The challenges of serology - towards reliable SARS-CoV-2 antibody assays

Sebastian M. Richter, Angela Zellmer, Manuel Hecht & Peter Rauch

With the ongoing worldwide spread of CoViD-19, reliable antibody detection is urgently needed across the globe. Since many of the currently available assays are not performing adequately[1,2], here we explain the technical challenges in the development of truly reliable antibody detection assays through the example of ELISAs.

Immunity against SARS-CoV-2 is thought to be conferred by highly specific and high-affinity IgG antibodies[3,4]. Not all recovered patients exhibit a robust neutralizing antibody response[5], making reliable assays for the detection of IgGs against SARS-CoV-2 an absolute necessity. These assays are intended to indicate immunization, either elicited by a prior infection or a vaccination, and to be widely used. For mass testing, however, an extremely high specificity, i.e. a low rate of false positive results, is essential. Even with perfect sensitivity, an apparently good specificity of 99 % and an optimistically estimated antibody prevalence of 5 %, a positive result means that the person tested has only an 84% probability of actually having specific antibodies against SARS-CoV-2[6,7]. Such uncertainty is simply unacceptable for tested individuals working in critical areas. Conversely, assays aimed at IgM or IgA antibodies are a possible tool to confirm an active infection, as these subtypes are detectable within the first week of symptom onset[3,8]. In this context, near perfect assay sensitivity, i.e. a low rate of false negatives, is of the utmost importance to identify all potentially infective individuals.
Using ELISAs as an example, we outline here the reason for the low reliability of many available infection diagnostics assays and the technical requirements for the development and mass production of truly reliable test procedures using established and modern solutions. After all, cost pressure should not be the justification for compromises at the expense of safety.

Four different causes of false positives in serology are known:

1. Inappropriate capture molecules,
2. Cross-reactivities and biochemical interferences,
3. Insufficient surface blocking in serological assays
4. Instability of the reagents.

Regarding 1) it should only be mentioned here that the Receptor binding domain (RBD) of SARS-CoV-2 is currently emerging as the most promising antigen for IgG immunity testing[4,5,9,10]. In assays for an active infection, the highly immunogenic nucleocapsid is probably the prime candidate[3,11]. Additionally, the post-translational modification patterns of the recombinant antigens should be identical to the endogenous protein to preserve all potential epitopes. Thus, prokaryotic expression systems, such as *E. coli*, are not suitable in this context.

Cross-reactivities and biochemical interferences

Even with optimal capture molecules, cross-reactivities and interferences are always to be expected. As an example, antibodies against other corona viruses may also bind to related epitopes of SARS-CoV-2 and lead to false positives[12]. These cross-reactivities are based on low to medium affinity antibody interactions and are not sufficient for immunity against CoViD-19. Moreover, recent years have seen a surge in patients with autoimmune diseases, like rheumatoid arthritis, who in many cases produce antibodies of unknown specificity that are able to cause interferences and false positives in serum-based assays. Many potentially fatal false positives can be prevented by using the LowCross technology[13].

As mentioned above, this boost in assay specificity is especially important when detecting IgG antibodies against SARS-CoV-2 antigens. LowCross-Buffer® replaces the sample dilution buffer and very reliably and independently of their molecular causes reduces low to medium affinity binding significantly, while high affinity binding events - the "true" signals of the assay - are not affected. The LowCross technology reduces cross-reactivities as well as many other forms of interference and has helped to optimize many diagnostic assays worldwide since its introduction. LowCross-Buffer® is ready-to-use and replaces assay diluents not only in ELISAs but also in lateral flow assays (used as chase or flow buffer), Luminex assays, protein arrays, automated high-throughput immunoassay systems and can be used in many other formats. In a pandemic, false positives should not be accepted as easily as in routine serology and LowCross-Buffer® can make an important contribution to the development of reliable diagnostic tools for SARS-CoV-2 antibodies. In our own serological assays, the use of LowCross-Buffer® results in highly specific detection of SARS-CoV-2 antibodies and avoids false positives obtained with other assay diluents.

Surface Blockers for Serology

Surface blocking is a special challenge for all serological assay formats. It is intended to avoid false positives - independent of the presence of an analyte - due to nonspecific binding of molecules to the surface. A serology-specific problem is that some serum and plasma samples can detach individual molecules from a previously dense blocking layer and allow the attachment of unspecific antibodies to the surface in an exchange reaction. The results are high background values if the samples are not sufficiently diluted. However, high sample dilutions of 1:100 or more can have the disadvantage of decreased assay sensitivity. This can be clinically relevant, because specific antibodies present in low concentrations may no longer be detectable. For some RBD-specific antibodies, even titers below 10 ng/mL can have neutralizing activity and hence convey protection against infections[5,14]. Sensitivity is of great concern also in the context of IgM or IgA assays for active infections where false negatives are to be avoided at all costs. Especially patients with asymptomatic or mild disease progressions often exhibit low antibody titers[15].
The use of undefined blockers such as milk powder or FCS is to be rejected entirely: Not only does blocking efficiency and hence assay performance suffer (Fig. 1), but the fluctuations in composition are also at the expense of batch consistency.

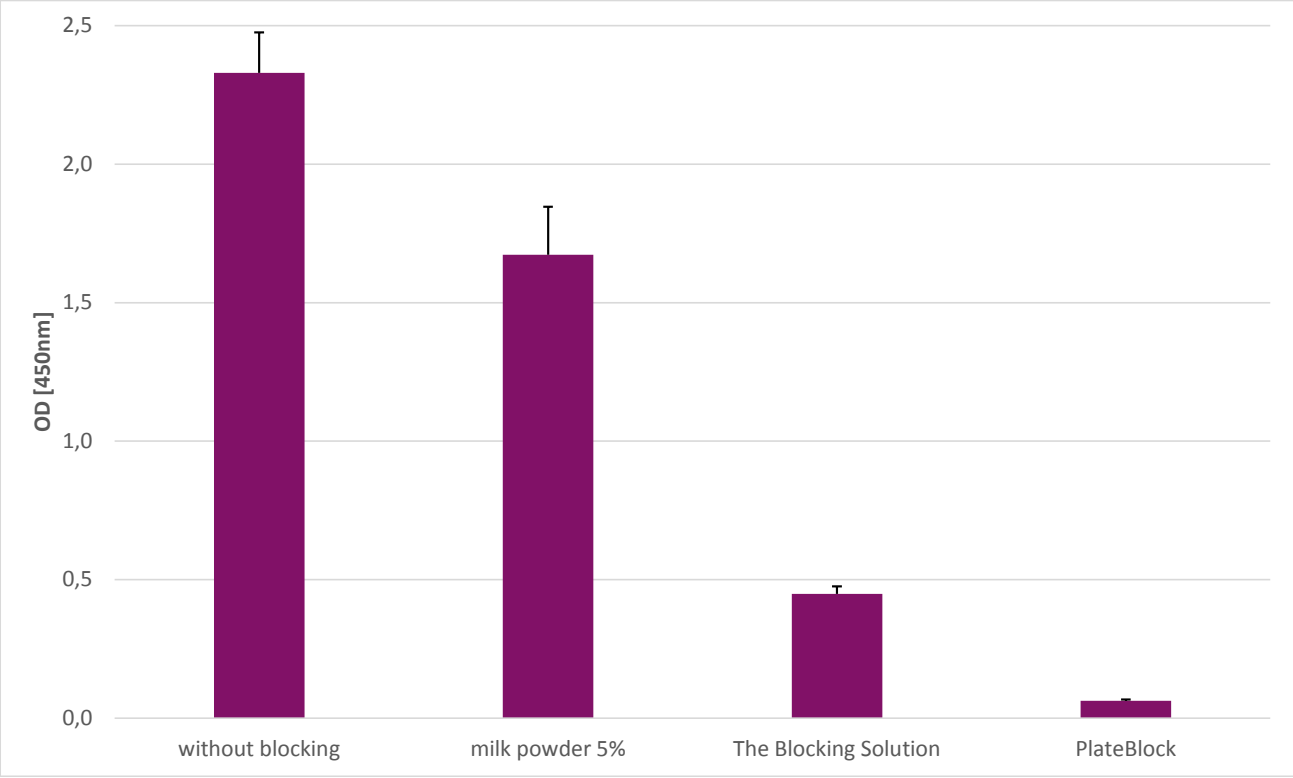

Fig. 1: Comparison of blocking reagents for serology: a Nunc MaxiSorp plate was saturated with different surface blockers and then incubated with a human pool serum diluted 1:10 in CANDOR's assay diluent Sample Buffer on the plate (n = 4 each; error bars correspond to one standard deviation). After washing, the accumulation of antibodies from the serum is detected with a peroxidase-labeled anti-human-antibody.

Latest when the assays are to be validated according to the EU regulation on in vitro diagnostics (IVDR), which will be in effect from May 2022, inappropriate surface blocking will inevitably cause issues. BSA and Casein are widely used blocking agents but face problems specifically in serologic testing: allergy-related antibodies (mostly IgEs but additionally other types such as IgGs have been described) against these animal proteins[16,17] can bind to the blocking layer and lead to false positive results. Solutions for the plate production of sandwich ELISAs, combining blocking and coating stabilization in one step, such as Liquid Plate Sealer® are well-known and commercially used for fast and efficient diagnostics kit production. Even such solutions are not sufficient in serological assays if they are not supplemented with a serological blocker[18], although they show good performance in classical non-serological sandwich ELISAs.
For these critical assays CANDOR has developed the PlateBlock™. The protein-free and animal-free PlateBlock™ was optimized to prevent these exchange reactions in the most comprehensive way. Experiments demonstrate a very good applicability in serology (Fig. 1). In combination with LowCross-Buffer® as sample diluent, PlateBlock™ shows very good performance in ELISA tests for neutralizing SARS-CoV-2 antibodies.

Reagent Stability

For a commercial test that can be used worldwide with high volumes during a pandemic, the stability of the reagents and the associated kit shelf life is crucial. For the labeled detector, a stabilizer based on LowCross® (LowCross® HRP-Stab) is available in addition to the well-established HRP-Protector™. If patient-mediated cross-reactivities between the detector and the blocking reagent were to occur, the use of LowCross® HRP-Stab is recommended. Nevertheless, in ongoing trials for SARS-CoV-2 IgG ELISA HRP-Protector™ has shown extremely good assay performance, when combined with LowCross-Buffer® as the sample diluent in a sequential assay protocol. Both conjugate stabilizer solutions allow shelf lives of several years, reduce the dependence on cold chains and show better assay performance compared to other commercial HRP stabilizers. Equally essential are coating stabilizers for the capture molecule to prevent the loss of native protein folding during storage and transport and thus to avoid misidentifications. CANDOR's Liquid Plate Sealer® product group offers outstanding stabilization compared to alternative solutions and has already been used on millions of samples. Experiments with our in-house SARS-CoV-2 assay have shown that, even when plates are stored at 4 °C, coated RBD of SARS-CoV-2 loses 50 % of its ability to capture specific antibodies within 2 weeks. This deterioration is easily and efficiently prevented by Liquid Plate Sealer® (Fig. 2). In combination with LowCross-Buffer® and PlateBlock™, Liquid Plate Sealer® enables excellent blocking and stabilized serological assays.

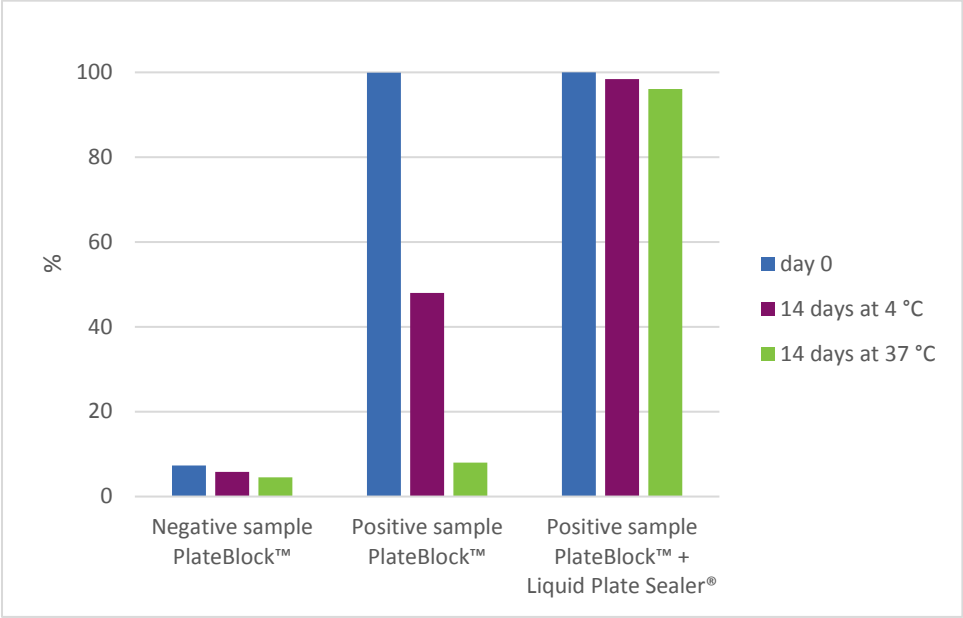

Fig. 2: Stabilization of the Receptor-binding domain (RBD) of SARS-CoV-2: a Nunc MaxiSorp plate was coated with 100 ng RBD and saturated with PlateBlock™. A subset of wells was stabilized with Liquid Plate Sealer®. Plates were stored for 2 weeks at 4 °C or 37 °C and then incubated with human serum samples. After washing, captured antibodies from the serum are detected with a peroxidase-labeled anti-human-IgG-antibody. Values are normalized to the maximum value at day zero.

Setup of an Ideal Serological ELISA

1. Coating of the carefully selected and produced capture antigen
2. Aspirate or tap the plate (do not wash)
3. Blocking with PlateBlock™
4. Aspirate or tap the plate (do not wash)
5. Stabilization with Liquid Plate Sealer®
6. Aspirate or tap the plate (do not wash), dry and store
7. Dilute the patient sample (1:10 to 1:50) in LowCross-Buffer® and incubate on the plate
8. After washing, incubate with detector conjugate stored in HRP-Protector™ (or LowCross® HRP-Stab)
9. Detect with substrate after further washing.

Notes:

- Wash steps during the assay protocol should not be omitted for faster ELISA execution.
- Avoid overdiluting of the serum sample.

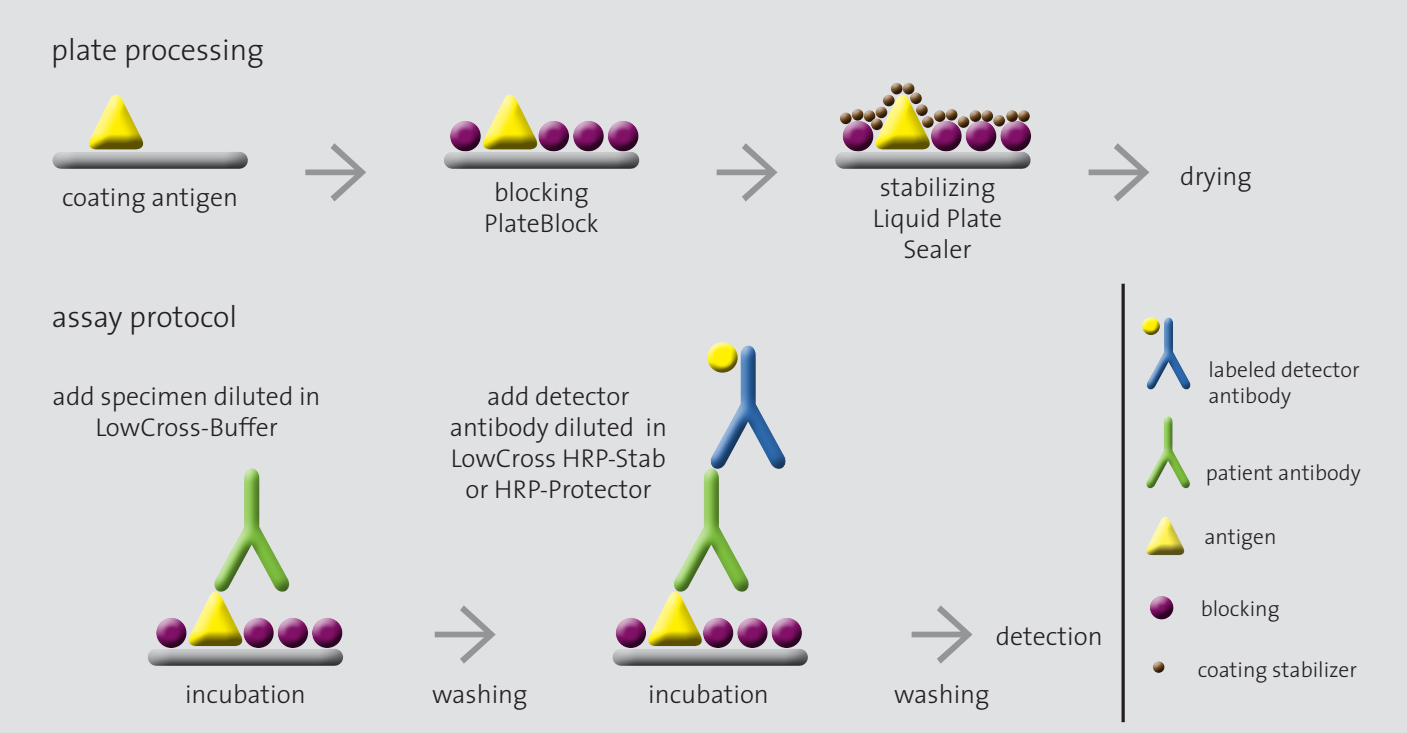

Fig. 3: Ideal ELISA setup for serology.

Conclusion

The development of reliable diagnostics for CoViD-19 is a complex challenge that can benefit not only from the expertise of virologists, but also from experienced practitioners in the field of immunoassay optimization. If the recommendations described here are employed on a wider scale, we expect that truly reliable antibody tests for SARS-CoV-2 will soon be available. CANDOR Bioscience is happy to provide advice to all interested parties because the current crisis can only be mastered together.

References

1. Lassaunière, R et al. (2020). Evaluation of nine commercial SARS-CoV-2 immunoassays. *Preprint at medRxiv*
2. Bond, K et al. (2020) Evaluation of serological tests for SARS-CoV-2: Implications for serology testing in a low-prevalence setting. *Preprint at medRxiv*
3. Long, QX et al. (2020). Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med*
4. Suthar, MS et al. (2020) Rapid generation of neutralizing antibody responses in COVID-19 patients. *Cell Reports Medicine*
5. Robbiani, DF et al. (2020) Convergent Antibody Responses to SARS-CoV-2 Infection in Convalescent Individuals. *Preprint at bioRxiv*
6. Richter, SM (2020). Specificity, positive predictive value and validation statistics in the context of CoViD-19. Free for download on *www.candor-bioscience.de; 4-2020*
7. Bryant, JE et al. (2020) Serology for SARS-CoV-2: Apprehensions, opportunities, and the path forward. *Science Immunology*
8. Randad, PR et al. (2020) COVID-19 serology at population scale: SARS-CoV-2-specific antibody responses in saliva. *Preprint at medRxiv*
9. Tan, CW et al. (2020). A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2-spike (RBD) protein-protein interaction. *Preprint at Research Square*
10. Premkumar, L et al. (2020) The receptor binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Science Immunology*
11. Burbelo, PD et al. (2020) Detection of Nucleocapsid Antibody to SARS-CoV-2 is More Sensitive than Antibody to Spike Protein in COVID-19 Patients. *J Infect Dis*
12. Khan, S et al. (2020) Analysis of Serologic Cross-Reactivity Between Common Human Coronaviruses and SARS-CoV-2 Using Coronavirus Antigen Microarray. *Preprint at bioRxiv*
13. Polifke, T & Rauch, P (2009). Affinity discrimination to avoid interference in assays. *IVD Technology*
14. Brouwer, PJM et al. (2020) Potent neutralizing antibodies from COVID-19 patients define multiple targets of vulnerability. *Preprint at bioRxiv*
15. Lynch, KL et al. (2020) Magnitude and kinetics of anti-SARS-CoV-2 antibody responses and their relationship to disease severity. *Preprint at medRxiv*
16. Chruszcz, M et al. (2013) Serum albumins - unusual allergens. *Biochim Biophys Acta*
17. Wal, JM (2004) Bovine milk allergenicity. *Annals of Allergy, Asthma & Immunology*
18. Polifke, T & Rauch, P (2020). The Covid-19 antibody test challenge. *www.bionity.com*

CANDOR Bioscience GmbH
Simoniusstrasse 39
88239 Wangen, Germany
www.candor-bioscience.com
support@candor-bioscience.de

CANDOR®
The ELISA Experts