

SARS-CoV-2 Immunoassays: Interference elimination by affinity discrimination

Sebastian M. Richter, Tobias Polifke & Peter Rauch

Like all immunoassays, tests for SARS-CoV-2 related antigens or antibodies can suffer from interference, leading to faulty results. In the context of a pandemic, these errors can have tremendous impacts as they can lead to the formation of new disease clusters and hence facilitate the viral spread. In the following we elaborate the molecular basis for the underlying interferences, how they can be prevented by modern affinity-discriminating assay diluents and how these diluents can help to develop more reliable antibody- and antigen-assays.

Immunoassays can answer the two most urgent questions in the context of the CoViD-19 pandemic: “Am I infected?” and “Am I immune?”. A good antigen test can help to identify currently infected people by detecting viral protein (antigens), typically in saliva and mucus samples. In contrast to the detection of viral RNA by RT-PCR, antigen tests are relatively inexpensive, quick and - if they come in lateral flow format - can be point-of-care diagnostics operated by a layperson. Reliable antibody tests indicate a past infection and, if based on the right viral antigen¹, can make predictions about a potential immunity against re-infection with SARS-CoV-2. In terms of their analytical requirements, these two types of immunoassays differ dramatically. For an antibody test, almost perfect specificity - i.e. a low rate of false positives² - is essential to prevent a tested person from potentially infecting others due to an ill-founded feeling of immunity leading to unsafe behavior by non-compliance with hygiene precautions. In contrast, antigen tests require a very high sensitivity - i.e. a low rate of false negatives - to detect all currently SARS-CoV-2 infected patients and prevent a further spread of the virus. In this context, false positive results, albeit unwanted, are acceptable to a certain degree, as they could be further confirmed by an RT-PCR test. What too often prevents these and many other immunoassays from achieving a better analytical performance are different kinds of molecular interferences that can negatively impact sensitivity and specificity. Interference is defined as the effect of a substance present in a sample that alters the correct value of the result³. Interferences are commonly observed and are a constant problem in all types of immunoassays, such as ELISA, lateral flow assays, bead-based assay systems (e.g. Luminex assays), protein arrays, Immuno-PCR, or automated high-throughput immunoassay systems. They are often dependent on the individual patient and hence vary greatly from sample to sample. Although interferences can be observed in any type of sample, they are more common in patients with severe co-morbidities, like rheumatoid disorders, obesity, and cancer. Interestingly, many of the co-morbidities associated with interferences are known risk-factors for a severe course of CoViD-19. This leads to an unacceptable situation where the most vulnerable individuals are also the most likely to receive faulty test results. If these types of interference-prone samples are not considered adequately and included in the validation process, antibody and antigen assays that fail when confronted with the most critical samples are the consequence.

Interfering antibodies - HAMA & co

A common cause for problems in immunoassays are interfering antibodies, like the well-known HAMA⁴ (Human anti-mouse antibodies), anti-animal antibodies in general, heterophilic antibodies or rheumatoid factors. Interestingly, since today's therapeutic monoclonal antibody drugs are almost exclusively humanized, the prevalence of real HAMA is steadily declining. Still, with the rise in the incidence of rheumatoid and allergic disorders in recent years, interferences caused by antibodies will remain of high clinical relevance also in the future and seem to further increase in prevalence. In general, these antibody interferences are mostly observed for immunoassays in sandwich format, i.e. based on capture and detector antibodies (fig. 1). Interfering antibodies can cross-bridge capture and detector antibodies, leading to high readings in the assay and hence

false positive results. Alternatively, they can block access of the antigen to the detector antibody or even agglutinate the detector antibody, resulting in false negative readings.

SARS-CoV-2 antibody assays are typically antigen-down assays with an anti-human detector antibody and are hence unaffected. In contrast, immunoassays for SARS-CoV-2 antigens often use the sandwich format and are potentially susceptible to interfering antibodies.

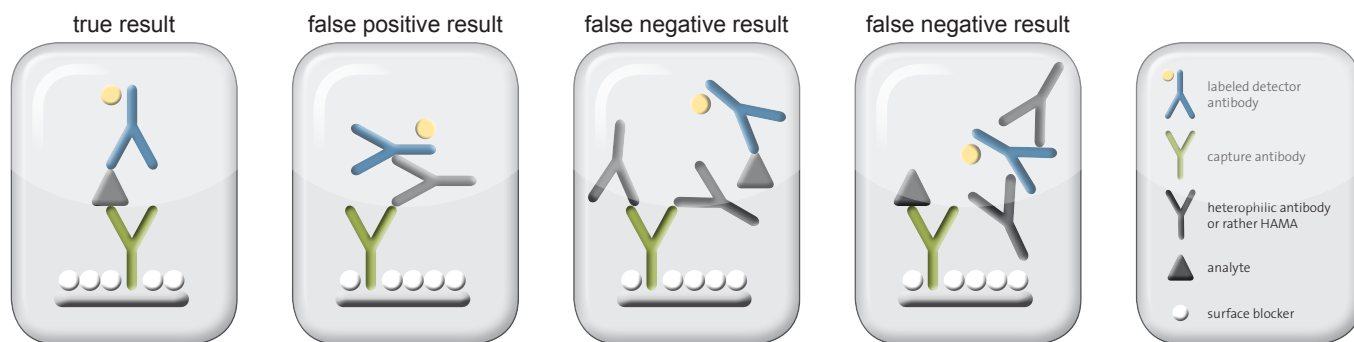


Figure 1: Interfering antibodies, like HAMA or rheumatoid factors, can result in false positives or false negatives

Whether interfering antibodies are the reason for faulty results can be investigated using commercially available ELISA Kits designed to identify heterophilic antibodies, HAMA and rheumatoid factors (often simply termed HAMA ELISA). After identification, HAMA-blockers are often used to solve the interference problem. The drawback is that more biologically active components - antibodies - are added to the assay. This might lead to new kinds of interferences. Besides, most producers offer several different HAMA-blockers, as there is no specific blocker that can be used for all assays. The most effective final concentration of the HAMA-Blocker also must be titrated carefully for each assay for cost reasons. This shows that very extensive testing is necessary to find the right HAMA-blocker for a given combination of assay and interfering sample. Moreover, despite the dominance of interfering antibodies in the relevant literature, many other types of interferences unrelated to antibodies are commonly observed in immunoassays.

Beyond HAMA: The many other types of interferences in immunoassays

Apart from antibodies, interference can be caused by endogenous substances in the sample, for example due to analyte masking, by cross-reactivities, or by general matrix effects, like high lipid and salt concentrations, viscosity, or pH. These types of interferences are highly relevant also in the context of SARS-CoV-2 immunoassays but remain completely unaffected by common HAMA-blockers.

Many endogenous substances, for example highly abundant serum proteins such as albumin, complement factors, lysozyme, alpha-1 antitrypsin, or fibrinogen, can interfere with immunoassays⁵. Due to its biological function, albumin is prone to binding many smaller analytes, but additionally other proteins. Hormones are often bound to transport carriers. These binding events can lead to a masking of the analyte, i.e. blocking the access of assay antibodies by steric hindrance. Low readings and false negatives are the result. Receptors, like albumin or C-reactive protein, bind many different endogenous substances as part of their biological function. Their broad range of binding partners requires a low binding-specificity which in turn can lead to unwanted interactions with assay antibodies or masking of the analyte. Lysozyme can non-specifically bind proteins with low isoelectric points and can therefore crosslink mainly negatively charged assay antibodies, leading to faulty readings⁶. These types of interference can affect both antibody and antigen tests for SARS-CoV-2 and cause faulty results. Not unlike serum, saliva and mucus contain a plethora of endogenous molecules that can interfere with immunoassays, like the mucins. Common to most of the interferences by endogenous substances is that they are based on promiscuous binding events and low-specificity interaction sites and hence are of low affinity.

Cross-reactions or cross-reactivities are defined as the binding of an antibody to other structures than the intended analyte due to similar, but not identical epitopes. The bound structures often exhibit great similarity to the target analyte, like metabolites of the analyte, chemically similar molecules, or proteins with a random amino acid sequence similarity or homology. When it comes to SARS-CoV-2, cross-reactivity is often discussed in the context of related corona viruses. SARS-CoV-2-antigen or antibody assays are completely useless if they also detect the antigens of - or antibodies against - other corona viruses. Importantly, if the interference is due to shared identical epitopes in SARS-CoV-2 and other corona viruses, it is by

definition not a cross-reactivity and cannot be resolved unless more suitable assay antibodies or antigens are used. As an example, the nucleocapsid of SARS-CoV-2 harbors many epitopes highly conserved in other corona viruses and hence would require careful selection of assay antibodies to be successfully used in antigen assays. In the case of a real cross-reactivity, the target of the cross reaction is only similar to the intended epitope. Consequently, the binding of the antibody to the cross-reactant is of lower affinity than to its intended target.

Nonspecific binding events are a type of interference that is closely related to cross-reactivities but differs from the latter in its molecular origins. If the cross-reactant is known and its potency can be quantified with a competing concentration of cross-reactant, then it is a bona fide cross-reaction. Nonspecific binding is caused by substances that are present at much higher concentrations than the target analyte. Apart from albumins or immunoglobulins from the sample, these can include the surfaces of microwell plates and blotting membranes, or spots of immobilized antibodies in protein arrays. Depending on the nature of the sample and the type of assay, this type of interference can cause problems also in assays designed to detect SARS-CoV-2 antibodies or antigens. As the name implies, nonspecific binding events are typically of low to medium affinity.

The term matrix effect is commonly used to describe the sum of all interference effects of the components present in a given sample that influence the measurement of the target analyte⁷. If an interference is known to originate from the sample but its molecular cause is unclear, it is called a matrix effect. The boundaries to other types of interference are sometimes blurry and some matrix effects may derive from sources discussed above. Matrix effects can be caused by the viscosity, pH, or salt and lipid concentrations of the sample. These physicochemical properties have an influence on the binding properties and specificities of antibodies or the solubility of certain analytes. As samples from patients with severe co-morbidities often display changes in these sample properties, matrix effects must be considered in SARS-CoV-2 immunoassays.

Labeling of detector antibodies or tracer analytes is a common practice in immunoassays. Labels can be enzymes, such as alkaline phosphatase (AP) or horseradish peroxidase (HRP), fluorescent dyes, radioactive isotopes, affinity tags, like biotin, or - in case of immuno-PCR - DNA. Unfortunately, these labels can change the properties of the labeled component and introduce new sources of interference. Fluorescent dyes are often very hydrophobic and can negatively impact the solubility of the labeled protein or even directly interact with other substances. Moreover, coupled dyes can change the binding properties of coupled proteins and hence either reduce the affinity of the interaction between assay antibodies and analytes or enhance nonspecific binding. This can lead to increased binding of the labeled protein to test surfaces, such as ELISA plastic surfaces, binding to endogenous proteins in the sample, or direct binding of the detector to the capture antibody. False positive results due to elevated background signals and worse signal-to-noise ratios are the consequence. Conversely, the binding of proteins to fluorescent dyes can lead to signal quenching and false negative readings. The labeling with enzymes or DNA can also cause interferences as they increase the surface and number of epitopes available for nonspecific interactions, for example with endogenous DNA binding factors. All these problems can be amplified if particularly small proteins, like the receptor binding domain (RBD) of SARS-CoV-2, are labeled. All forms of immunoassays, from sandwich formats for antigen detection to antigen-down assays for antibodies, are prone to these labeling effects. Assay formats in which several labeled components are present in the solution simultaneously, like protein arrays, are especially vulnerable due to the high complexity of potential nonspecific interactions. These interferences due to the labeling of assay components are again caused by low to medium affinity interactions.

Interference elimination by affinity discrimination

These Interferences, derived from cross-reactivities, matrix effects, endogenous substances, disorders, labeling and most interfering antibodies, share one common denominator: they are caused by low to medium affinity binding events⁸. In contrast, the specific interaction between a good antibody and its epitope is of high affinity. However, even low affinity binding events in sufficient quantity can lead to high assay readings, creating specific-looking false positive results, or high backgrounds and bad signal-to-noise ratios. A potential approach is using high stringency assay diluents to lessen the impact of these problematic low to medium affinity interactions. However, these highly stringent conditions also negatively impact high affinity binding and hence the correct signals of the assay. High stringency buffers are thus not a helpful option.

The only way to reduce low and medium affinity interactions while not obstructing the desired high affinity interaction is affinity discrimination. This mode of action was successfully introduced by CANDOR's LowCross-Buffer[®] 15 years ago and has since eliminated interferences in millions of samples. If used for sample and antibody dilution, these affinity discriminating solutions can remove all interferences based on low or medium affinity binding while maintaining specific signals. Hence, they greatly reduce the background, improve the signal-to-noise ratio, and enhance the reproducibility of your results (fig. 2). In contrast to HAMA-blockers, which only prevent interference from interfering antibodies, LowCross-Buffer[®] is able to eliminate all kinds of interferences discussed above. Therefore, such an affinity discriminating diluent can be used in any

assay challenged by interferences. A time-consuming closer investigation of the molecular basis or a careful selection and titration of different HAMA-blockers is not necessary. It is important that all binding reactions during the course of the assay take place under affinity selective conditions. Since many diagnostic kits require the storage of detector antibodies for an extended period, CANDOR also offers LowCross-HRP®, which combines the advantages of affinity discrimination with the functionality of a long-term stabilization of peroxidase-linked detector antibodies.

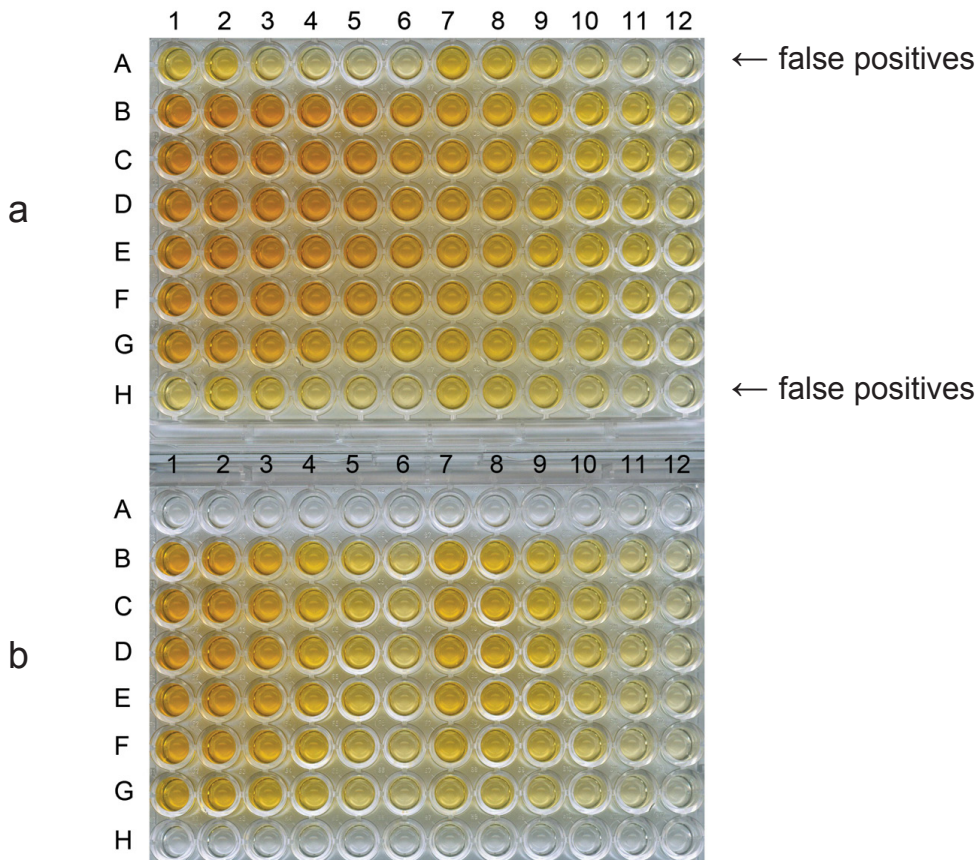


Figure 2: Prevention of false-positive binding using LowCross-Buffer® in an ELISA against guinea-pig IgG. With the standard assay buffer (a), false positives in the specificity control (row A) and in the blank values (row H) spoiled the interpretations and evaluation. The use of LowCross-Buffer® (b) prevented all false-positive signals. Assay performed by Dr. C. Specht (PARA Bioscience GmbH (now vivo Science GmbH), Gronau, Germany)

The positive effects of affinity discrimination can be visualized by comparing a generic antigen-down assay to detect antibodies against the RBD of SARS-CoV-2 performed with the standard diluent PBS-T/BSA with an enhanced protocol featuring CANDOR products, including LowCross-Buffer® as assay diluent (fig. 3). Whereas the maximum signal of the positive sample is almost unchanged, background and sample-to-sample variation of the negative samples are greatly improved, leading to a better detection limit. The reduction in background and variation of negative samples and the concomitant improvement in the signal-to-noise can prevent many false positive results. As mentioned above, false positives, for example from antibodies against related corona viruses, are to be avoided at all costs in SARS-CoV-2 antibody assays to not give the individual a false sense of security. Moreover, the superior antibody assays produced with affinity-discriminating diluents can also help in guiding the development of vaccines against SARS-CoV-2.

Antigen assays are often complicated by the complex matrix of mucus and saliva and by the fact that the assays deal with a mixture of intact and fragmented viral particles. Moreover, different assay formats, from the classic sandwich to double-ACE2-based assays, are employed. Still, false negative results must be reduced to a minimum to prevent an infected person from becoming careless due to being “confirmed virus-free”. Many potentially lethal infections could be spread this way. Affinity discrimination is able to improve assay performance irrespective of the sample matrix and the exact format used, simply due to preventing many unidentified sources of interference. Hence, including LowCross-Buffer® early during development can prevent many hurdles during assay development and validation. This not only saves time and money for the developer but will also help the general public by making reliable antigen assays available more quickly.

Affinity discrimination works by creating an affinity cut-off. Interactions with affinities lower than the cut-off are reduced while higher affinity binding is unaltered. Therefore, the concept works best when the assay antibodies have a very high affinity for the target analyte and the cut-off can be set high. For these high affinity antibodies, CANDOR offers the LowCross-Buffer® STRONG, which prevents even more unwanted interactions than the classic variant. Conversely, some assay formats, like in autoimmunity testing, must rely on lower affinity interactions and cannot be conducted with the cut-off set by the classic LowCross-Buffer®. For these assays, LowCross-Buffer® MILD can be beneficial. Still, for most applications, LowCross-Buffer® in its classic formulation is the perfect balance between interference suppression and true signal conservation.

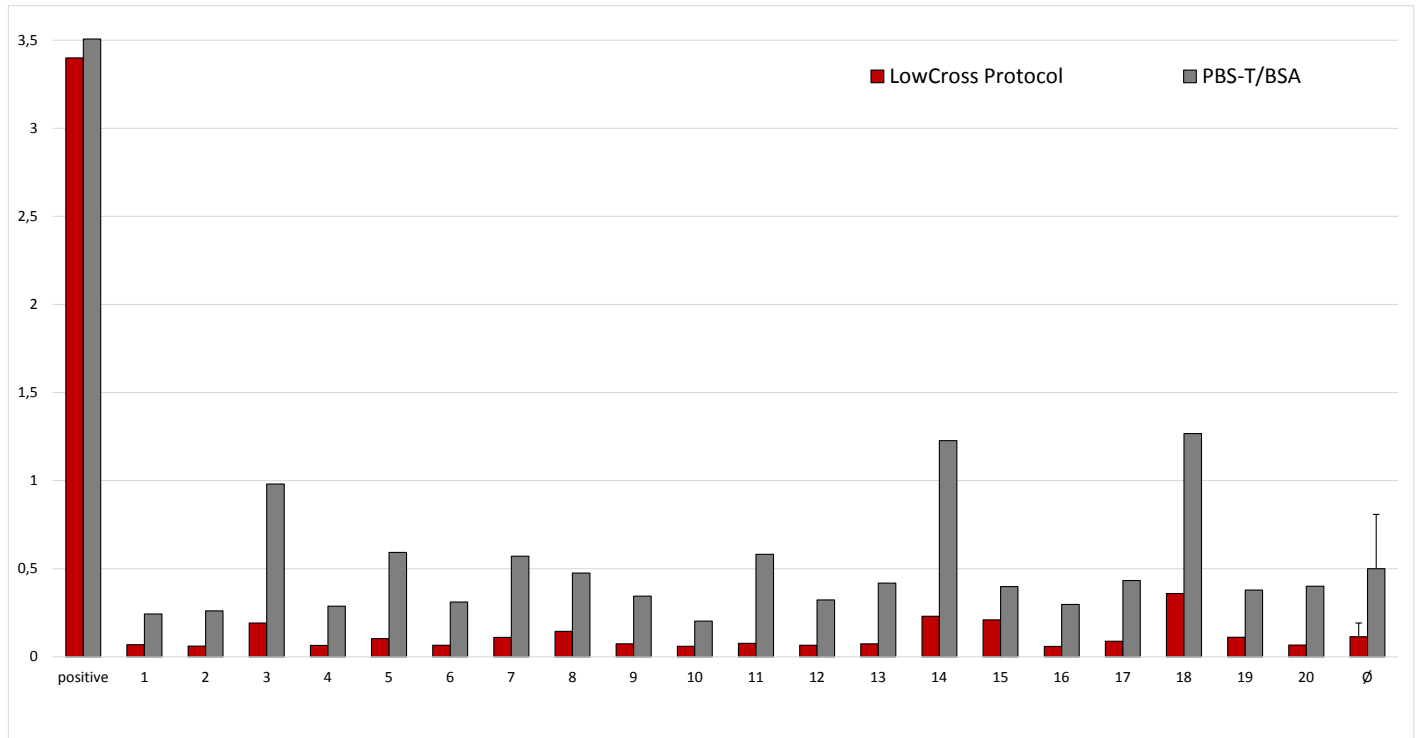


Figure 3: Improving an RBD-based SARS-CoV-2 antibody ELISA using LowCross-Buffer. Comparison of negative samples (# 1 – 20; Ø is arithmetic mean of 1 - 20) LowCross protocol vs. the standard diluent PBS/Tween/BSA (PBS-T/BSA). Samples were analyzed either using the CANDOR solutions, including LowCross-Buffer, or a standard PBS-T/BSA protocol. The LowCross protocol allows for less average background, less sample-to-sample variation, a better signal-to-noise-ratio, and a better detection limit due to a decreased cut-off. (Samples provided by in.vent Diagnostica GmbH, Henningsdorf, Germany.)

Conclusion

Affinity-discriminating buffers have the potential to deal with many different interferences without the need for detailed information about their molecular origins. Assay performance and reliability are increased without the need for additional processing steps. These improvements are not only essential in the highly critical context of SARS-CoV-2 immunoassays, but will also help save time and cost when diagnostic kits are to be validated according to the EU regulation on in vitro diagnostics (IVDR), which will be in full effect from May 2022. For the cost of a few cents per sample, affinity-discriminating diluents can greatly contribute to improving immunoassays in the future, no matter if they are developed in the context of the SARS-CoV-2 pandemic or for other purposes in human or veterinary diagnostics, in food testing, or bioanalytics. CANDOR maintains the highest quality standards for all products, which are “made in Germany” in an EN ISO 9001 and EN ISO 13485 certified production facility.

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