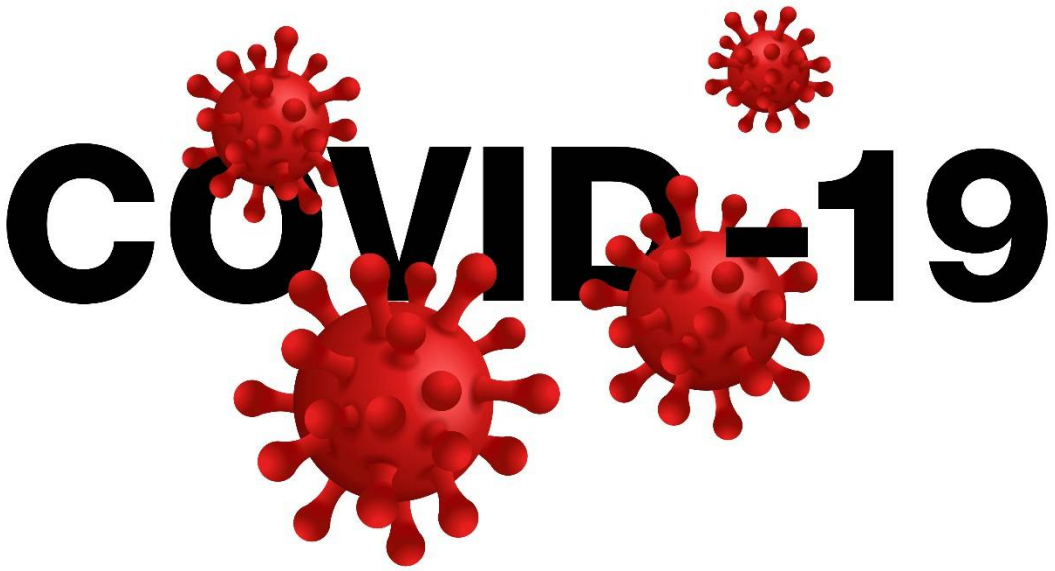

COVID-19 Serology Testing

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(version2.0)

allermetrix

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Executive Summary

The COVID-19 epidemic has created chaos in the medical field including how to utilize newly developed laboratory testing to determine patient status. One of the tests that has been totally discounted is the COVID-19 antibody assay. Qualitative substandard assays initially allowed by the FDA were recalled and there has been no truly quantitative test to receive EUA, (Emergency Use Authorization). A quantitative antibody test that correlates with protection from infection is a tool that can be used to identify those in need of vaccination and boosters. It could also serve as an indication of "healthiness" for clinicians in assessing patient COVID-19 immune status. Allermetrix has developed and offered quantitative testing for IgG to the most important SARS-CoV-2 antigens since July 2020. The test was submitted for EUA in July 2020. Allermetrix quantitative specific IgG assays to the receptor binding domain (RBD), S1 subunit of the spike protein (S1), and the nucleocapsid protein (N protein) are calibrated to the WHO international standard and are reported in units of μg/mL, and binding antibody units (BAU), the international standard unit.

Several antibody tests are available, and they are not at all equivalent. It is important to understand the antigen(s), antibody type, and formats to evaluate assays. It is also important to know if the assay is qualitative, semi-quantitative, or quantitative. Each has its place in the field, but only quantitative assays calibrated to an international standard will provide harmonized results that can be compared between labs and used to determine a correlate of protection.

Correlates of protection from infections have been determined for several viral infections such as hepatitis, influenza, measles, rubella, and polio. Peer reviewed studies have indicated that IgG anti-RBD correlates with neutralizing antibody titer and virus neutralization *in vitro*. It has also been shown to correspond to protection. This antibody test is likely to be correlated to protection from infection.

This monograph will explain the immunochemistry of these antibody tests and the methods used for measurement. Allermetrix assay data will be presented for recovered COVID-19 patients and vaccinated individuals as well as antibody results for 4 of the variants of concern, the alpha, beta, gamma, and delta variants in both groups.

Laboratory developed tests and procedures are designed, developed, and validated by highly skilled laboratory professionals who work in special "highly complex" laboratories. Only about 2 % of the laboratories in the US are designated as highly complex by the Centers of Medicare and Medicaid Services (CMS). Well-constructed, highly characterized assays in these laboratories help clinicians to determine how to best guide their patient through the COVID-19 epidemic. The time has come for quality, quantitative antibody testing to be recognized and used.

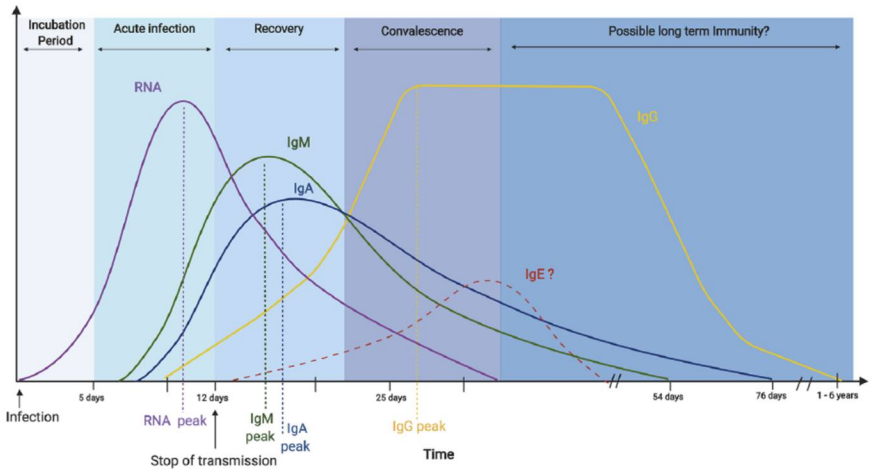
COVID-19 Serology Testing Overview

Introduction

The COVID-19 pandemic and associated information overload seems to have confused many people. The FDA and CDC have changed course several times since the outbreak and continue to lead an unsteady path back to some semblance of normality. The use of testing has been of paramount importance to identify people with COVID, but identification of people who are immune to COVID has been elusive. Important for this and other potential pandemics is some way to assess immunity. For other viral diseases laboratory tests have been used to assess an individual's protection from being infected after vaccination.

Current laboratory testing for COVID-19 is defined by 3 general analytes, viral RNA, viral antigen, and antibodies to virus. When discussing COVID-19 the clinical disease is COVID-19, but the cause of the disease is a virus that is called SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2). The most common testing for presence of the virus detects viral RNA. These tests, called RT-PCR or Nucleic Acid Tests (NAT), identify the specific virus RNA in samples taken from people suspected of having the disease. It is important to understand that viral RNA is only one component of an infectious virus, which has proteins and other components that together make the virus infectious. The immune system is constantly fighting the virus to keep it from infecting cells, using a plethora of tools to do so. NATs detect RNA from dead viruses as well as live "infectious" viruses without any differentiation. Viral antigen tests identify specific proteins that are components of the virus. These tests also do not differentiate dead from live viruses. Antibody tests do not detect any components of the virus, but rather demonstrate that one's immune system has responded to some viral component. These tests are evidence that the immune system has identified and responded to a foreign and unwelcomed visitor.

Over the course of COVID-19 disease these analytes are present in patient samples at different times. Symptoms of COVID-19 usually start 3-5 days after infection and resolve within 14-20 days. The viral RNA and antigens are generally detectible from 3-5 days after infection to 30 days after infection. Antibodies to the virus start to appear 7-14 days after infection and persist for months and may be detectable for years.



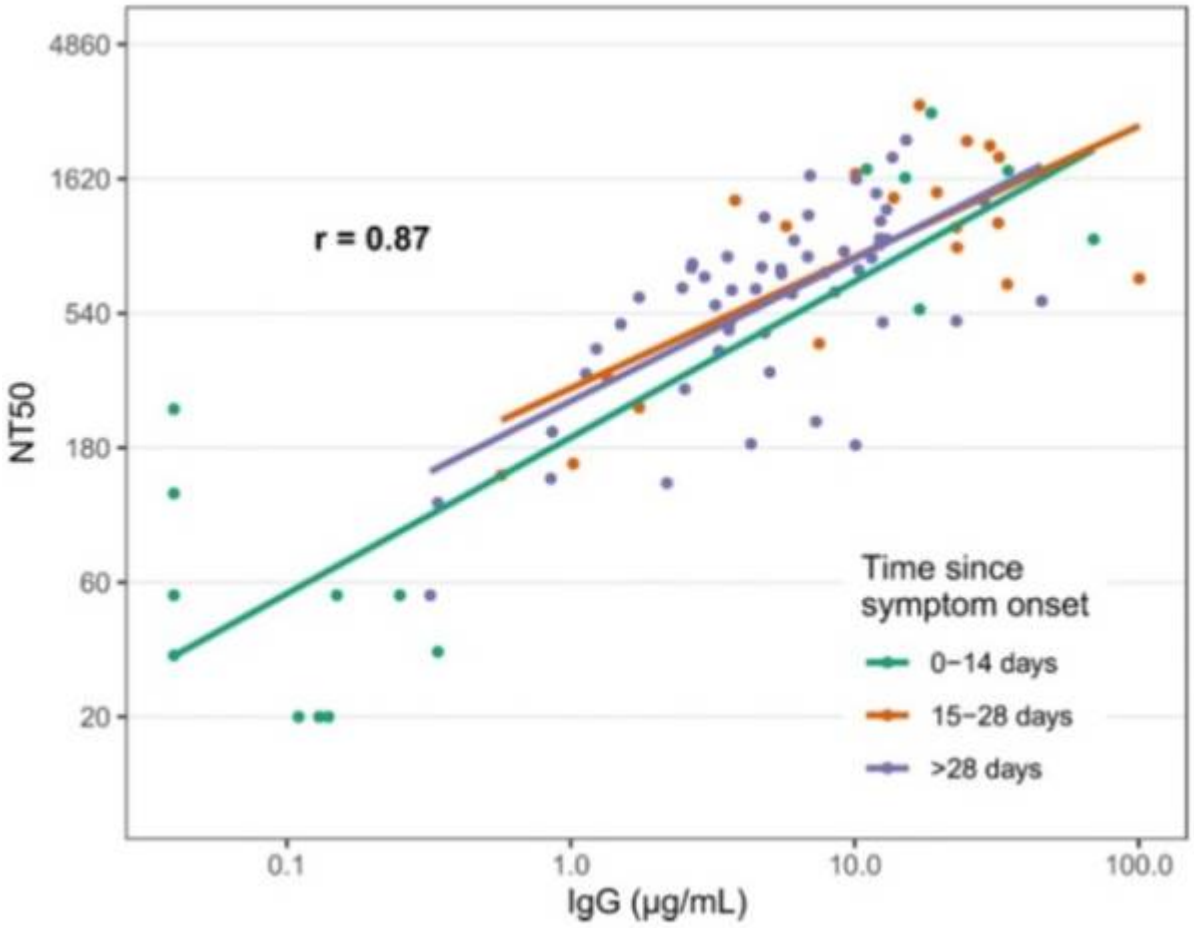


Figure 1. 50% neutralizing titer (NT50) correlation to IgG anti-RBD. The overall repeated measures correlation coefficient (r) is shown. Lines represent simple linear models for each time period. (from Iver et al.)[iv]

There are 5 antibody isotypes, IgM, IgG, IgA, IgE, and IgD. Within the IgG isotype there are 4 subclasses, 1-4, and IgA has 2. The antibody binding site defines the target for the immune system, but the isotype defines how the immune system deals with that target. The most abundant type of antibody is IgG, and it has several ways to block, remove, and destroy viruses. IgA antibodies tend to be in mucus membranes and act to block viruses from infecting cells. The IgM isotype is the first antibody to encounter virus and begins a cascade of events including working with T cells to create IgG and IgA antibodies. IgE antibodies are the least abundant circulating antibody and targets allergens and parasites. IgD antibodies are mostly membrane bound and not involved in active anti-viral targeting. Targeting the SARS-CoV-2 virus is predominantly done by IgM, IgG and IgA. IgM and IgA viral responses tend to wane after about 30 – 45 days. IgG responses are durable long-term antibodies that can be detected for months and even years after infection.

Antibodies deal with the target in several ways through effector functions which are in part determined by the isotype and subclass. IgM antibodies and most IgG antibodies activate complement when bound

to targets. Complement activation will prepare the targets to be consumed by macrophages that will destroy the viruses with enzymes. IgG also will bind to other leukocytes like neutrophils that will engulf the virus.

Antibody tests for COVID

Many different antibody tests are available for COVID-19 and they are all different and generally not comparable, which makes interpretation of test results confusing. Tests vary by the antigens to which they detect antibodies, the type of antibody (IgG, IgM, IgA) they detect, and the format of the test. Each of these attributes are important to understand for interpretation of the results.

The antigens that are used for antibody detection include the whole spike protein, the S1 subunit of the spike protein, the S2 subunit of the spike protein, the receptor binding domain (RBD) of the spike protein, and the nucleocapsid protein. These antigens are all recombinant proteins for the available tests and can be manufactured in many ways. The methods of creating recombinant proteins use vectors that are modified to produce the proteins of interest. Not all methods create the same protein because different vectors modify the proteins differently (e.g. glycosylation). For the purposes of this discussion, we will not go into detail on this point and only emphasize that **the source material used for the antigen is important for test performance.**

The antibodies detected are in some ways dependent on the assay format, and can identify IgG, IgM or IgA antibodies in some formats. In other formats the type of antibody is not identified and only the presence of antibodies is reported. This can be important because not all antibodies persist *in vivo*. As mentioned earlier, IgM and IgA antibodies tend to wane after 30-45 days. IgG antibodies persist much longer and, in some cases, can be detected months and years after an infection.

Serologic Measurements

There are three types of results reported for serological measurements. The simplest type of assays indicate the presence or absence of specific antibodies. These assays are qualitative and will vary in sensitivity from assay to assay. Qualitative assays merely demonstrate that antibodies are present or not.

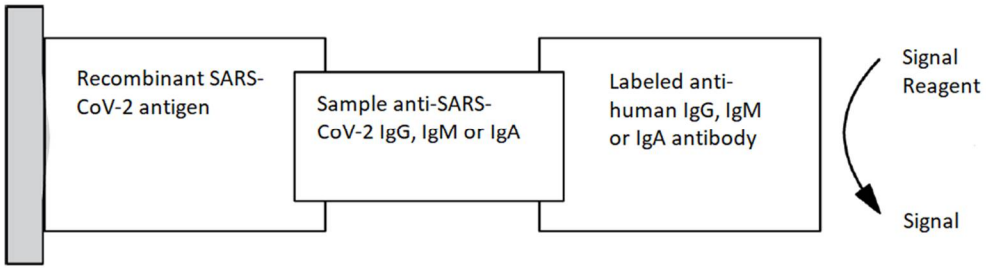
Semi-quantitative assays will indicate if antibodies are present and whether one sample has more than another. Semi-quantitative assays do not demonstrate how many antibodies are present but will indicate if antibody levels are higher or lower than an internal reference point. Semi-quantitative assays cannot be compared because each uses a different internal reference point. The result is reported as a number, but the numbers are not scaled. For example, if one serum results in a value of 2 and another a value of 4, it does not follow that the higher serum has twice as many antibodies as the lower one. There is no simple relationship between these readings. Additionally, a result of 2 in one semi-quantitative assay, may result in a number like 100 in a different method because of different internal reference points and units of measurement.

Quantitative tests scale and have calibrators that can be compared to some primary calibration source. Additionally, consensus standards created by organizations like the WHO can be used to harmonize quantitative tests. Allermetrix serological assays are quantitative and calibrated to the WHO

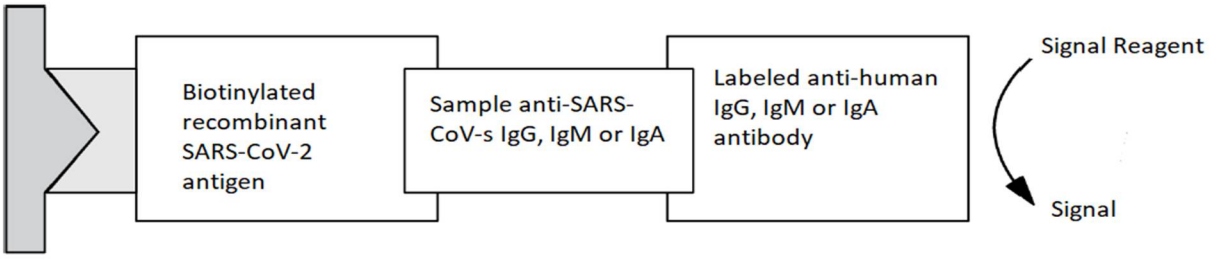
international standard. Test results are reported in µg/mL and binding antibody units (BAU), the international consensus standard unit, which allows comparison to other quantitative methods calibrated to the international standard.

ELISA format

Several different formats for testing antibodies are used. One of the most common formats is Enzyme-Linked Immunosorbent Assay (ELISA). The classical ELISA format affixes the antigen to a surface, most often a plastic microplate well. The sample containing antibody is added to the well and reacted with the solid-phase antigen. After some incubation time the sample is washed away and a detection reagent is added. After another incubation the detection reagent is washed away. The detection reagent generally is specific for a type of antibody (IgG, IgM, IgA) and is covalently bound to a signal generating compound. Traditionally it is an enzyme that converts a non-colored substrate to a colored substrate that can be measured with a spectrophotometer. The substrate is added after washing away the detection reagent and if color develops, it demonstrates the presence of antibody to the antigen on the solid phase. Some modifications of the detection reagent have included using fluorescent compounds or enzymes with substrates that create light. These modifications change the detection mechanism, but the assay dynamics remain the same.

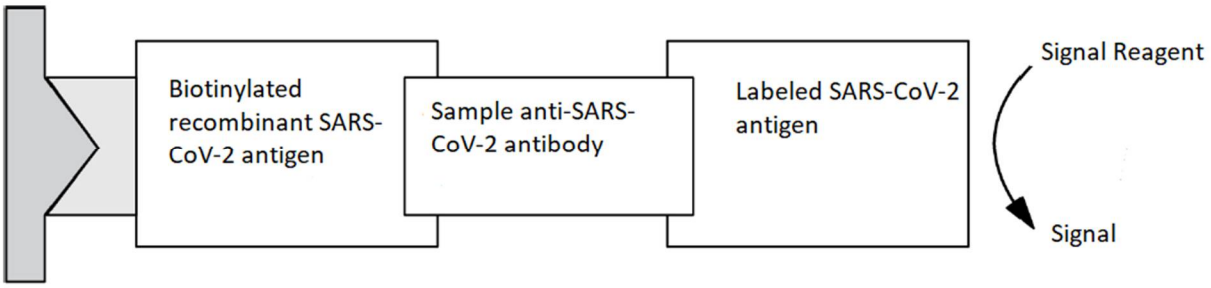


One modification of ELISA uses antigens that are biotinylated rather than adsorbed to the solid phase **(this is the technology Allermetrix utilizes)**. Biotinylation of the antigens chemically link a small molecule, biotin, to the antigen, and the solid phase is coated with a protein, streptavidin, which binds biotin very strongly. **Biotinylated antigens are more like the natural form of proteins than those adsorbed to solid phase.** Adsorption to solid phase will denature proteins making them different from the natural form and change the way in which they react with antibodies. The differences between solid-phase and biotinylated antigens have been demonstrated in many studies.



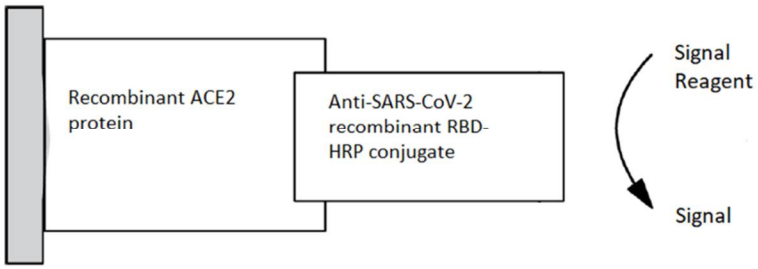
Antibody Complex Capture

Antibody capture techniques react the sample with the same antigen in 2 forms. One form is captured and is biotinylated, the other form includes a reporter molecule. When the sample reacts, antibodies that have at least 2 identical antigen binding sites will form immune complexes that include antibodies and both forms of the antigen, biotinylated and reporter molecule linked. This mixture is added to a solid phase that is coated with streptavidin, which captures the whole immune complex. The signal is generated with the reporter molecule and more signal indicates more antibody complexes. This technique does not identify the type of antibody, only that antigen specific complexes are present.



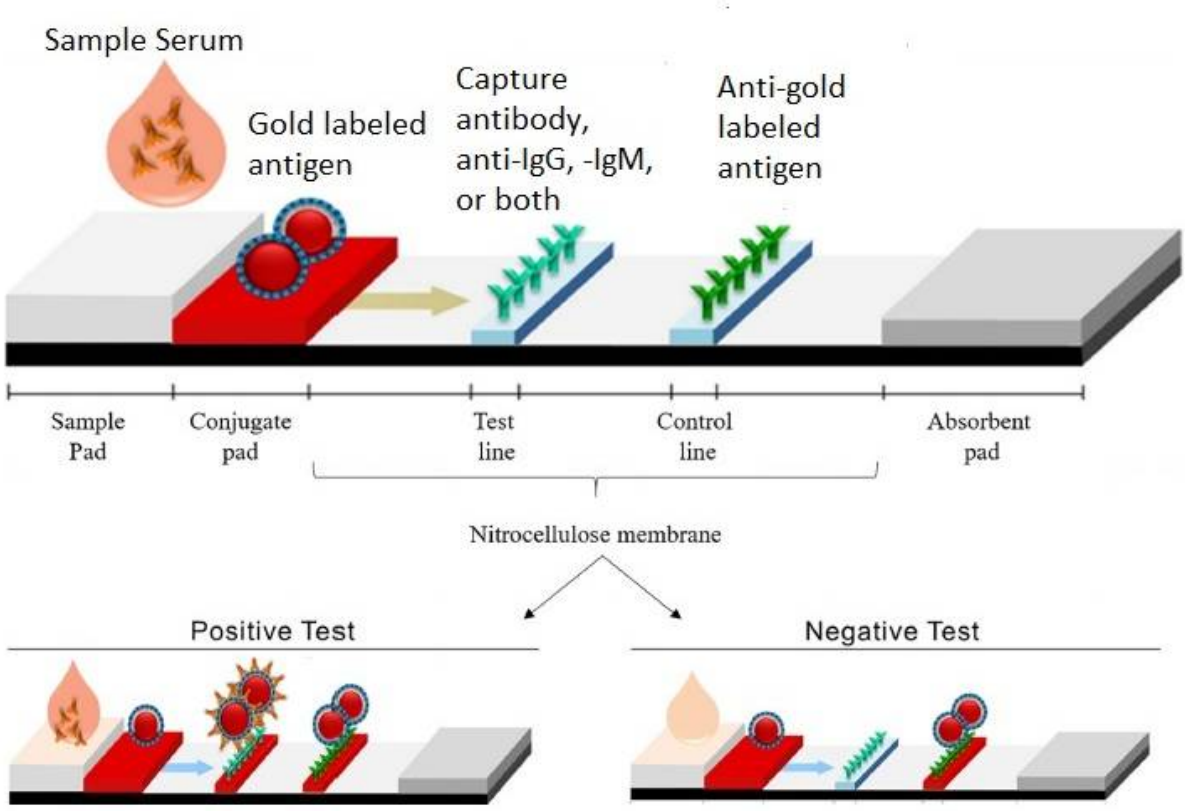
Neutralizing Antibody Inhibition

The neutralizing antibody inhibition format has some similarities to the antibody complex capture technique. The sample is mixed with a single form of the antigen, in this case it is the RBD because it binds to the cell receptor, angiotensin converting enzyme 2 (ACE 2), when the virus infects cells. RBD is linked to a reporter molecule. The sample and the RBD are mixed to allow the antibodies to form complexes with the RBD-reporter. The solid phase has recombinant ACE 2 adsorbed onto it and the sample-RBD complex is added after incubating for an allotted time. The sample-RBD complex mixture is washed away, and signal is generated from the reporter molecule. In this assay there is a control to which no sample is added which represents 100% binding of the RBD-reporter to ACE 2. Each sample signal is compared to the 100% signal and reported as the amount of inhibition of the 100% control.



Lateral Flow

Lateral flow techniques use antigen linked to colloidal gold and non-covalently "painted" onto a membrane. The membrane also has anti-human IgG, IgM or both covalently linked to it at selected areas. For test control, antibody to the antigen is also linked to the membrane at a selected position. The sample is added to one end of the device and it flows laterally through the membrane, mixing first with the labeled antigen and then through the anti-human antibody region(s) and finally through the anti-antigen region. Antibodies are detected if a visible line develops over the anti-human antibody region(s), and over the anti-antigen region. If a line does not develop over the anti-antigen region the test is invalid.



Measurement of Antigen Specific Antibodies

As well as many formats, there are several types of results from these antibody assays. Some tests merely report that antibodies are present or not present, qualitative testing. Others report semi-quantitative results which can rank the results, but do not measure how many antibodies are present. Quantitative test results measure how many antibodies are present.

Traditional antibody testing for reactive samples uses the titering technique, which tests several dilutions of a sample in an assay. The titer is the highest dilution at which the sample is positive in the test. Results are reported as a dilution like 1/50 or 1/5000. Titering is a good method to determine the strength of a response, but each labs technique is different and titer values are not comparable. Also, the precision of repeated measurements for titering is generally considered within one dilution, or at least a 2-fold difference. When measuring precision, the coefficient of variation (cv) is most commonly calculated. In titering assays this value would be 100-200%.

Quantitative antibody testing can use a consensus standard so that different laboratory results can be compared using a common standard. The WHO has issued a consensus standard for COVID-19 serology and can be ordered by any laboratory. **The precision of quantitative antibody tests is 10 to 20 fold better with coefficient of variation (cv) of 15% or lower depending on the assay. The advantages of quantitative testing are important and necessary for a universally useful laboratory test to determine antibody levels to the SARS-CoV-2 antigens.**

Allermetrix COVID-19 Serology Tests

Allermetrix developed and is performing quantitative COVID-19 serology tests for IgG to the SARS-Cov-2 antigens, RBD, S1 subunit, and Nucleocapsid protein. Each of these 3 tests can be performed on serum or plasma (CPD). The WHO first international standard for anti-SARS-COV-2 immunoglobulin (human) 20/136 has been evaluated in the Allermetrix tests. The consensus value for the WHO standard is 1000 binding antibody units (BAU) per mL, and 250 IU of neutralizing antibody activity (NAA). Table 1 demonstrates how BAU/mL convert to ug/mL in the Allermetrix IgG assays.

IgG COVID-19 Serology Conversion Table

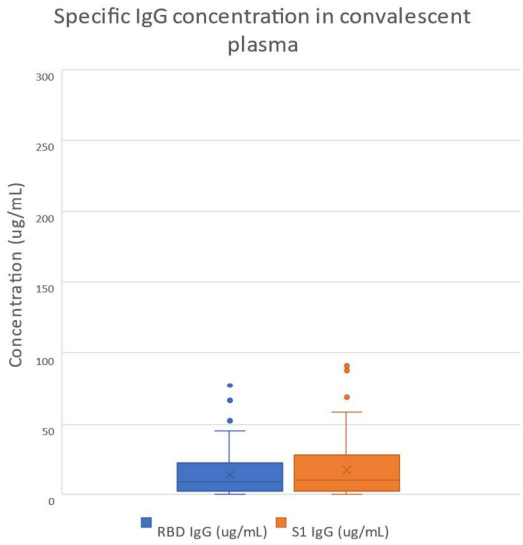
	ug/mL	CV	BAU/mL	BAU/ug
RBD	53.46	8.03%	1000	18.71
N Protein	139.56	9.11%	1000	7.17
S1 subunit	55.06	10.86%	1000	18.16

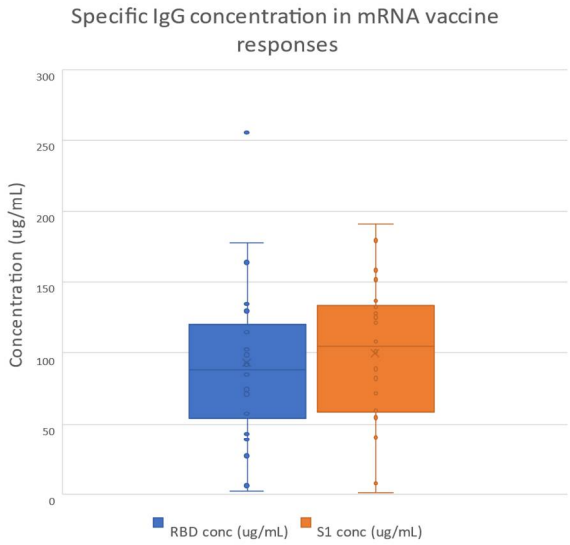
Antibody Concentrations in Recovered and Vaccinated People

Recovered COVID-19 patients have antibodies that react with viral antigens which protect them from subsequent infections. These convalescent antibodies are directed mainly at the spike and nucleocapsid proteins. Only the spike protein is used for vaccination. Antibodies from vaccinated individuals will only

react with the RBD and S1 proteins, except for those who have recovered from COVID-19 and been vaccinated, who will also have antibodies to the N Protein.

In a study looking at 96 convalescent plasma and 22 sera from individuals vaccinated with one of the mRNA vaccines the **levels of antibody were 10-fold higher against the RBD and S1 spike subunit in vaccinated individuals.**





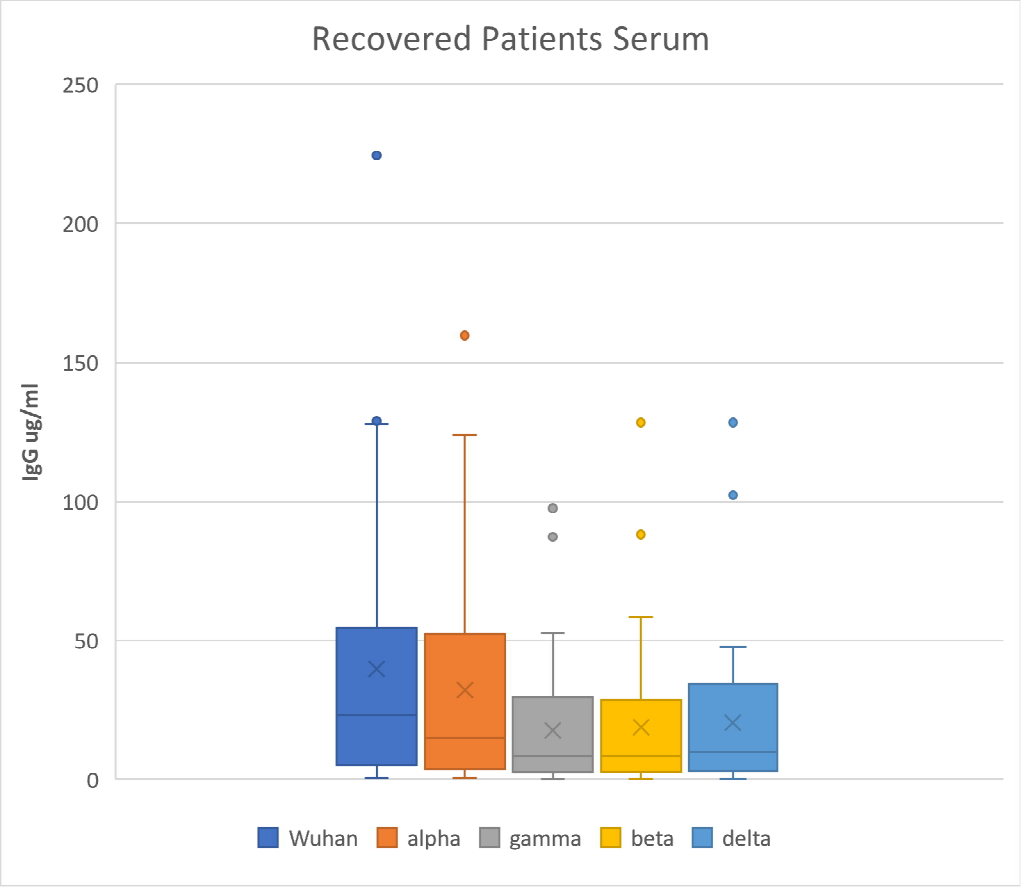
n=96	RBD	S1
Median IgG concentration (ug/mL)	8.90	10.13

n=22	RBD	S1
Median IgG (ug/mL)	88.55	104.6

SARS-CoV-2 Variants

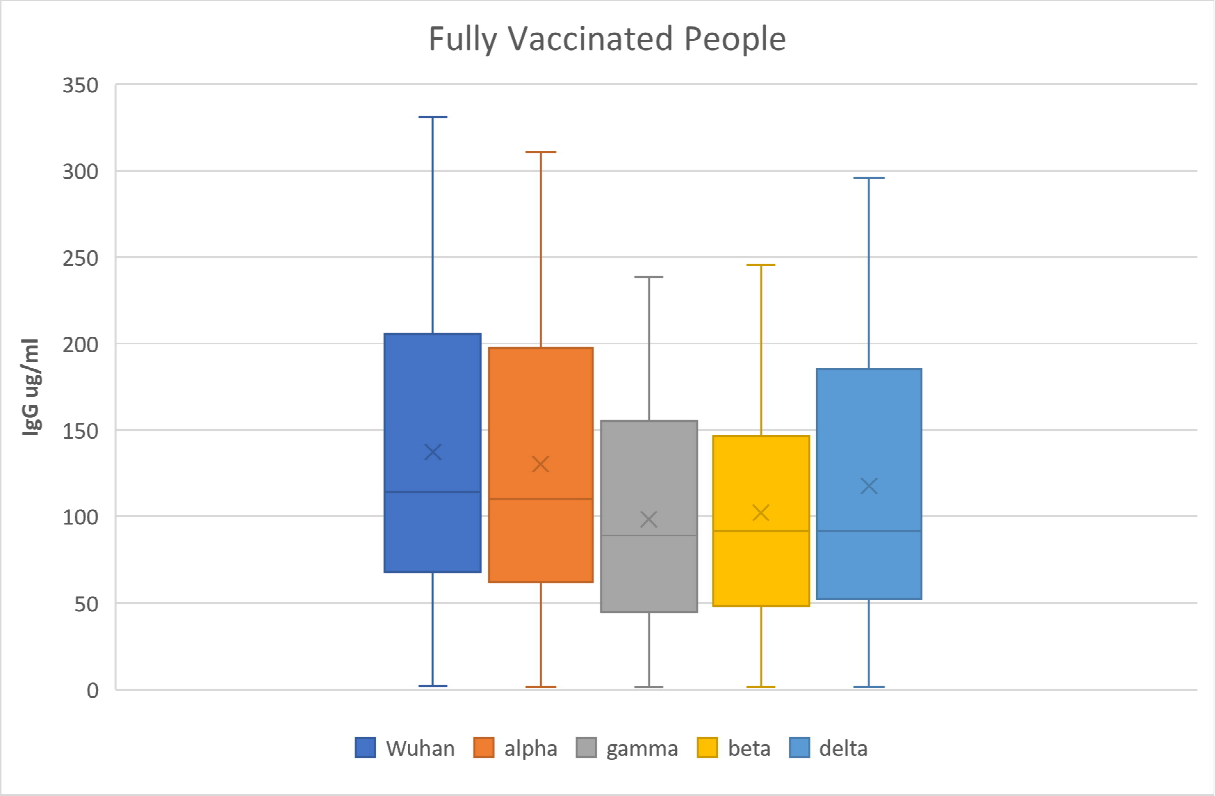
Many SARS-CoV-2 variants have been described. There are three classifications of variants, variants of interest, variants of concern, and variants of high consequence. There have been several variants of interest and currently there are four variants of concern because they are more infectious than the original Wuhan strain. At this time there are no variants that have been classified as having high consequence. Variants are changes in the viral RNA that result in changes in the proteins expressed by the virus or ability of the RNA to be replicated or transcribed into proteins. The changes in RNA or proteins may be detrimental to or enhance features that change the way the virus interacts with the host. Current variants of concern include the alpha, from the UK, beta, from South Africa, gamma, from Brazil and Japan, and delta, from India. Each of these variants have some change in their proteins that make them more infectious. Only the alpha variant has been associated with possibly more severe disease, the other three variants have not.

Allermetrix has the recombinant RBD antigens for all four of the variants. In studies with both recovered and vaccinated patients we have demonstrated antibody reactivity with all the variants. In the figure below.



n = 36	Wuhan	Alpha	Beta	Gamma	Delta
Median conc (ug/mL)	23.01	15.06	8.39	8.42	9.96
% of Wuhan antibody concentration	100%	65%	36%	37%	43%

Antibody concentrations were determined to the original Wuhan, Alpha, Beta, Gamma, and Delta RBD proteins. Median antibody levels are indicated by the solid line in each of the filled columns. The X indicates the mean value for the group. The percent of the Wuhan concentration is calculated by dividing the mean value for each of the variants by the Wuhan concentration.



n=19	Wuhan	alpha	beta	gamma	delta
Median conc (ug/mL)	114.42	110.32	91.71	89.24	91.58
% of Wuhan antibody concentration	100%	93%	75%	72%	84%

Antibody concentrations were determined to the original Wuhan, Alpha, Beta, Gamma, and Delta RBD proteins. Median antibody levels are indicated by the solid line in each of the filled columns. The X indicates the mean value for the group. The percent of the Wuhan concentration is calculated by dividing the mean value for each of the variants by the Wuhan concentration.

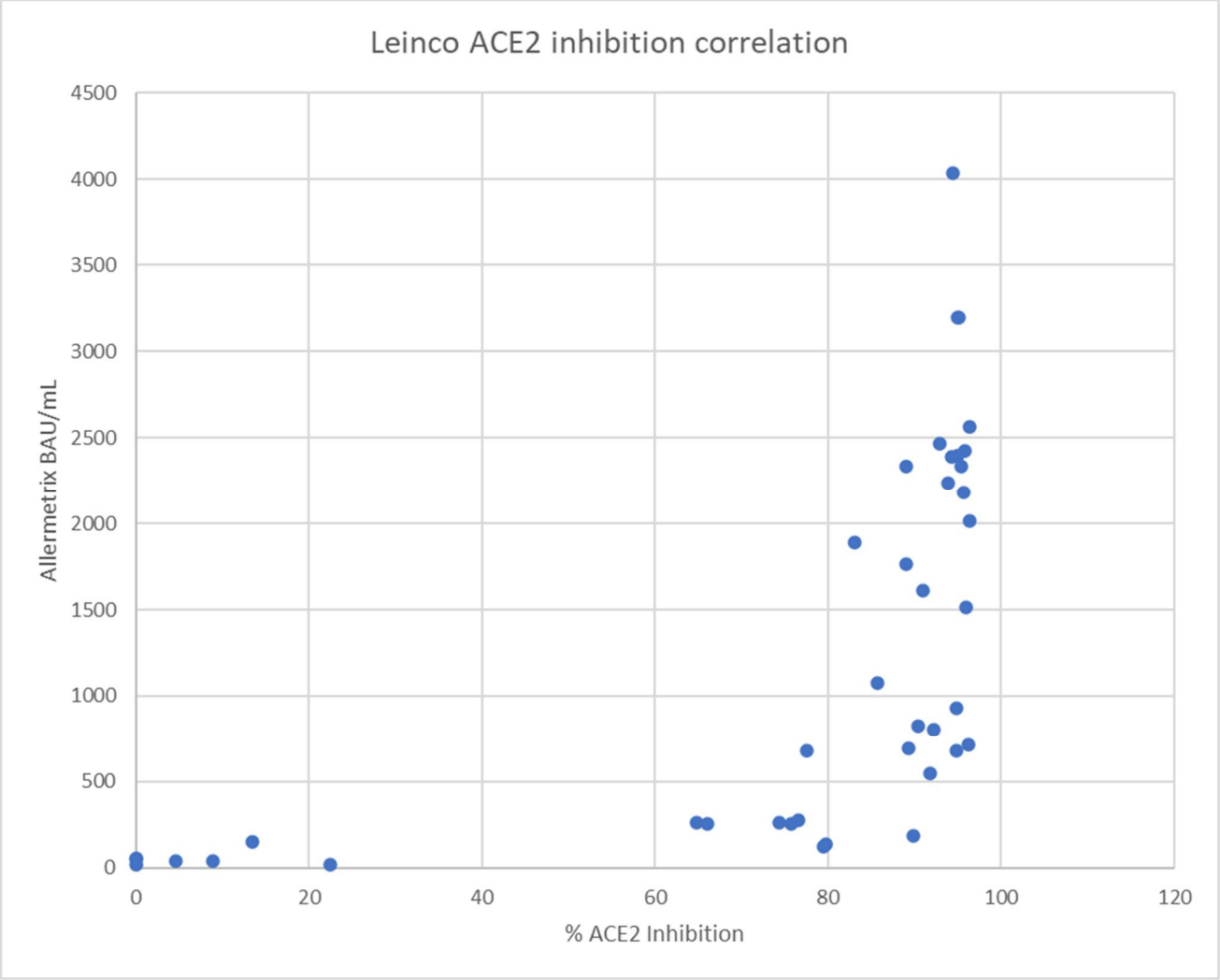
The antibody reactivity to the variants is higher to the variants for vaccinated people than for those who have recovered from COVID-19. This however does not indicate that recovered patients are more vulnerable to reinfection. In fact, studies have indicated that natural infection by SARS-CoV-2 may be more protective than vaccination. Longitudinal studies will help demonstrate how long protection from vaccine or natural infections will last.

Measures of Protection from Viral Infections using Antibody Measurements

There are no laboratory tests that can unequivocally demonstrate that any person is immune to viral infections. What researchers have done is to correlate antibody activity with the risk of being infected by a virus. These measures are called correlates of protection and have been determined for several bacterial and viral vaccines (Table 1). Although these levels have not been determined for COVID-19, there is evidence that anti-RBD antibodies can confer protection[i]

There are several test types that are used, and the most common ones are viral neutralization titer and ELISA. Viral neutralization titers are technically more complicated than ELISA and much more expensive. For COVID-19 several studies have demonstrated that IgG antibodies to the RBD correlate well with viral neutralization studies.[ii,iii,iv,v] As more studies are completed and published, correlates of protection will be determined for COVID-19 using IgG anti-RBD antibody levels. Another ELISA assay for COVID is the neutralizing antibody inhibition test. These assays also correlate with the quantitative IgG anti-RBD assay and may also become a correlate of protection for COVID-19.

Correlation of Allermetrix IgG anti-RBD to ACE2 receptor inhibition:



Forty-one sera, 14 from patients recovered from COVID-19 and 24 from known vaccinated individuals, and 3 of unknown status were assayed for IgG anti-RBD and inhibition of RBD binding to ACE2 using the Leinco assay.

Virus inhibition is important for blocking viral entry into the cell, but the human polyclonal response to the vaccines will also create specific antibodies to clear viruses and viral particles. These other antibodies can be directed to areas of the RBD that do not inhibit ACE2 binding or to other areas of the

spike protein. Measurement of IgG to the S1 subunit of the spike protein is also important to compare to the RBD antibody levels because the S1 antibodies may be more effective at clearing viruses in concert with other antibodies.

Conclusions

Not all COVID-19 serology tests are the same, and each can serve purposes in the clinical arena. However, only quantitative antibody tests have the promise of identifying a correlation to protection from infection[vi]. Serology correlates of protection (COP) have been published and show that both spike and RBD IgG are correlated with protection with AstraZeneca[v] . A similar preprint has been published for Moderna vaccines[vii]. Allermetrix quantitative serology tests measure the adaptive immune response to COVID-19 infections and vaccinations, which may determine the likelihood of being protected from infection as more research is published.

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