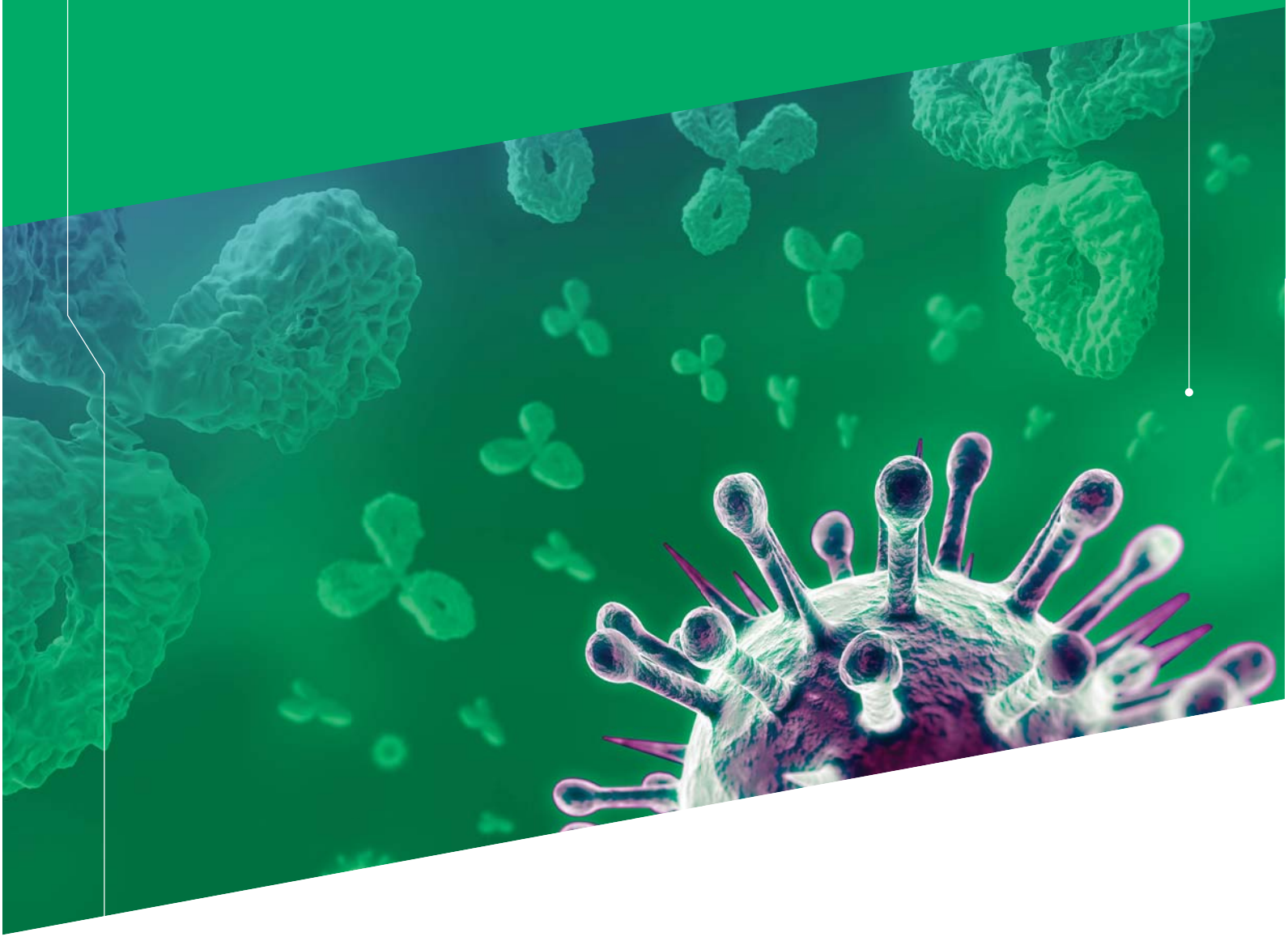
COVID-19 RESEARCH METHODS



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INTRODUCTION

The coronavirus disease 2019 (COVID-19) is caused by the highly contagious SARS-CoV-2 virus. [1] Some infected individuals are asymptomatic while others experience very severe symptoms that can result in death (Figure 1). [2] More specifically, those over 55 years old or have underlying conditions are especially vulnerable to developing serious illness. [3] To date, no effective treatment or vaccine exists to fight COVID-19. In this eBook, the different research tools to detect and study COVID-19 infection – from single protein interactions to systemic host responses – are discussed. The SARS-CoV-2 life cycle is also briefly reviewed. For a general overview of COVID-19, please read our blog, COVID-19 Biology 101.

Figure 1. Fever is a common symptom of COVID-19. Non-contact infrared thermometers are often utilized in screening patients for COVID-19.

THE SARS-COV-2 LIFE CYCLE

Like other coronaviruses, the genome of SARS-CoV-2 contains ten or more open reading frames (ORFs) encoding some 29 proteins. Roughly two thirds of the viral RNA are contained within the first two, called ORF1a and ORF1b. [4] These are translated into two large non-structural polypeptides (NSPS) that are then cleaved into multiple smaller NSPS. [5] Based on the functions of their SARS-CoV and MERS-CoV homologs, the NSPS are thought to hijack the membrane structures of the host rough endoplasmic reticulum, rearranging them into double-membrane vesicles (DMVs) wherein viral transcription takes place. [6] The other one-third of the viral genome contains ORFs for the 4 principal structural proteins: spike (S-protein), nucleocapsid (N-protein), envelope (E-protein), and membrane (M-protein), along with several accessory proteins with as yet unclear functions (Figure 2).

The S-protein represents the key to viral entry into the host cell. First, the virus's S-protein's receptor binding domain (RBD) on the S1 domain attaches to the cell surface protein angiotensin-converting enzyme (ACE-2). Subsequently, the S-protein's S2 domain engages the type II transmembrane protease (TMPRSS2) to accomplish a crucial cleavage step known as priming, which allows fusion of the S-protein with the cell membrane. [7]

Inside the cell, the virus converts intracellular membrane structures into DMVs which serve as factories for viral RNA replication, transcription, and virus particle assembly. [8] As the viral particles are built, nascent N-proteins insert into the host membrane, forming a nucleocapsid structure. Finally, the virus particle-containing vesicles fuse with the plasma membrane and mature virions are released. [4]

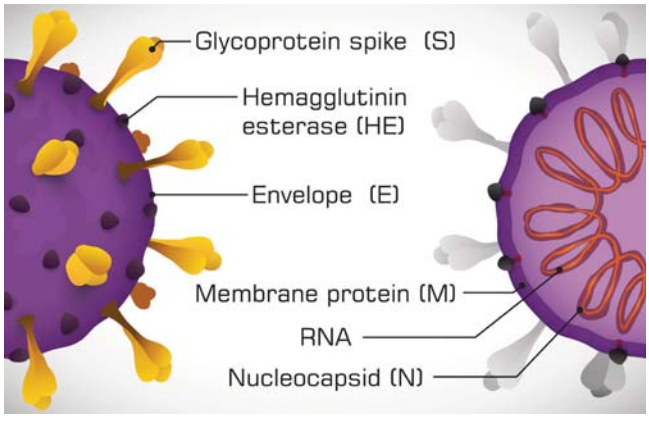


Figure 2. Structure of the SARS-CoV-2 virus

REAGENTS

Recombinant Proteins

Due to their critical roles in viral entry, the S-protein, ACE2, and TMPRSS2 proteins are of particular interest to the COVID-19 research community. [8] The N-protein, which encapsulates the viral RNA, is also studied often because it assists in viral assembly, RNA synthesis and folding, and virus budding. Moreover, it affects host cell responses, including cell cycle and translation. [9]

As described below, SARS-CoV-2 proteins and human proteins are utilized in different assays. For example, these proteins may be used to detect COVID-19 antibodies or as standards to quantify unknown protein levels in samples. They could also serve as positive control samples in western blots or, possibly, used in cell culture experiments. Although the proteins could be isolated directly from the source, producing "recombinant" proteins *in vitro* using synthetic genes and cells is often preferred due to its affordability, flexibility, and ability to produce large quantities. Another potential advantage of making proteins recombinantly is that the researcher does not need to work with samples containing live virus.

RayBiotech has synthesized recombinant N-protein, S-protein S1 subunit, S-protein RBD, S-protein S2 subunit, and human ACE2 in different expression systems, fusion tags, and formats (i.e., purified, unpurified), providing flexible options depending on the study's objective and budget. [10] Proteins expressed in E. coli are cheaper, whereas human HEK293 cells are more likely to have native conformations with post translational modifications. [11, 12] Fusion tags can be used for protein detection and purification. For protein interaction experiments, a small histidine tag (~1 kDa) that is unlikely to block binding epitopes would be more appropriate than the Fc tag (~25 kDa). Unpurified proteins are an excellent, affordable option as western blot controls. For a more in-depth review of how recombinant proteins are produced and employed in various proteomics applications, please read our blog, "Recombinant Proteins in Research."

Notably, both we and others have observed that expression of the full-length S-protein is extremely poor, even in human HEK293 cells. [13] To address this issue, only specific domains or regions of the S-protein are expressed (e.g., S1 domain, S2 domain, RBD) to improve expression. In addition, some commercial manufacturers and researchers do not rely on recombinant expression at all, but chemically-synthesize short peptides (~15 amino acids) representing the S-protein.

Recombinant Proteins (continued)

Proteins and peptides that are produced either in E. coli or chemically, respectively, will not have post translational modifications (PTMs) or native conformations; these proteins may not provide accurate data in functional studies. For example, an analysis of the S-ACE2 crystal structure by Shang et al. suggested that specific glycan moieties may also assist in S-ACE complex formation. [14] Thus, proteins expressed in human HEK293 cells, which have the ability to glycosylate and fold the proteins in their native structure, may be more appropriate for functional research (Figure 3). [10]

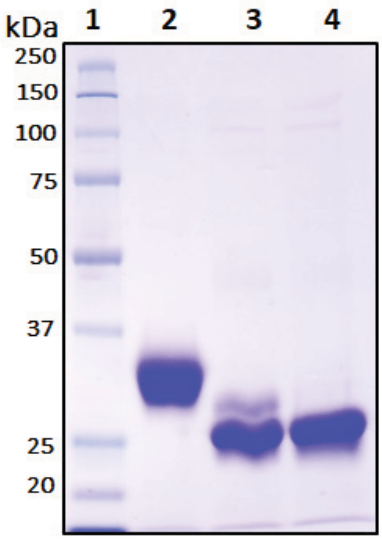


Figure 3. HEK293 cells can glycosylate recombinant proteins. Purified SARS-CoV-2 S-protein RBD proteins were untreated (lane 2) or deglycosylated under native (lane 3) or reducing (lane 4) conditions. Deglycosylation resulted in a mobility shift to its expected size (25 kDa) compared to the protein standard ladder (lane 1).

Antibodies

Antibodies, or immunoglobulins (Ig), are proteins produced by B cells in the adaptive immune system to enable the specific detection of invading pathogens, or antigens. [15] They have a "Y"-like structure where each "Y" unit is comprised of two small "light" chains and two large "heavy" chains that are linked together through disulfide chains (Figure 4). The stem of the "Y" is referred to as the constant region, which is similar within an antibody isotype (i.e., IgA, IgD, IgE, IgG, IgM isotypes) and confers different biological functions; for example, which immune cells will be stimulated when the antibody binds to its antigen. Antibody isotypes also differ based on the number of conjoined "Y" units, binding affinity, functional location, and half-life. The two arms of the "Y" are highly variable from one antibody to the next and confer specificity to an antigen.

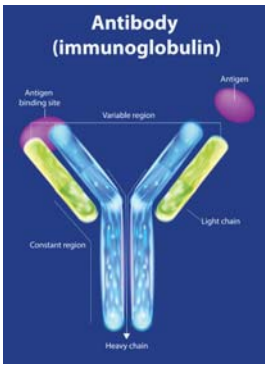


Figure 4. Antibody structure

Antibodies are a common reagent in research assays because they bind to a specific antigen with high affinity. To generate antibodies, the antigens – either full-length proteins or selected peptide sequences – must be produced. Chemically-synthesized peptides can be produced within a few weeks, but as mentioned above, they will not have tertiary structure or PTMs and may not be immunogenic enough to stimulate antibody production. While recombinant proteins don't have these disadvantages, the turnaround time from gene synthesis to purified protein is at least two months. Animals are then inoculated with the target several times over weeks to months to stimulate antibody production. Polyclonal antibodies comprised of a mixture of antibodies that bind to different epitopes on the same target can be obtained within 3 months from the first inoculation. Monoclonal antibodies, which bind to only one epitope, take at least 5 months to produce. Antibodies can also be recombinantly expressed using synthetic genes and cells. [16] These recombinant antibodies represent the variable region and, as such, the sequence of the variable region must be known.

Antibodies (continued)

Following the initial outbreak of COVID-19, no antibodies specific to SARS-CoV-2 were available. Thus, researchers used antibodies to SARS-CoV, the virus responsible for the SARS outbreak in 2003, because SARS-CoV has ~65% homology with SARS-CoV-2. [17, 18] These antibodies, therefore, had to bind to sequences shared by both SARS-CoV and SARS-CoV-2. Importantly, antibody binding may still be limited by differences in tertiary structure between the two viruses. Also, mutated regions specific to SARS-CoV-2 that may responsible for the virus's unique pathology cannot be targeted.

In response to the COVID-19 pandemic, RayBiotech has produced rabbit polyclonal antibodies to the SARS-CoV-2 S-protein's RBD (cat no. 130-10759, 130-10784), N- protein (cat no. 130-10760, 130-10785), and S-protein's S2 subunit (cat no. 130-10761) (Figure 5). Mouse monoclonal antibodies are also available for the SARS-CoV-2 N-protein (cat no. 130-10779, 130-10780, 130-10781, 130-10782), and the S-protein's RBD (130-10798), with S2 subunit coming soon.

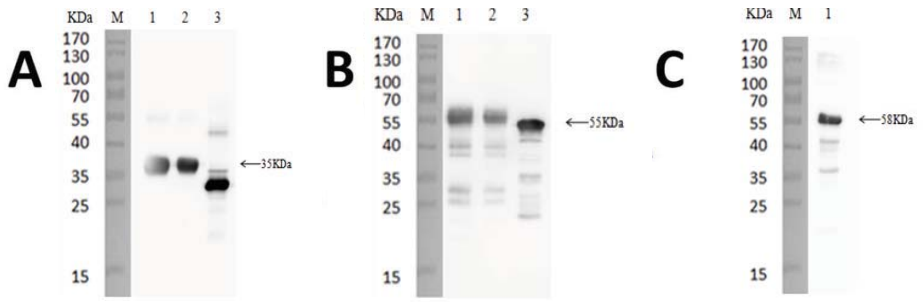


Figure 5. Western blot analysis of rabbit polyclonal antibodies specific to the SARS-CoV-2 proteins. (A) S-protein RBD, (B) N-protein, and (C) S2 subunit using recombinant proteins expressed in human HEK293 cells (lanes 1 – 2) and E. coli (lane 3). M = marker; protein standard ladder

Aptamers

Aptamers are single-stranded nucleic acid-based affinity reagents that are ~70 nucleic acids in length (Figure 6). [19] Aptamers can bind proteins, peptides, viruses, and small molecules, although the turnaround time for these targets is longer. [20, 21] Advantages of aptamers compared to antibodies include smaller size, no lot-to-lot variability, higher specificity, does not need as much antigen (100 µg protein versus > 3 mg protein), and more affordable to produce. Aptamer identification ranges from 3 months to 7 months, such that smaller targets have a longer turnaround time. A primary disadvantage of aptamers compared to antibodies is that their binding affinity is generally lower.

In an experiment by Lee and Zeng, a DNA aptamer specific to the Zika virus was immobilized on a plate and used to capture the virus, while a second "reporter" aptamer targeting a different epitope of the Zika virus was added in a subsequent step. [22] Using this sandwich approach (i.e., aptamer-virus-aptamer), Lee et al. was able to detect 1 ng/ml of Zika virus in human serum.

Target detection using aptamer-based assays can be colorimetric, fluorescent, chemiluminescent, electrical, or light-dependent (e.g., surface plasmon resonance, interferometry). A more thorough review of the use of aptamers in detecting and treating viruses is provided by Zou et al.'s article "Applications of Aptamers in Virus Detection and Antiviral Therapy". [23] Learn more about RayBiotech's custom aptamer service here.

For more information about aptamers and how they compare to antibodies, please see our blog, "Aptamer Selection & Development."

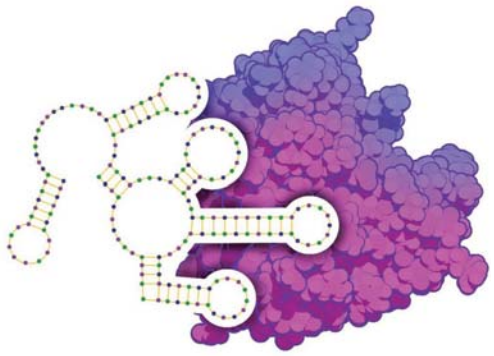
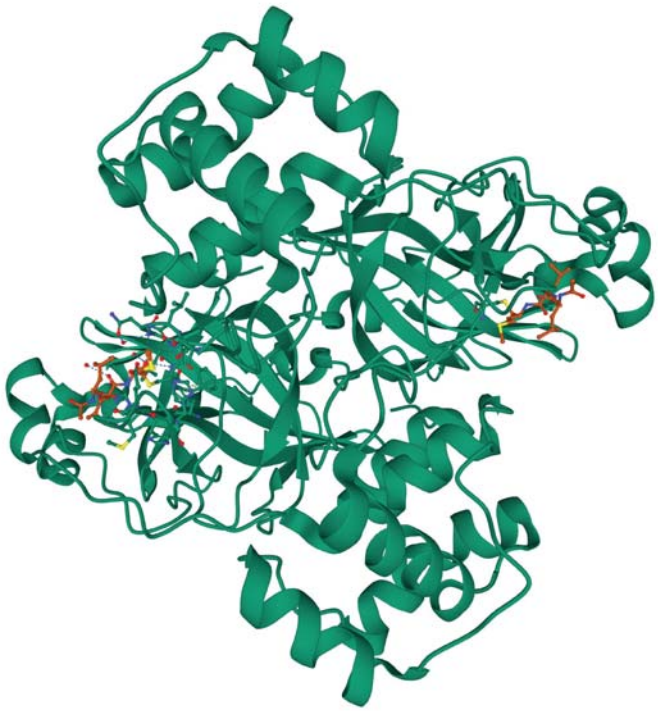


Figure 6. A schematic depicting a DNA aptamer adopting a tertiary structure to bind specifically to a protein

Small Molecules

Small molecules are natural or artificial organic compounds that are < 900 daltons, which bind to and affect the function of specific biological macromolecules. [24, 25] Small molecules that can impede the SARS-CoV-2 life cycle is a major focus of COVID-19 research. For instance, small molecules that inhibit the interaction between the SARS-CoV-2 S-protein and the human ACE2 receptor could be used to treat COVID-19 (see "Binding Assay" for screening potential inhibitors). RayBiotech offers a large catalog of over 5,000 high-quality small molecules, some of which are known inhibitors of different viruses. For instance, Lopinavir is a potent HIV protease inhibitor, but is also known to inhibit the coronavirus main protease 3CLpro (cat no. 331-11643). Chloroquine diphosphate (cat no. 331-11962) and hydroxychloroquine sulfate (cat no. 331-21357) inhibit malaria species from binding to the ACE2 receptor. In addition to the small molecule inhibitors of viruses that may work with SARS-CoV-2, RayBiotech has small molecule libraries for high throughput screening.



Patient Blood

Blood samples from COVID-19 patients are important in both assay and therapy development. These samples include dried blood spots or cell-free blood fluid, which includes serum (without coagulation factors) and plasma (with an anti-coagulant like EDTA [ethylenediaminetetraacetic acid], heparin, or citrate) (Figure 7). First, they can be used to test and validate antibody-based assays for research and *in vitro* diagnostics. These results can then be compared to samples collected from patients without COVID-19 (i.e., negative controls). Importantly, viral RNA cannot be detected in these sample types (see "Viral RNA Detection"). Second, plasma from COVID-19 patients that contain neutralizing antibodies – or antibodies that inhibit viral infection – may also prove to be effective convalescent plasma therapy (i.e., plasma transfusion) to treat COVID-19. [26, 27] Such therapy is considered investigational. There are two major limitations to using patient plasma to treat COVID-19 patients: 1) medical complications can occur and 2) there is limited supply since a person can only donate so much plasma. However, the identification of the neutralizing antibodies and their specific binding epitopes may help direct scaled-up monoclonal or recombinant antibody production to enable widespread treatment.

Figure 7. Serum and plasma from COVID-19 patients can be used to develop assays and treatments

Serum and EDTA-treated plasma from patients with and without confirmed COVID-19 infection are available from RayBiotech. Normal samples were either collected before the COVID-19 outbreak or were confirmed to not have COVID-19 based on patient exposure history, clinical symptoms, and antibody serology tests (cat no. NEGSMPL). Over 100 COVID-19 patients confirmed via viral RNA detection and clinical symptoms are also available to choose from. These include patients with varying levels of IgM and IgG to SARS-CoV-2 proteins (cat no. CoV-PosPCR) and patients with high titer IgM, IgA, or IgG antibodies (cat no. CoV-PosM, CoV-PosA, CoV-PosG). Sample sets from 10 normal samples and 20 COVID-19 patients are also available for researchers who want to analyze serum or plasma from numerous patients (cat no. CoV-PosSet).

VIRAL RNA DETECTION

qRT-PCR

Direct detection of virus particles in a patient is usually accomplished by quantitative polymerase chain reaction (qPCR). [28] In brief, the basic PCR method quickly amplifies segments of DNA through an iterative process of building new strands of DNA from the original DNA template. Amplification of a specific target, such as the SARS-CoV-2 virus, is accomplished by using two short DNA sequences called "primers" that bind to and flank a specific gene region. These primers essentially act as initiation points for amplification. This cumulative synthesis is accomplished through alternating cycles of heating and cooling in the presence of DNA polymerase, deoxynucleoside triphosphates (dNTPs), and primers, where the DNA strands melt at the high temperature and "anneal and extend" at low temperature. The exponential template amplification combined with the high polymerase efficiency enables over a billion copies to be synthesized in 30-40 cycles (a few hours) (Figure 8). For RNA-based viruses, an initial reverse transcription (RT) step to transcribe the RNA into a DNA template for PCR amplification is required.

To obtain quantitative data (i.e., number of viral copies within a sample), a fluorescent oligonucleotide probe is added into a basic PCR reaction. The probe is designed to hybridize within the target sequence and is subsequently cleaved by the 5′ nuclease activity of the polymerase. After cleavage, the fluorophore emits a detectable light signal due to release from a quencher. The entire reaction is carried out in a thermal cycler equipped with a fluorescent detector, allowing both amplification and signal detection to occur simultaneously. High throughput analyses are possible with 96-well and 384-well plates (Figure 9).

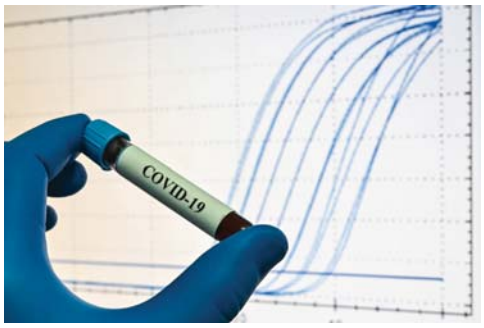


Figure 8. qRT-PCR is used to detect the SARS-CoV-2 virus that is responsible for COVID-19 infection. This method targets and amplifies specific regions of the SARS-CoV-2 RNA genome for quantitative analysis.

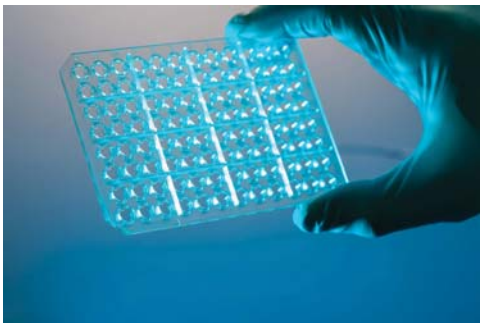


Figure 9. RT-qPCR can be high throughput with 96-well plates. 384-well plates can also be used, but are not as common.

qRT-PCR (continued)

The SARS-CoV-2 RNA is present in appreciable amounts in the upper and lower respiratory tract during infection. For this reason, pharyngeal swabs and bronchoalveolar lavage fluid (BALF) specimens are typically used for diagnosis of COVID-19. One of the first COVID-19 PCR-based diagnostic test kits was developed by the U.S. Centers for Disease Control (CDC). [29] The test includes primer-probe sets targeting the N1 and N2 regions of the N-protein, as well as human ribonuclease P (hRNP), which serves as an internal amplification control. On March 15, 2020, the CDC received an Emergency Use Authorization (EUA) from the U.S. Food and Drug Administration (FDA), permitting the use of this test for COVID-19 diagnostics.

The need for COVID-19 testing is expected to continue throughout 2020, underscoring the importance of accurate, reliable testing. To help meet the demand, RayBiotech has developed two different COVID-19 Real Time RT-PCR Nucleic Acid Detection Kits. One kit (cat no. PCR-COV) conforms to the specifications of the CDC's FDA-authorized test, and can be performed in a high-complexity CLIA laboratory with a fluorescence qPCR instrument capable of reading the FAM or equivalent channel. The second kit is a high-throughput version (cat no. PCR-COVHT) which contains a primer-probe panel targeting the ORF-1a/b, N-protein, and hRNP (Figure 10). This kit has 2 special features: 1) the primer-probe panel is highly specific to SARS-CoV-2, with no expected false positives from other coronaviruses or human microflora, and 2) the primer-probe panel is provided as a single mixture, requiring only 1 well to analyze both viral targets and RNP. Thus, one 96-well plate can accommodate up to 94 samples, a negative control, and a positive control, compared to CDC-conforming kit (PCR-COV), which allows 32 samples per plate (Figure 8). This format enables higher testing capacity at a lower cost.

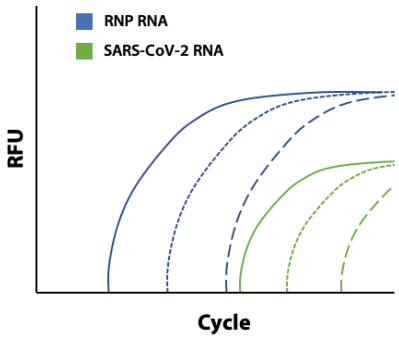


Figure 10. Multiplex detection of SARS-CoV-2 and human genes by qRT-PCR. Dashed and solid lines represent varying concentrations of RNA. RNase P (RNP) detected in JOE channel (533 nm); SARS detected in FAM channel (494 nm). RFU = relative fluorescent units.

Isothermal RNA Amplification

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a type of PCR that amplifies RNA without the need for thermal cycling. [30] This is accomplished using specialized primers that enable continuous amplification of nucleic acids at 60°C. RT-LAMP is faster (< 1 hour) than traditional qRT-PCR with a colorimetric endpoint readout (Figure 11). The color of the PCR solution is dependent on pH. As more DNA is amplified, the solution will turn from pink to yellow. Thus, detection of viral RNA can be performed by eye. The results can also be measured using a plate reader capable of measuring absorbance at 440 nm (yellow) and 560 nm (pink). A higher optical density (OD) at 440 nm compared to the negative control therefore indicates that viral RNA is present in the sample. The main advantages of using RT-LAMP over traditional RT-PCR are that it is easy to perform and only requires a heat block. It is also highly specific because it amplifies multiple regions of the same gene.

The RT-LAMP approach was first developed in 2000 by Notomi et al. [30] Since then, it has been used to detect a wide array of viruses, including the Zika virus, Sorghum mosaic virus, Sugarcane mosaic virus, Ebola virus, West Nile virus, and HIV. [31 – 35] RT-LAMP assays have also been developed to detect SARS-CoV-2. The assay from RayBiotech (cat no. RT-LAMP) targets 6 different sequences on the nucleocapsid gene that are unique to SARS-CoV-2. In addition, as few as 250 viral copies can be detected in as little as 20 minutes.

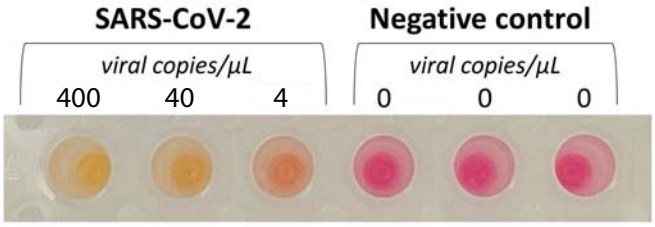


Figure 11. Colorimetric detection of SARS-CoV-2 RNA using RT-LAMP. The presence of SARS-CoV-2 viral RNA results in a color change from pink to yellow. The intensity level in the yellow spectrum is proportional to the amount of viral RNA present in the sample.

ANTIBODY DETECTION

Rapid Antibody Tests

Current testing for the SARS-CoV-2 virus is limited in throughput and sampling time. However, results using lateral flow technology – the same type of technology used for home pregnancy tests – can be obtained within 10 minutes after sampling. Such devices are largely utilized in the field to detect common viral infections, either by measuring viral antigens or anti-viral antibodies. [36, 37] For example, this technology is widely utilized for point-of-care to diagnose influenza type A and B from nasal swabs. [38 – 40] Lateral flow kits against COVID-19 have been developed and are either serology based (detecting host antibodies), or in some cases, antigen based (detecting specific viral proteins).

For antibody detection, biological fluid (often serum or finger prick blood) is added to the sample pad (Figure 12). The sample pad includes two detection antibodies: one antibody specific to a control antigen and one antibody that binds to human antibodies. The sample, along with the detection antibodies, will migrate across the test window, onto which a positive control antigen (PCA) and one or more SARS-CoV-2 antigens are immobilized as a "positive line" and "test line(s)," respectively. The presence of a control line representing the interaction between the PCA-PCA antibody should appear during every analysis. A "test line" will also be visual should antibodies in the samples be present to the SARS-CoV-2 antigen(s). In addition to producing rapid results, the test is easy to perform and does not require an instrument! Different antibody isotypes can be detected using lateral flow technology.

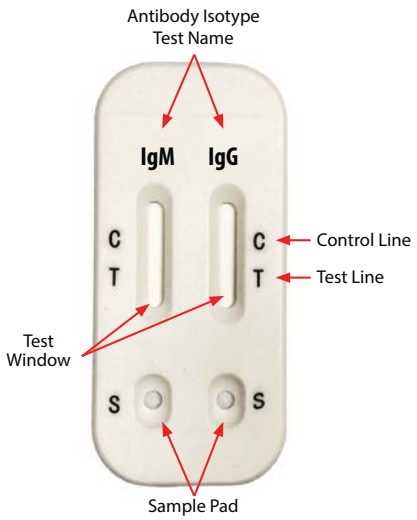


Figure 12. The different part of lateral flow technology uses to detect COVID-19 IgM and IgG antibodies

Rapid Antibody Tests (continued)

Serology measurements rely entirely on a host's antibody response to the virus rather than detecting the virus itself. This results in delayed timing for antibody detection since antibodies are generally detectable 3 – 10 days post infection whereas the virus can be detectable usually within 1-3 days post infection. [41 – 43] Although relying on the host immune response is a disadvantage of this technology, there are several advantages to using this approach. First, antibody samples can be collected from numerous places in and around the body. Tests that detect pathogens directly must take samples directly from the pathogen's location. Second, antibodies to specific pathogens can be detected long after the patient has recovered, thus enabling retrospective evaluations of infected individuals after viral clearance. In comparison, assays that detect the virus or viral antigens are only relevant during active (or acute) infection. Finally, lateral flow tests offer a rapid and affordable method to screen populations without any special equipment.

As with any *in vitro* diagnostics test, false negatives and positives can occur (Tables 1 – 2). False negatives often occur when the antibody levels are below the detection limit of the rapid test, either because the timing of the test missed the appropriate "window" for detection (i.e., taken too early or too late) or the individual has not developed antibodies to the specific SARS-CoV-2 antigens immobilized on the strip. False positives can happen if the individual was infected with another virus and developed antibodies to regions that were homologous to the SARS-CoV-2 antigens immobilized on the lateral flow device.

From a research perspective, large-scale population COVID-19 screens are incredibly lacking, as initial studies have already shown that more people have been infected than been reported. For example, a small study in California that measured antibody levels to SARS-CoV-2 antigens estimated that there were likely 28-55 times more infected people than the number of confirmed cases in the state. [44] Such data are important in evaluating the spread of COVID-19, the true number of infected individuals, viral severity, and associated death rates. Moreover, exposure studies, either through actual infection or potential vaccines, will provide valuable information about COVID-19. This could be accomplished by evaluating host immune responses to the virus in large population groups. For example, titering viral neutralizing antibodies in patient samples (e.g., serum).

RayBiotech offers rapid testing of IgM and IgG antibodies to SARS-CoV-2 in serum, whole blood, and finger prick blood (cat no. CG-CoV-IgM/IgG). For finger prick samples, a lancet set (lancets, alcohol swabs, adhesive bandages) can also be included with the COVID-19 IgM/IgG rapid test (cat. CG-CoV-IgM/IgG-FP).

Rapid Antibody Tests (continued)

Test Results			Clinical significance
PCR*	IgM**	IgG**	
-	-	-	Patient does not have COVID-19
-	-	+	Patient may have had COVID-19, but the virus has cleared. Patient has likely gained immunity to COVID-19.
-	+	-	Patient is in the acute phase of COVID-19 and contagious (false negative PCR results)
-	+	+	Patient has COVID-19 and is contagious (false negative PCR results) or patient is in the recovery phase of COVID-19
+	-	-	Patient has COVID-19, but antibody levels are below the detection limit
+	-	+	Patient may be in late or recurrent stage of infection
+	+	-	Patient is in the acute phase of COVID-19 and contagious
+	+	+	Patient has COVID-19 and is contagious

Table 1. Clinical significance of qRT-PCR and antibody serology tests

Possible reason	Explanation
Assay not performed properly	Refer to the manufacturer's instruction manual. Running the test differently than what is outlined in the manual can result in inaccurate data.
Antibody level below detection limit	• IgM and IgG levels change over time, and their profiles in response to COVID-19 are still being ascertained. For example, a false negative result with the IgM rapid test may due to the IgM phase ending (as the IgG levels increase). • Individual IgM and IgG responses will vary. To increase accuracy, both IgM and IgG analyses should be performed.
Antibodies not present *(false negative)*	• The patient may not have generated antibodies against the SARS-CoV-2 antigen used in the test. • SARS-CoV-2 virus is mutating. Patients may have antibodies to a mutated region of SARS-CoV-2 antigen.
Antibody crossreactivity *(false positive)*	The patient infected with another virus may have developed antibodies to homologous sequences shared between that virus and SARS-CoV-2.
Unknown cause	In vitro diagnostics tests are not accurate 100% of the time. Sometimes the reasons for false results are unclear.
Test not working properly	The test is working properly if the control line is present.

Table 2. Possible causes for false positives and negatives with rapid antibody serology tests using lateral flow technology

Indirect ELISA

Indirect enzyme-linked immunosorbent assays (ELISAs) enable the semi-quantitative measurement of antibodies in biological fluids *in vitro*. These tests employ immobilized antigens coated on a 96-well plate, which are bound by their specific "primary" antibody during sample incubation. After washing unbound sample from the plate, antigen-antibody complexes are detected using a secondary anti-human antibody conjugated to horse radish peroxidase (HRP). In the presence of 3,3',5,5'-tetramethylbenzidine (TMB) substrate, the HRP produces a blue color that is proportional to the amount of bound antibody. The HRP-TMB reaction is halted with the addition of sulfuric acid, resulting in a blue-to-yellow color change. The intensity of the yellow color that is proportional to the amount of bound antibody is then measured at 450 nm. (Figure 13). This method has high sensitivity since multiple secondary antibodies can bind to a single primary antibody. [45]

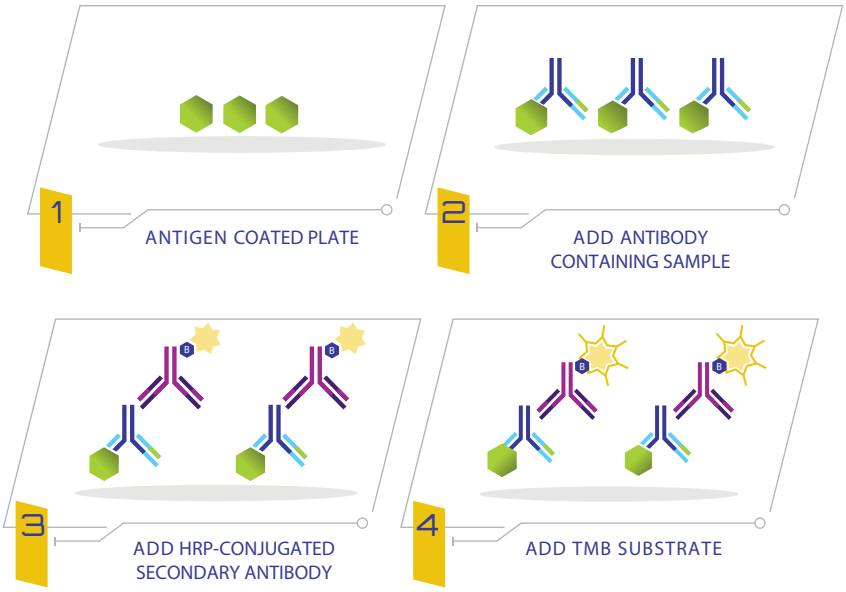


Figure 13. Antibody detection using indirect ELISA

Indirect ELISA (continued)

Compared to qRT-PCR, indirect ELISA is a less complex procedure that uses testing equipment that is often more affordable and available than a qPCR instrument (i.e, plate reader). Antibody-based testing with indirect ELISAs has been used to 1) diagnose patients who are more than 1 week post symptom onset, 2) determine potential immunity and risk of infection, 3) advance contact tracing, and 4) understand the extent of COVID19 spread and immunity in communities through epidemiological studies. [46 – 48] These epidemiological studies are particularly important for fighting COVID-19 while minimizing economic impact. For example, public health interventions that help reduce the transmission rates of COVID-19 (e.g., school and business closures, limiting sizes of gatherings and restricting movement, physical distancing) have had severe social and economic costs.

In response to the COVID-19 pandemic, indirect ELISAs for the detection of COVID-19 antibodies have been developed. With the different antibody serology tests on the market, a number of studies assessing their performances have been conducted. In one example, a meta-analysis on antibody-based tests for COVID19 investigated their accuracies in diagnosing COVID-19. [48] Using data from 38 studies and 7848 individuals, all methods were shown to have high specificity, with some ELISA-based assays reaching levels around 99%. In addition, ELISA-based methods had higher sensitivity (90%–94%) than other types of assays, suggesting that ELISA tests are a good option for COVID- 19 assessment. The positive rate for IgG and IgM antibody detection was < 60% within 10 days post symptom onset; this percentage increased thereafter. [49] Notably, the positive rate for IgM detection significantly decreased 35 days post symptom onset. These data suggest that the antibody isotype and timing should be carefully considered to optimize the diagnostic accuracy.

In addition to diagnostic uses, indirect ELISAs have helped understand the immune system's response to COVID-19 and used in drug development. Zhang et al. used an indirect ELISA to measure IgA and IgG antibodies to assess antibody kinetics of COVID-19 among asymptomatic carriers who can still transmit the disease. [50] This information is invaluable since asymptomatic people are rarely tested for COVID-19, yet they contribute to the transmission of COVID-19. This subset of infected people makes it extremely difficult to control the spread of COVID-19. Similar to previous reports, the authors of this study found that while asymptomatic, pre-symptomatic, and symptomatic patients all showed a rapid increase in IgG within seven days of symptom onset.

Indirect ELISA (continued)

However, asymptomatic patients were characterized by low levels of IgM, but high levels of IgG. These noted differences in sub-populations of COVID-19 patients could have important implications for control strategies. Indirect ELISAs are also perfectly suited to determine neutralizing antibody titers in plasma samples for convalescent plasma therapy in a high throughput and cost-effective manner (see "Patient Blood"). In a study by Harvala et al., virus neutralizing antibody titers and reactivity in several ELISA-based antibody tests had robust associations. [51] Their study demonstrated the possibility of scaling up production of convalescent plasma containing potentially therapeutic levels of anti-SARS-CoV-2 neutralizing antibodies in a cost-effective and timely manner.

As explained above, indirect ELISAs are important in COVID-19 diagnosis, understanding the immune system's response to COVID-19, and identifying plasma samples that may be used to treat other patients. RayBiotech has developed indirect ELISAs to detect IgA, IgG, and IgM antibodies specific to the SARS-CoV-2 N-protein (cat no. IEQ-CoVN-IgA, IEQ-CoVN-IgG, IEQ-CoVN-IgM) and S-protein RBD (cat no. IEQ-CoVS-IgA, IEQ-CoVS-IgG, IEQ-CoVS-IgM).

Protein Array

Multiplex antibody detection or epitope mapping can be achieved using protein arrays. Here, proteins or peptides are immobilized on a solid substrate, which is often a glass slide (Figure 14). For COVID-19 research, patient samples (e.g., serum) or a purified antibody can be incubated with the protein array, during which "primary" antibodies will bind to their antigens. Patient sample analyses can help shed light on the immune response and which proteins or protein regions are the most immunogenic. [18] Protein arrays can also be used as an epitope mapping assay, such that the binding location of purified antibodies can ben ascertained when peptide fragments are immobilized. Information obtained from protein arrays can direct the development of treatments and more accurate antibody tests. RayBiotech has developed a COVID-19 protein array for the semi-quantitative detection of IgM and IgG antibodies in serum or plasma to the SARS-CoV-2 S-protein (cat no. PAH-SASP-G1). This array contains three SARS-CoV-2 proteins (S-protein RBD, S-protein S2 subunit, full-length N-protein), 11 peptides representing unique sequences of the SARS-CoV-2 S-protein, and 3 peptides with homologous regions shared between SARS-CoV-2 and SARS-CoV-2 S-proteins.

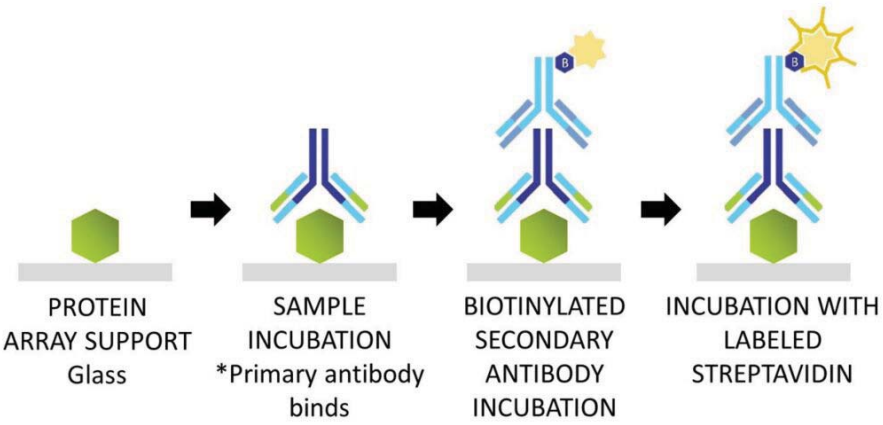


Figure 14. General schematic of the steps involved in processing a protein array

PROTEIN DETECTION

Sandwich ELISA

Sandwich-based ELISAs enable the quantitative measurement of soluble proteins in biological fluids *in vitro*. [52] These tests employ a specific capture antibody coated on a 96-well plate (Figure 15). Samples are pipetted into the wells, during which the antibody binds to the target proteins (i.e., antigens) present in the samples. A biotinylated detection antibody that binds to a separate epitope on the target protein is added, such that the protein is "sandwiched" between the capture antibody and detection antibody. An HRP-streptavidin is added, which then binds to the biotinylated detection antibody. In the presence of TMB substrate (and eventually sulfuric acid), color development occurs. Sample antigen concentration is determined by extrapolating the color intensity to a standard curve produced by a series of diluted purified antigen at known concentrations.

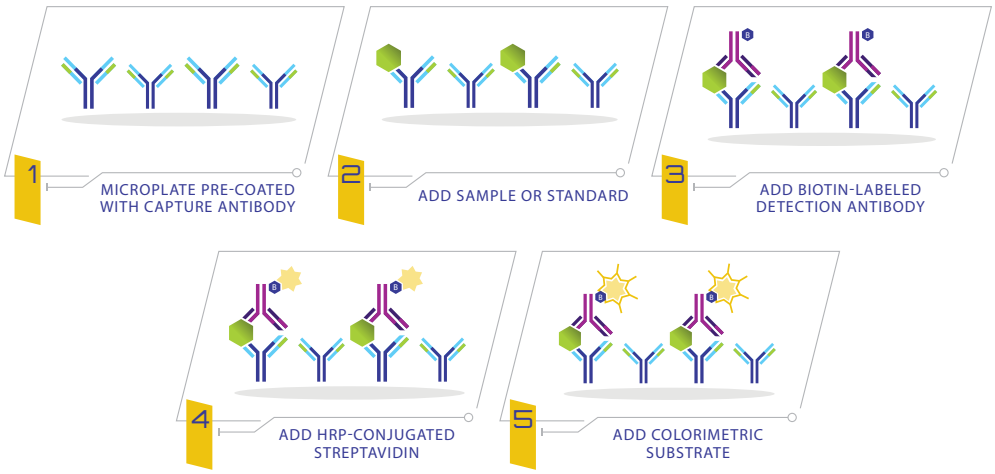


Figure 15. Antigen detection using a sandwich-based ELISA

Sandwich ELISA (continued)

Liu et al. investigated the diagnostic feasibility of using ELISAs to detect the SARS-CoV-2 N- and -S proteins. [49] Their results showed that the overall positive rates and sensitivity of the S-based ELISA was higher than the N-based ELISA. Notably, sandwich-based ELISAs that detect SARS-CoV-2 antigens can only be used during acute infection.

RayBiotech offers sandwich-based ELISAs to the SARS-CoV-2 N-protein (cat no. ELV-COVID19N) and S-protein S2 subunit (cat no. ELV-COVID19S2) as pre-coated 96-well plates. An ELISA to human ACE2 is also available (cat no. ELH-ACE2). Any sandwich-based ELISA can be converted to a PCR-based format called "immuno-PCR ELISA" (IQELISA™) in which a DNA barcode is attached to the detection antibody and amplified via qPCR using specific primers (Figure 16). The level of amplification is compared to a standard curve to analyze sample antigen concentration quantitatively.

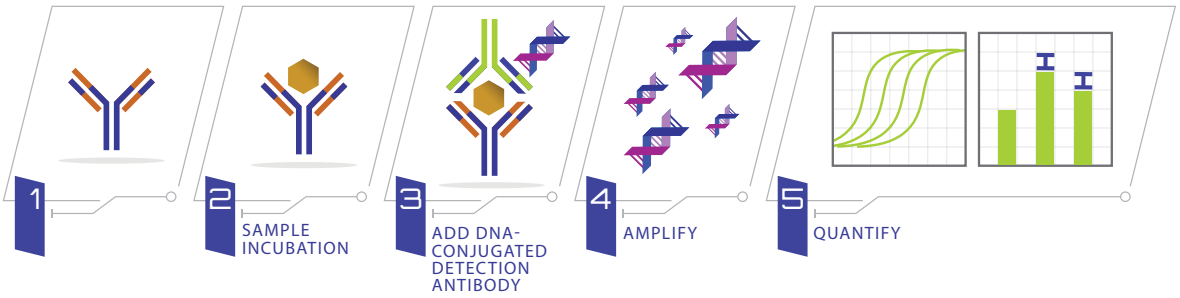


Figure 16. Antigen detection using a sandwich-based immuno-PCR ELISA (IQELISATM)

Antibody Arrays

Multiplex antibody arrays have historically been used for protein profiling, biomarker discovery, and drug development. [53 – 55] These arrays allow a broader view of protein activity than can be obtained with single-target ELISAs in a more efficient and cost-effective manner while maintaining the high sensitivity and specificity of an immunoassay. In short, antibody arrays immobilize capture antibodies on a substrate like a membrane, glass slide, or beads in an addressable format. After a blocking step, samples are incubated with the arrays. Nonspecific proteins are then washed off, and the arrays are incubated with a cocktail of biotinylated detection antibodies, followed by a streptavidin-conjugated fluorophore or other compatible detection system. Signals are then visualized for multiple antigens in a single sample simultaneously (Figure 17). [56] These sandwich-based arrays that utilize an antibody pair can provide either quantitative or semi-quantitative data. Another type of semi-quantitative array biotinylates the protein sample rather than utilize a biotinylated detection antibody. Although not quantitative, an advantage of these "labeled" arrays is that only one antibody per target is necessary; there is no required for an antibody pair (i.e., capture and detection antibodies that bind to the same target but at different epitopes).

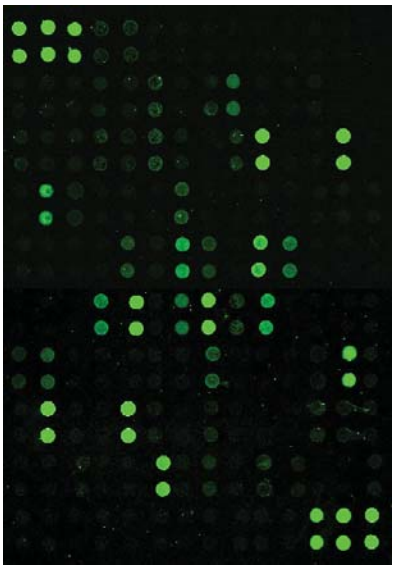
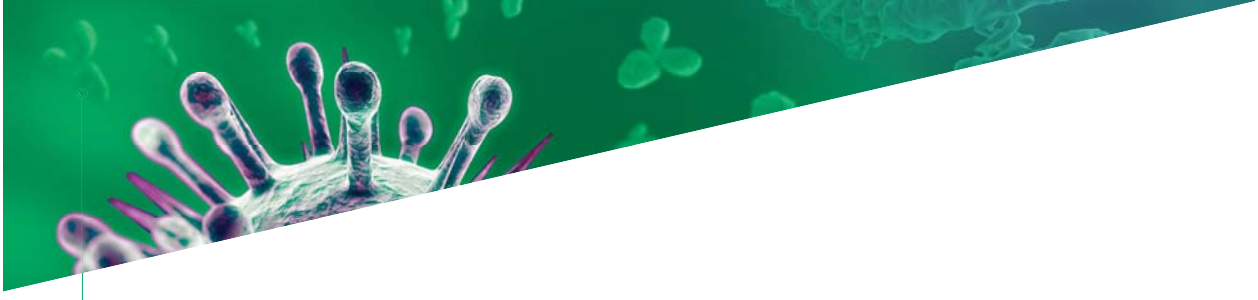


Figure 17. False-colored image of an antibody array after analysis. Each antibody to a specific antigen was spotted in quadruplicate. The green signal intensity is proportional to the amount of antigen in the sample.

Antibody arrays have been utilized in COVID-19 research to profile disease pathology and progression, which may help identify potential therapies or vaccine strategies. For instance, many COVID-19 patients with severe symptoms have a dysregulated immune response to the infection where T-cells, monocytes, and macrophages accumulate in the lung epithelia and result in the overproduction of pro-inflammatory cytokines. [57] This "cytokine storm" causes damage to the lungs, and ultimately other organs via cytokine entry into the bloodstream. Analyses of these inflammatory factors in severe cases could prove useful for monitoring of disease progression and determining prognosis.



Antibody Arrays (continued)

Protein profiling can help identify the precise drivers of this immune dysregulation, which in turn may help guide optimal immunomodulatory treatments. In one study, Hou et al. used a high-density antibody array to assess the proteomic changes in serum proteins throughout the SARS-CoV-2 infection. [58] A large set of differentially-expressed immune markers were identified between COVID-19 patients and influenza patients. They also noted significant correlations between neutrophil and lymphocyte levels with the CCL2 and CXCL10-mediated cytokine signaling pathways. These and other studies have shown that the serum proteomic profile of severe COVID-19 patients has a notable increase of specific cytokines (IL-2, IL-7, IL-10, G- CSF, IP-10, MCP1, MIP1α, TNF) compared to non-COVID or otherwise infected patients. These data are valuable for understanding COVID-19 pathogenesis, biomarker discovery, and guiding optimal anti-inflammation treatments.

COVID-19 vaccine development is being conducted concomitantly. Early testing of COVID-19 vaccines in mice caused an immune response resulting in lung or liver damage. [59] Thus, assessing the immune system's response to a potential vaccine is required to optimize the delivery platform, vaccine, or adjuvant. One study investigated the bronchoalveolar immune microenvironment in COVID-19 patients to determine the underlying mechanisms of COVID-19 pathogenesis, which is critical for identifying optimal vaccines. BALF from patients with severe COVID-19 symptoms contained higher proportions of macrophages and neutrophils and lower proportions of myeloid dendritic cells, plasmacytoid dendritic cells, and T cells than those with moderate infection. The same study by Liao et al. also measured several cytokines and chemokines in the BALF of COVID-19 patients using a bead-based antibody array. [60] A number of inflammatory cytokines were increased in severe cases compared with moderate cases, including IL-8, IL-6, and IL-1β, suggesting that macrophage recruitment of inflammatory monocytic cells and neutrophils into the lungs can play a role in COVID-19 severity. These studies underscore the need for assessing the immune response to vaccines or therapies. Such data can guide treatment by identifying potential biomarkers of patient response or mitigate potential complications.

Antibody arrays for analyzing a specific set of antigens are available from RayBiotech, including the Human Inflammation Array Q1 (cat no. QAH-INF-1) and the Human Inflammation Array Q1 (cat no. QAH-INF-3) that measures 10 and 40 human inflammatory factors quantitatively, respectively. The Human Immune Response Array Q1 (cat no. QAH-IMR-1) detects 40 human immune response-related biomarkers. Large-scale protein profiling and biomarker discovery studies are possible with high density antibody arrays, that can simultaneously measure as many as 1,000 human proteins quantitatively (cat no. QAH-CAA-X00) or 2,000 human proteins semi-quantitatively (cat no. AAH-BLG-2000). Full testing services employing high-density antibody arrays for the quantitative detection (click here) or the semi-quantitative (click here) are available for human, mouse, rat, porcine, and bovine studies. For a more detailed list of RayBiotech's available arrays, please click here. Custom antibody arrays can also be built based on the researcher's particular protein panel-of-interest.

NEUTRALIZING ASSAY

Binding Assay

A critical step of COVID-19 infection is when the virus enters human epithelial cells, which is enabled by the interaction between the SARS-CoV-2 S-protein's RBD on the surface of the viral particle and the ACE2 receptor on the surface of human cells. Thus, **molecules that inhibit formation of the S-ACE2 complex *in vitro* could be effective treatment for COVID-19 *in vivo*.** *In vitro* binding assays generally immobilize the S protein or ACE2 onto a solid substrate, such as a 96-well plate, and then probe with the ACE2 or S protein ("probe"), respectively, in the presence of a potential inhibitor (Figure 18). The S-ACE2 interaction is then detected using an anti-probe antibody labeled with HRP, which produces a blue color in the presence of TMB that is proportional to the amount of S-ACE2 complex (Figure 19). The HRP-TMB reaction is stopped with sulfuric acid, resulting in a blue-to-yellow color change. The intensity of the yellow color is then measured at 450 nm. Successful inhibition of the S-ACE2 interaction is reflected when the optical density (OD) with the inhibitor is lower than the OD without the inhibitor (i.e., only S + ACE2).

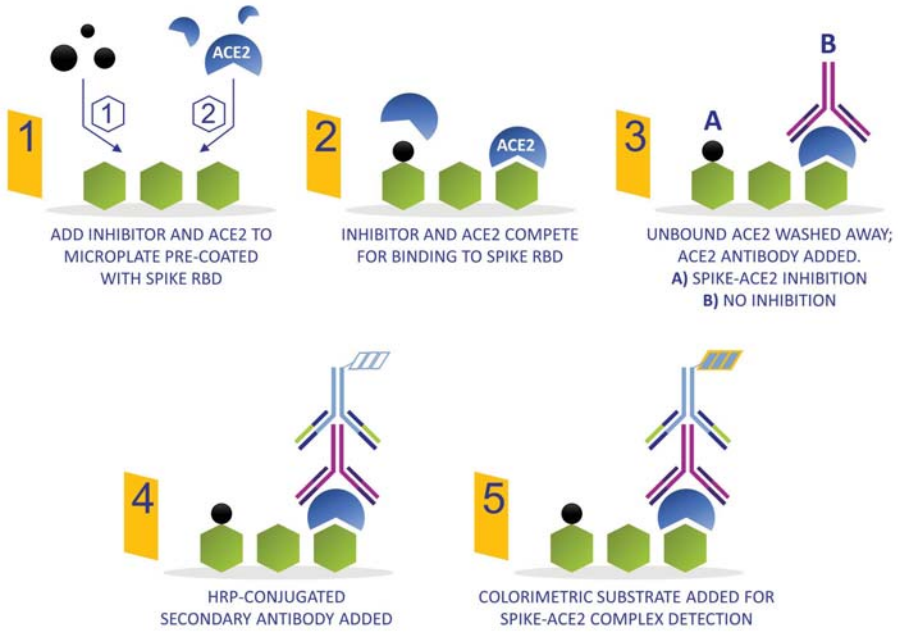


Figure 18. Schematic of an *in vitro* binding assay to study the interaction between the S-protein and ACE2.

Binding Assay (continued)

One recent study investigated the effect of the highly potent ACE2 inhibitor, MLN-4760, on the SARS-CoV-2 S-ACE2 interaction. [61] Although MLN-4760 alters ACE2 conformation, including the residues involved in binding to the RBD, MLN-4760 did not inhibit S-ACE binding. Using classical molecular dynamics simulations, peptide inhibitors of the S-ACE2 complex were designed and simulated based on crystal structures of the RBD and ACE2. [62] Peptides identified with this work and other types of simulations should be followed up with *in vitro* and in vivo binding assays.

Given the recent emergence of SARS-CoV-2, most binding studies have examined the interaction of ACE2 with the novel coronavirus, SARS-CoV, which was responsible for the first SARS outbreak in 2003. Ho et al. used plate-based *in vitro* binding assays to screen the ability of 14 peptides representing the SARS-CoV S protein to inhibit S-ACE2 complex formation. [63] Three of the peptides blocked the S-ACE2 interaction *in vitro* in a dose-dependent manner, whereas one of them also blocked the infectivity of S protein pseudotyped retrovirus in cells. Hong et al. also used an *in vitro* binding assay to examine the neutralizing ability of 27 mouse monoclonal antibodies to the S protein. [64] The majority of conformation-dependent antibodies (23/25) could inhibit S-ACE2 formation, whereas both antibodies targeting linear epitopes did not. This supported other data collected by them using cell-based ELISA and competitive binding assays. Their data suggests that the S protein elicits the immune response to generate antibodies to conformational epitopes.

Plate-based *in vitro* binding assays are rapid (< 1 day), simple to use, and require a common laboratory instrument (i.e., plate-based reader). Furthermore, they offer a safer alternative compared to working with live virus (biosafety level 3) and a more affordable option compared to working with pseudoviruses that require multiple plasmids and cell lines (biosafety level 2). Finally, *in vitro* binding assays such as RayBiotech's COVID-19 Spike-ACE2 Binding Assay Kit (cat no. CoV-SACE2) enable the high throughput screening of hundreds to thousands of different molecules simultaneously. These molecules could include small molecules, peptides, proteins, aptamers, or antibodies (see "Patient Blood"). Identification of molecules capable of inhibiting of the S-ACE2 interaction *in vitro* can be further investigated using cell-based assays and, eventually, within clinical trials.

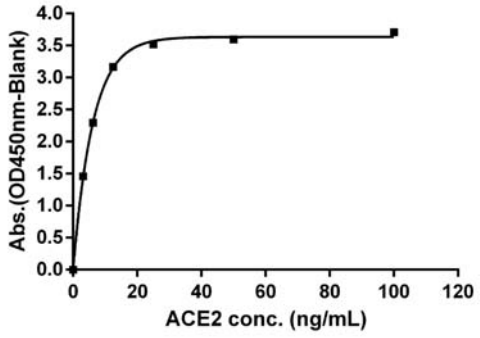
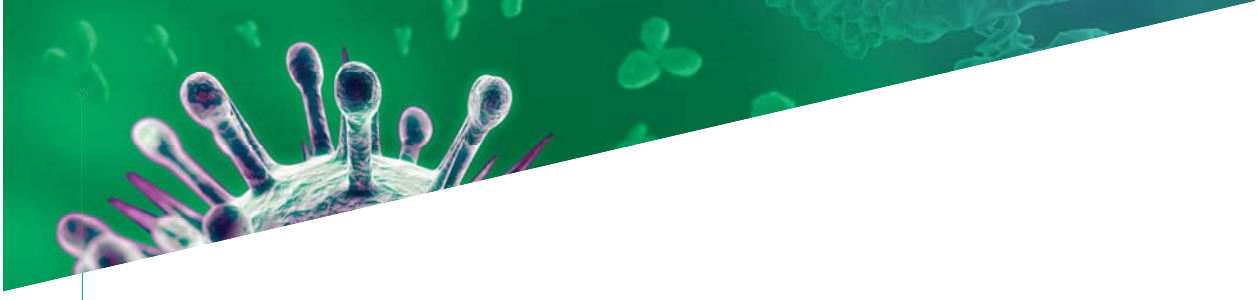


Figure 19. Measurement of serially-diluted human ACE2 protein using the S1-ACE2 binding assay



CONCLUSIONS

This eBook covered numerous reagents and platforms for COVID-19 diagnosis and research, all of which can be handled at biosafety level 2 or below. COVID-19-related antibodies, proteins, and patient serum are integral to nearly all currently-developed assays, and are likely to play an important role in the development of even higher throughput methods with higher sensitivity and specificity. The ELISAs and binding assay discussed here are affordable and require a plate reader capable of measuring absorbance at 450 nm, which is a common piece of laboratory equipment. The RT-LAMP assay and COVID-19 IgM/IgG rapid tests enable the detection of viral RNA and COVID-19 antibodies, respectively, with either no instrument at all or only a heat block! Presence of these targets result in a color change that is visual by eye. In addition, they are easy to use so require minimal training. All of the PCR-based tests detect SARS-CoV-2 RNA taken from the site of viral entry and propagation, and can provide valuable information regarding a patient's COVID-19 infection status and infectivity. The antibody arrays can help shed light on the host response to COVID-19, either with specific protein panels, such as inflammatory markers associated with COVID-19 severity and prognosis, or high density panels for a more unbiased approach.

This eBook does not contain an exhaustive list of the different methods that can be employed. For example, cell-based ELISAs, biolayer interferometry, and flow cytometry can be used to study the S-ACE2 interaction in the presence of potential inhibitors. [64, 65] Protein profiling of human responses can be obtained using mass spectrometry (see our blog, "A Comparison of Antibody Arrays and Mass Spectrometry in Protein Profiling and Biomarker Research"). The SARS-CoV-2 live virus or pseudotype virus are also essential tools in COVID-19 research. [7] Undoubtedly, all of the research methods provide valuable information in fighting COVID-19.

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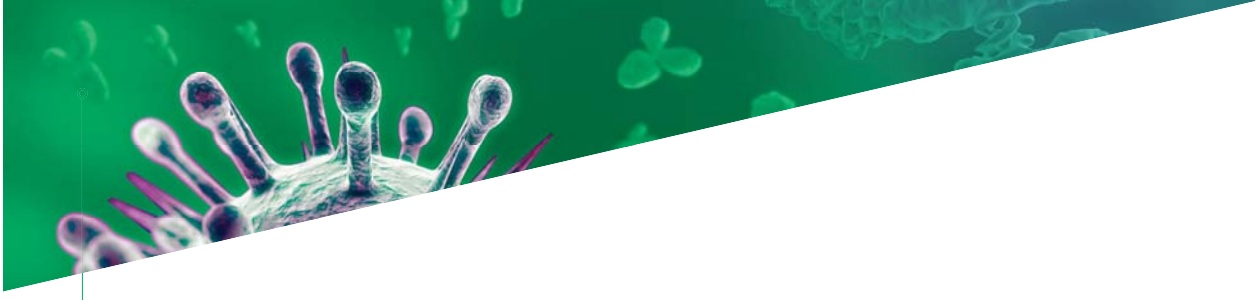

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