manual Procedure the Strips have to be sealed in each step with the supplied adhesive sealing foil to avoid massive evaporation during the incubation step. When the Kit is used in combination with an automated ELISA processor do not use the adhesive sealing foil.*

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. Gently mix and then pipette 100 µl ACE2/SAV^{HRP} complex conjugate into each well, with the exclusion of A1 for the Blank Operations, and cover with the sealer. Check that this pink/red colored component has been dispensed properly in all controls and samples wells.

Important note: *Be careful not to touch the plastic inner surface of the well with the tip filled with the Complex 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 10 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 11 to stop the enzymatic reaction. Addition of acid will turn the negative control and negative 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).

Important notes:

- *Ensure that no finger prints are present on the bottom of the microwell before reading. Finger prints could generate false positive results on reading.*
- *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.*

M2. NEUTRALIZING TITRATION ASSAY:
The titration of the neutralizing bioactivity of a sample, positive in the previously described screening assay, is recommended to determine the strength of such antibody to neutralize the binding between RBD (the “virus”) and its receptor ACE2.

1. Prepare the conjugate complex ACE2-SAV^{HRP} as reported in section H. Place one strip per each sample to be titrated in the microwell holder.
2. Dispense 100 ul of Negative Control NC in all the wells of the strip (from A to H).
3. Dispense 100 ul of sample in the well A and mix 5 times the content of the well (1:2 dilution) by aspiration/dispensation.
4. Dispense the last aspirated 100 ul in the adjacent well B and mix 5 times the content of the well (1:4 dilution) by aspiration/dispensation.
5. Repeat the dilution operation up to well G included (1:128 dilution). Discard the last 100 ul aspirated. The remaining well H is used as 100% Binding Control (B_0).
6. Dispense 50 ul of DILAS in all the wells; the color of the well turns to blue.
7. Proceed with the incubations and the operations reported above for the Screening Assay from point # 6 to point # 13 Do not blank the reading!

Important Note:
In case the titer of a sample goes over the last point of dilution (1:128) - making impossible to provide a titer – it is suggested to apply the following protocol:

- *Dispense 200 ul of Negative Control (NC) in the well A and then 100 ul of NC in all the other wells of the strips (from well B to H).*
- *Dispense 20 ul of the sample in well A.*
- *With a new tip set to 100 ul mix its content by 5 cycles of aspiration/dispensation (dilution 1:10).*
- *Aspirate then 100 ul sample from A and start the dilution as reported before.*
- *The new values of dilutions are:*
  1:10 – 1:20 – 1:40 – 1:80- 1:160 – 1:320 – 1:640
- *Proceed then as reported in the previous protocol.*

M3. FOLLOW-UP OF VACCINATION
The following assay procedure has to be applied specifically for the determination of **neutralizing antibodies** developed upon vaccination with a vaccine able to stimulate the production of antibodies (IgG&IgA&IgM) to the Receptor Binding Domain (or RBD) of SARS-CoV-2 Spike 1 antigen.
This method will rule out samples whose titer of neutralizing antibodies is below 1:10 or lower than 50 IU/ml and point out those vaccinated individuals for which the vaccine has stimulated a good or excellent titer of antibodies capable to prevent RBD from binding to ACE2 and therefore from developing the infection.
The assay method below has to be followed:

1. Dissolve the lyophilized ACE2-Biot antigen (CONJ 1) with 4 ml of the CONJ 2 as reported in chapter H.
2. Dispense 75 ul Assay Diluent (DILAS) in all the wells, except A1.
3. Dispense **25 ul of NC** (0 IU/ml in well) in wells B1+C1 and then pipette up and down for 5 times with the tip immersed into DILAS to mix. The color of the solution turns slightly to pale bluish green.
4. Dispense 10 ul of **PC** (to obtain 400 IU/ml) in wells D1+E1+F1 and mix as described above.
5. Then dispense 25 ul of **PC** (1000 IU/ml) in wells G1+H1 and mix as reported above.
6. Dispense then **25 ul of sample** in the other proper sample-wells (from A2 forward) and pipette up and down for 5 times with the tip immersed into DILAS to mix. The color of the solution turns slightly to pale bluish green.
7. Dispense 100 ul of NC in all the wells, with the exclusion of A1 used for blanking. The color of the wells turns to blue.
8. Cover the microplate with the sealer, shake gently the plate for 10-15 seconds and then incubate for **60 min at +37°C**.
9. Wash according to what described in the section I.3.
10. Gently mix the complex ACE2-RBD on vortex and then dispense 100 ul in all the wells, except A1. Recover the

microplate with the sealer and incubate for **45 min at +37°C**.

11. Wash according to what described in the section i.3

12. Dispense 100 ul TMB in all the wells, A1 included. Incubate the plate **at r.t. for 10 min** in the dark

13. 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 negative control and negative samples from blue to yellow/brown.

14. Read OD_s at 450nm/620-630nm blanking on well A1.

Important Note: *In the follow-up of vaccination usually high titers of antibodies are generated. In order to obtain a corrected evaluation of IU/ml, samples in this case should be previously diluted 1:10 in Assay Diluent (ex.: 20 ul sample + 180 ul DILAS). After mixing on vortex 25 ul of the diluted samples are applied. Calculated IU/ml of the diluted samples on the calibration curve have finally to be multiply by 10 to determine the final IU/ml in the original sample.*

N. ASSAY SCHEME:

The table reports the scheme of the **screening assay**:

Screening Assay			
Step	**Reagents&Method**	**Operations**	**Wells**
1	DILAS	50 ul	All but A1
2	Negative Control	100 ul	B1+C1+D1
3	Positive Control	100 ul	E1
4	Samples	100 ul	F1 ➔
5	**1st incubation**	**60 min**	
	Temperature	+37°C	
6	Wash step	n° 5 cycles with 20'' of soaking OR n° 6 cycles without soaking	All
7	Complex Conjugate	100 ul	All but A1
8	**2nd incubation**	**45 min**	
	Temperature	+37°C	
9	Wash step	n° 5 cycles with 20'' of soaking OR n° 6 cycles without soaking	All
10	TMB/H2O2	100 ul	All
11	**3rd incubation**	**10 min**	
	Temperature	r.t.	
12	Sulphuric Acid	100 ul	All
13	Reading OD	450nm /620-630nm	

An example of dispensation scheme is reported below for the Screening Assay:

Microplate

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

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

In the following table a scheme for **Neutralization Titration** is reported:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	*1:2*											
B	*1:4*											
C	*1:8*											
D	*1:16*											
E	*1:32*											
F	*1:64*											
G	*1:128*											
H	NC											

Legenda: NC = Negative Control

The table below reports the scheme of the method for the **follow-up** of vaccinated individuals:

Follow-up quantitative assay			
Step	**Reagents&Method**	**Operations**	**Wells**
1	DILAS	75 ul	All but A1
2	Negative Control (NC)	25 ul	B1+C1
3	Positive Control (PC ➔400)	10 ul	D1+E1+F1
4	Positive Control (PC ➔1000)	25 ul	G1+H1
5	Samples	25 ul	A2 ➔
6	Negative Control (NC)	100 ul	All but A1
7	**1st incubation**	**60 min**	
	Temperature	+37°C	
8	Wash step	n° 5 cycles with 20'' of soaking OR n° 6 cycles without soaking	All
9	Complex Conjugate	100 ul	All but A1
10	**2nd incubation**	**45 min**	
	Temperature	+37°C	
11	Wash step	n° 5 cycles with 20'' of soaking OR n° 6 cycles without soaking	All
12	TMB/H2O2	100 ul	All
13	**3rd incubation**	**10 min**	
	Temperature	r.t.	
14	Sulphuric Acid	100 ul	All
15	Reading OD	450nm/620-630nm	

An example of dispensation scheme is reported below for the vaccinated follow-up method:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S1										
B	0	S2										
C	0	S3										
D	400	S4										
E	400	S5										
F	400	S6										
G	1000	S7										
H	1000	S8										

Legenda: 0 = Negative Control 400 = 10 ul PC 1000 = 25 ul PC

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.

Check	Requirements
Negative Control	1.500 < OD450nm value < 3.000
Positive Control - screening assay method - follow up of vaccination (25 ul/well)	 **Co/S** > 5 NS% > 60%

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. CALCULATION OF THE CUT-OFF

In the screening and in the titration assay methods test 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 ($_{mean}$NC).

$$_{mean}NC / 2 = Cut\text{-}Off\ (Co)$$

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

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

Q1. Screening Assay (Qualitative)
Test results are interpreted as ratio of the Cut-Off value (**Co**) and the sample OD450nm/620-630nm (**S**), or **Co/S**, according to the following table:

SCREENING ASSAY

Value	Neutralizing Antibodies
Co/S < 1	Negative
1 < **Co/S** < 5	Low Positive
5 < **Co/S** < 10	Medium Positive
Co/S > 10	High Positive

A **negative** result indicates that the subject has not developed neutralizing antibodies to RBD/Spike antigen or that their titer is below the limit of detection of the assay. The subject should be vaccinated.
A **positive** result indicates that the subject has developed neutralizing antibodies to RBD/Spike antigen after vaccination or natural SARS-CoV-2 infection.
A low or medium positive result is indicative of a poor or moderate development of neutralizing antibodies. It would be suggested to vaccinate the individual anyway when possible if it has not already been done.
High positive results are indicative of high development of neutralizing antibodies. Values of Co/S > 10 are usually obtained in individuals submitted to vaccination at the end of treatment. It is suggested to keep monitored the RBD/Spike Value of the antibodies to follow up the status of the antibodies.

Q2. Titration Assay (quantitative)
In the titration assay, in order to better correlate to titers obtained in the "in vivo" neutralization assay (VNT or pVNT), the value of Co is calculated from the well H of the strip (**N**egative **C**ontrol used for the dilutions of the sample) as follows:

NC / 2 = Cut-Off (Co)

Results are interpreted as reported in the table below:

Co/S	Interpretation in Titration
< 1	Negative for neutralizing antibodies
≥ 1	Positive for neutralizing antibodies

Determine at which dilution (titer) a value of the Co/S ≥ 1 is reached. When such dilution has been identified, results are then interpreted as follows:

Titer	Interpretation in Titration
1:2	Low titer
1:4	Medium titer
1:8	
1:16	
≥ 1:32	High Titer

Individuals showing a high titer value of neutralizing antibodies might be considered potential candidates as donors of hyper immune-plasma in immunotherapy.

If a **correlation to the "in vivo" system** is required apply the following calculation to turn ELISA titers into titers VNT or pVNT ones:

ELISA titer x K ≃ "in vivo" titer

A K value of 10 has been calculated from comparison studies to provide a correlation to VMT or pVNT with an acceptable degree of approximation, despite the two system are quite different.
Example: ELISA titer 1:4 ≃ VNT titer 1:40

Important notes:
- *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
- *Any positive result should be in principle confirmed by an alternative method capable to detect IgG antibodies to SARS-CoV-2 RBD/SPIKE (example: Dia.Pro's code COV19GSPIKE.CE or confirmation ELISA code COV19CONF.CE) before a diagnosis is formulated.*
- *When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer.*
- *At the present time no indication is given by any international entity (WHO, CDC, NIH and national MOHs) on the titer that antibodies should have to make consider an individual "protected" from a secondary infection.*

Q3. Follow-up of vaccination
Calculate the mean OD450nm of NC and then the Percentage of Neutralization of the sample (NS%) with the following formulation:

$$NS\% = 100 - \left[\frac{OD_{450nm}\ Sample}{{}_{mean}OD_{450nm}\ NC} \times 100 \right]$$

Data are then interpreted as reported in the following table:

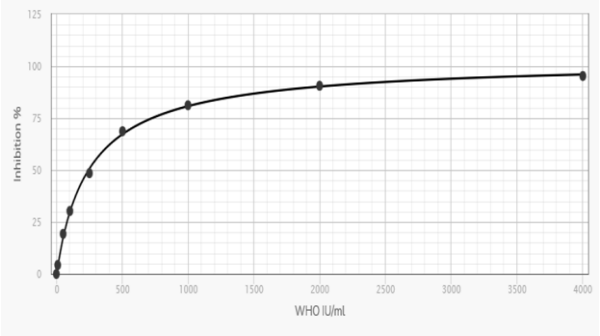
% of Neutralization (N_{sample} %)	Neutralizing activity	WHO IU/ml range
< 20%	Low or not reactive (*)	< 10
20% < NS% < 30%	Moderate	10 - 100
30% < NS% < 60%	Good	100 - 400
60% < NS% < 100%	Excellent	> 400

Important Note (*): *In this case the individual should be retested in the screening assay reported in IFU to verify whether it is negative or low reactive for antibodies to SARS-CoV-2 RBD. Anyway low reactive individuals are likely to be at risk of infection by SARS-CoV-2*

Important Note (**): *For binding antibody assays, an arbitrary unitage of binding antibody units (BAU/ml) can be used to assist the comparison of assays detecting the same class of immunoglobulin with the same specificity (e.g.: anti-RBD IgG, anti-NCP IgG, etc.). 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 anti-SARS-CoV-2 immunoglobulin (human), NIBSC code 20/136 (Version 2., Dated 17/12/2020*

In the quantification of antibodies to RBD in WHO IU/ml calculate the mean OD450nm values of NC, PC400 and PC1000 and use a curve fitting system to draw a point-to-point curve connecting 0 IU/ml, 400 IU/ml and 1000 IU/ml.
On this curve calculate the concentration of antibodies in IU/ml by means of their OD450nm readings.
Values lower than 100 IU/ml and higher than 1000 IU/ml are not precise. Therefore for such values provide an interpretation of:
< 100 IU/ml and > 1000 IU/ml, respectively.

The graphical representation of a standard curve obtained with the First WHO International Standard for anti-SARS-CoV-2, NIBSC code 20/136 (4-parameters interpolation) is reported in the figure below:



Note: *Do not use this graphical representation for the calculation of results of samples.*

R. PERFORMANCES

An extract of the Design Dossier produced for CE marking is available upon request and reports all the studies conducted on the devise to proove its validity as an IVD for testing neutralizing antibodies to SARS-CoV-2.

Analytical Sensitivity

The First WHO International Standard for anti-SARS-CoV-2, NIBSC code 20/136, used to define its limiting dilution, is detected positive (**Co/S** ≥ 1) up to a final concentration of 15 arbU/ml in the screening assay.

Diagnostic Sensitivity

The diagnostic sensitivity of the device was evaluated by testing a panel of anti RBD/SPIKE tested positive in the DiaPro's ELISA code COV19GSPIKE.CE.
Results are reported below for two studies (present in the Design Dossier of the product) where total 150 IgG positive samples were tested.

TRUE POSITIVES	150
FALSE NEGATIVES	0
TOTAL SAMPLES	150
SENSITIVITY %	**100**

The product meets the assigned requirement of ≥ 98% diagnostic sensitivity, value defined for all the other DiaPro's serological markers of COVID-19.

Recently NIBSC/WHO 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 are reported in the table below in comparison to the DiaPro's ELISA for anti SPIKE1&2 IgG (REF):

Sample #	NEUTR Co/S	REF result	Sample #	NEUTR Co/S	REF result
1	1,3	pos	20	32,8	pos
2	2,0	pos	21	16,3	pos
3	36,8	pos	22	51,5	pos
4	20,6	pos	23	64,3	pos
5	29,1	pos	24	0,5	neg
6	3,4	pos	25	0,5	neg
7	4,7	pos	26	0,5	neg
8	38,6	pos	27	0,5	neg
9	25,3	pos	28	0,4	neg
10	27,1	pos	29	0,4	neg
11	8,1	pos	30	0,4	neg
12	8,9	pos	31	0,6	neg
13	10,8	pos	32	0,5	neg
14	8,6	pos	33	0,5	neg
15	29,1	pos	34	0,5	neg
16	6,0	pos	35	0,4	neg
17	8,5	pos	36	0,5	neg
18	42,9	pos	37	0,5	neg
19	2,9	pos			

Analytical Specificity

The **cross-reactivity** was studied by examining the following:

1. **Other respiratory infective microbes and viruses**: pre-pandemic samples coming from infections or other respiratory viruses or bacteria, or vaccination of influenza certified positive for : Parainfluenza Virus 1-3 (PIV), Influenza A, Human coronavirus (hCoV229E, hCovOC43, hCoV HKU1,hCoV NL63, hCoVHKU1/NL63+), SARS-CoV 1, Rinovirus, Respiratory syncytial virus (RSV), Adenovirus, Parvovirus B19, Coxackye virus, Micoplasma Pneumoniae, Chlamydia Pneumoniae.
2. **Unrelated agents**: pre-pandemic specimens certified positive for infective agents that might be present significantly in the population for which the product is intended, such as : Herpes Virus (CMV, EBV and HSV), Toxoplasma, Rubella, H.Pylori, HCV, HIV, Syphilis, Plasmodium species and minor others.. Samples were tested to verify absence of interference in the detection of IgG to SPIKE antigens.
3. **Known most important and frequent interfering substances**: Hemoglobin, Bilirubin, Protein, Triglycerides, Rheumatoid Factor, Antinuclear Antibodies (ANA), Autoantibodies (TPO), IgG to E.coli (for recombinant antigens used for coating), pregnant females, human anti-mouse antibodies, abnormal level of liver enzymes and other common organ-specific pathologies.

The results are summarized in the tables below.

Other respiratory infective microbes and viruses (pre-pandemic samples)

Category	gender	age	ACE2/RBD		
			N° of samples tested	Pos	Neg
PIV 1-3 Ab+	Male&female	25-40	11	0	11
Influenza A+B (vaccinated 10/2019)	Male&female	25-40	12	0	12
Anti H. influenza Ab+	Male&female	25-40	5	0	5
hCoV 229E Ab+	Male&female	25-40	5	0	5
hCoV OC43 Ab+	Male&female	25-40	5	0	5
hCoV HKU1 Ab+	Male&female	25-40	5	0	5
hCoV NL63 Ab+	Male&female	25-40	5	0	5
hCoV HKU1/NL63 Ab+	Male&female	25-40	5	0	5
SARS-CoV 1 Ab+	Male&female	25-40	5	0	5
Rinovirus Ab+	Male&female	25-40	5	0	5
RSV Ab+	Male&female	25-40	5	0	5
Adenovirus Ab+	Male&female	25-40	5	0	5
M.pneumoniae Ab+	Male&female	25-40	5	0	5
C.pneumoniae Ab+	Male&female	25-40	5	0	5

Unrelated microbes

Category	gender	age	ACE2/RBD		
			N° of samples tested	Pos	Neg
CMV IgM+	Male&female	25-40	5	0	5
CMV IgG+	Male&female	25-40	5	0	5
EBV EBNA IgG+	Male&female	25-40	5	0	5
EBV VCA IgM+	Male&female	25-40	5	0	5
HSV1&2 IgG+	Male&female	25-40	5	0	5
Toxo IgG+	Male&female	25-40	5	0	5
Rub IgG+	Male&female	25-40	5	0	5
H.Pylori IgG+	Male&female	25-40	5	0	5
HCV Ab+	Male&female	25-40	5	0	5
HIV Ab&Ag +	Male&female	25-40	5	0	5
HBsAg+	Male&female	25-40	5	0	5
HBsAb+	Male&female	25-40	5	0	5
Syphilis Ab+	Male&female	25-40	5	0	5
Malaria Ab+	Male&female	25-40	5	0	5
Coxackye virus Ab+	Male&female	25-40	5	0	5
Parvovirus B19 Ab+	Male&female	25-40	5	0	5

No false positive reaction was observed.

Additional studies on potential interferences
A panel of 88 plasma, stored **frozen** at - 20°C in bags, collected in 2017 were thawed, cleaned by filtration to remove visible particles of fibrin and tested on the devise.
Results are summarized in the following table:

NEGATIVE	88
POSITIVE	0
TOTAL SAMPLES	88
SPECIFICITY %	**100**

The same 88 samples were tested for antibodies to herpes family viruses including EBV and Helicobacter pylori, positive in almost all human sera&plasma to verify whether their present could interfere with the assay.

Analyte	N° of positive samples
HSV 1&2 IgG	88/88
CMV IgG	86/88
VCA IgG	87/88
EBNA IgG	85/88
HP IgG	71/88

Despite all the 88 samples in most of cases were positive in their respective assays, none turned out positive for IgG to Spike, assuring a specificity ≥ 98%, as required by the DiaPro's Essential Requirements in compliance to our CE Quality Sytem.

Interfering substances
Pre-pandemic samples characterized for the presence of potentially interfering substances were tested, before and after "spiking" with high titer neutralizing sample.

Potential interfering substance	Concentr.	Samples Before spiking			Samples After spiking		
		N° of samples tested	Pos	Neg	N° of samples tested	Pos	Neg
Hyper Haemoglobin	≥500 mg/dl	5	0	5	2	2	0
Total bilirubin	≥20 mg/dl	2	0	2	1	1	0
Serum protein	≥15g/dl	2	0	2	1	1	0
Triglyceride	3000 mg/dl	4	0	4	2	2	0
RF+	≥ 2500U/ml	5	0	5	2	2	0
ANA IgG+		7		7	2	2	0
TPO IgG+		2	0	2	1	1	0
Anti-E coli IgG+		4	0	4	2	2	0
Pregnant women	> 3 months	11	0	11	2	2	0
Transamisase	pathological	2	0	2	1	1	0
Dialysed patients		2	0	2	1	1	0

No interference were observed in pre-pandemic interfering samples.
No false negative result was found in spiked samples assuring no interferences of such substances in positive sample testing.

Diagnostic specificity
A panel composed of 440 sera, prescreened negative for IgG to RBD/SPIKE with the DiaPro's specific ELISA, were tested.
Results are reported below:

TRUE NEGATIVES	440
FALSE POSITIVES	0
TOTAL SAMPLES	440
SPECIFICITY %	**100**

Accuracy Of Measurement
Repeatability (**intra-assay**) of the device was studied by examining 1 border sample closed to the cut-off and 1 high positive one in the same run in 16 replicates.
Summary of results is reported in the table below:

	border	positive
MEAN	0,941	0,101
STD DEV	0,060	0,007
CV%	6,4	6,8

Reproducibility (**inter-assay**) was determined by examining n° 1 high positive sample (**HP**) with index 4.0 < Co/S < 8 and n° 1 border sample (about 1 Co/S)
These samples were examined in **three times** for a total number of 24 replicates each.
Results are reported below.

High Positive

	TEST 1	TEST 2	TEST 3	
	0,102	0,094	0,097	
	0,104	0,095	0,101	
	0,114	0,094	0,101	
	0,100	0,093	0,098	
	0,117	0,094	0,100	
	0,114	0,094	0,100	
	0,109	0,098	0,100	
	0,100	0,092	0,103	
				mean values
Mean	0,108	0,094	0,100	0,101
STD DEV	0,007	0,002	0,002	0,003
CV%	6,4	1,9	1,9	**3,5** Between

Border Positive

	TEST 1	TEST 2	TEST 3	
	0,950	0,919	0,987	
	0,885	0,885	0,965	
	0,933	0,919	1,020	
	0,873	0,896	1,021	
	0,968	0,938	1,065	
	0,998	0,927	0,997	
	0,886	0,875	0,993	
	0,832	0,857	0,992	
				mean values
Mean	0,916	0,902	1,005	0,941
STD DEV	0,056	0,028	0,030	0,038
CV%	6,1	3,1	3,0	**4,0** Between

Finally, 40 positive samples titrated in VNT in a COVID-19 center of excellence were titrated in the devise and turned all positive for neutralizing antibodies with a correlation in titer of acceptable approximation.
Other 40 samples negative in the VNT assay turned all negative in the devise.
Results are summarized in the table below:

ELISA	"in vivo"	
	negative	positive
negative	40	0
positive	0	40

S. LIMITATIONS
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) iper IgM samples that by nature tends to aggregate upon freezing&thawing and become "sticky",

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

Values in WHO IU/ml obtained with this device might not correspond to IU/ml or BAU/ml of systems detecting antibodies to RBD and not their biological activity.

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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.

Manufacturer:
Dia.Pro Diagnostic Bioprobes S.r.l.
Via G. Carducci n° 27 – Sesto San Giovanni (MI) – Italy

CE