Serological ELISA, Detection of IgG antibodies against the RBD of SARS-CoV-2

0.5 µg/ml *SARS-CoV-2 Spike S1 Protein (RBD)* (trenzyme GmbH, Germany) was diluted in 1-fold *Coating Buffer pH 7.4* (CANDOR Bioscience GmbH, Germany, # 120, 10-fold-solution) and 100 µl of the solution was added to each well of 96-well ELISA plates *Nunc MaxiSorp* and incubated overnight at 2 - 8 °C. The wells were emptied without washing and blocked with 200 µl *PlateBlock™* (CANDOR Bioscience GmbH, Germany, # 112) per well by incubating for 2 hours at room temperature. The wells were emptied without washing and stabilized with 200 µl *Liquid Plate Sealer® animal-free* (CANDOR Bioscience GmbH, Germany, # 163) per well for 2 - 15 min at room temperature. *[The stabilization of the RBD is necessary if the coated ELISA-plates must be stored for a longer period of time. RBD contains structural epitopes that denature within a few days after immobilization if not stabilized with Liquid Plate Sealer® animal-free. If the plates are used directly after immobilization, stabilization is not necessary].*

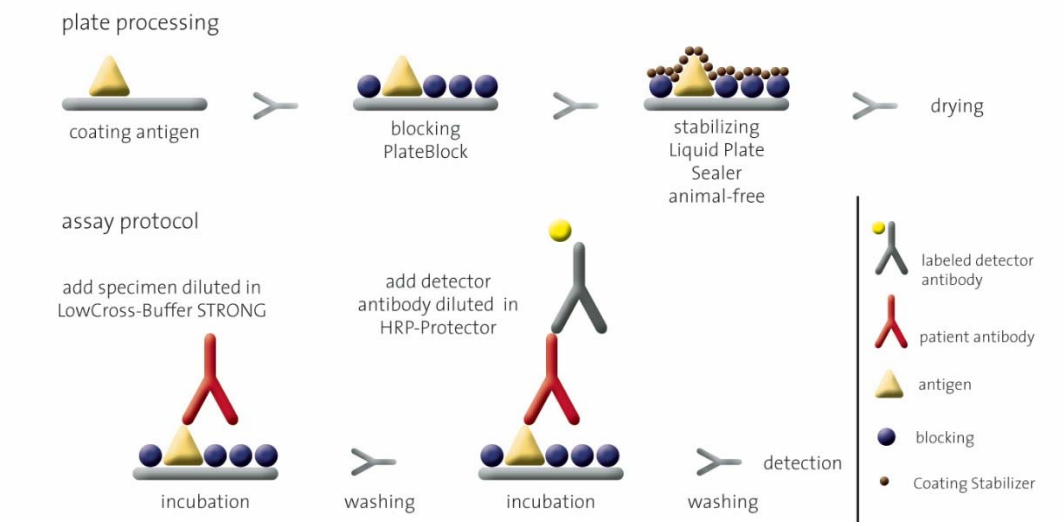
Coated plates were emptied without washing and air-dried and sealed in aluminium pouches and stored at 2-8°C until use. The plates are stable for many months because of the stabilizing effect of *Liquid Plate Sealer® animal-free*.

If the plates are not stabilized, they must be used immediately after blocking.

Human plasma or serum samples were diluted 1:100 with *LowCross-Buffer® STRONG* (CANDOR Bioscience GmbH, Germany, # 102). Note: Each serum sample should be tested in duplicates. 100 µl of appropriately diluted sample was added to each well of the coated plate and incubated for 2 hours at room temperature with constant agitation on a shaker. The wells were emptied and washed three times with 300 µl per well with 1-fold *Washing Buffer TRIS™* (CANDOR Bioscience GmbH, Germany, # 145, 10-fold-solution).

100 µl of appropriately diluted (0.25 µg/ml) *goat anti-human IgG peroxidase conjugate* (Jackson ImmunoResearch, USA, #109-035-098) in *HRP-Protector™* (CANDOR Bioscience GmbH, Germany, # 222) was added to the respective wells and incubated for another 1 hour at room temperature with constant shaking. The wells were emptied and washed three times with 300 µl per well with 1-fold *Washing Buffer TRIS* (CANDOR Bioscience GmbH, Germany, # 145) before addition of 100 µl per well TMB substrate solution *SeramunBlau® slow 50* (Seramun Diagnostica GmbH, Germany, # S-150-TMB). After 5 - 10 min of light-protected incubation, the chromogenic development was stopped using 50 µl 1M H_2SO_4 per well. Optical density (OD) was measured at 450 nm wavelength in a microplate spectrophotometer.

Summary



1. Coating of the carefully selected and produced capture antigen
2. Aspirate or tap the plate (do not wash)
3. Blocking with 200 µl *PlateBlock™* for 2 h
4. Aspirate or tap the plate (do not wash)

Optional stabilization steps (if plate storage is required):

   a. Stabilization with *Liquid Plate Sealer® animal-free* for 2 - 15 min
   b. Aspirate or tap the plate (do not wash), dry and store

5. Dilute the patient sample (1:100) in *LowCross-Buffer® STRONG* and incubate 100 µl per well on the plate
6. Wash three times with 300 µl 1-fold *Washing Buffer TRIS™* per well
7. Incubate with detector conjugate stored in *HRP-Protector™*
8. Detect with substrate after further washing.

Comments on protocol:

This protocol can be a basis for similar assay protocols.
Aspects which can be changed without necessarily making the assay results less reliable:
Coating Stabilizer: *Liquid Plate Sealer® animal-free* (CANDOR Bioscience GmbH, Germany, # 163) can be exchanged by *Liquid Plate Sealer®* (CANDOR Bioscience GmbH, Germany, # 160). Both have been tested with same results in the assay and stability testing is ongoing with same results as well. So both solutions can be exchanged by each other.
Secondary antibody: The secondary antibody can be exchanged by other secondaries. However, be aware that the choice of the secondary is critical. A secondary for a SARS-CoV-

2 IgG-ELISA (the same applies for any other serological IgG ELISA) should not only be specific and of high affinity for human IgG, it should also be pre-adsorbed against other human antibodies such as IgA, IgM and the like. The reason is that otherwise an IgG-ELISA, intended to detect immunity (to speak in general) would also show IgA-antibodies, which are not a sign of long-term immunity, but a sign of an infection. The same applies vice versa to IgA- or IgM- detecting serological assays. Choice of pre-adsorbed secondaries is absolutely crucial for clinical correctness of results. There are only very few antibody producers with good experience in pre-adsorption. During in house testing at CANDOR, we found several commercially available secondaries, which were labelled as pre-adsorbed against other antibodies (and maybe indeed were pre-adsorbed by the producers) but additionally detected the wrong isotypes of human antibodies. This potential non-specificity has to be tested in specific assays. It is not a question of cross-reactivity, but a question of having a mixture of antibodies specific to IgG, specific to IgA, specific to IgM and so on. Due to this, secondaries with bad pre-adsorption show very high affinity binding to the other isotypes of human antibodies. If one likes to save for such additional testing, we recommend using the secondary mentioned in the protocol.

Conjugate diluent: *HRP-Protector™* (CANDOR Bioscience GmbH, Germany, # 222) is used in this protocol instead of *LowCross® HRP-Stab* (CANDOR Bioscience GmbH, Germany, # 222). Both options are possible with this CoViD-19 IgG ELISA, since we so far did not find any samples for which results differed between the two. *HRP-Protector™* is mentioned in the protocol due to its slightly lower cost. Using *LowCross® HRP-Stab* enables to avoid a specific kind of interference, which can only occur in the last step of the sequential assay protocol. This is by far not the major cause for false positives in serological assays and very rare. The protocol was developed for commercial kit producers worldwide, which are - due to the high volumes of the Covid-19 IgG ELISA to be produced during the next months - under high cost pressure. Hence the decision to mention *HRP-Protector™* instead of *LowCross® HRP-Stab*. For applications with lower volumes, such as in research settings or in clinical testing for drugs or vaccines, we highly recommend using *LowCross® HRP-Stab* for even better safety of the protocol.

Substrate: Other substrates may also work well with this ELISA protocol. Choosing an appropriate substrate is a task for itself, but exchange will be possible without risking worse reliability, if the selected substrate has similar characteristics. The substrate is not the most critical component for a reliable immunoassay but can have deleterious effects if not selected carefully.

ELISA plate: Other ELISA plates may potentially perform well enough. Our testing showed supremacy of the chosen plate for this specific antigen.

Other changes: We do not recommend any other change to the protocol.
The worst option would be an exchange of surface blocker and/or assay diluent, as both changes would definitely lead to high numbers of false positives.

If the protocol should be adapted to IgA ELISA, changes will be necessary not only in terms of the appropriate antigen, but additionally in terms of the appropriate diluents, stabilizers and secondaries, of course. The main reason is that IgG assays need premium specificity whilst IgA assays need premium sensitivity. Currently we are preparing a similar protocol for high-quality anti-IgA ELISAs for CoViD-19 for optimum detection of ongoing infection in people. If you are interested in this protocol, please ask your distributor to be informed as soon as this protocol is available.

Kind regards

Your team of CANDOR Bioscience – The ELISA experts