Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Alternative Methods to Detect Severe Acute Respiratory Syndrome Coronavirus 2 Antibodies

Rashmi Patel, MBBS, MS[a], Siddharth Khare, PhD[a], Vinay S. Mahajan, MBBS, PhD[b,c,*]

KEYWORDS

• Serology • Immunoassay • ELISA • SARS-CoV-2 • Electrochemical detection • Luminescence • Neutralization assay

KEY POINTS

- Lateral flow assays (LFA) and enzyme-linked immunosorbent assays (ELISA) are the cornerstones of SARS-CoV-2 serologic diagnosis.
- A comprehensive serologic analysis involves determining the response to multiple viral antigens, and antibody characteristics, such as isotype and neutralization potential.
- Technical advances in photonics, electrochemistry, protein design, luminescence probes, and nanotechnology have been applied to serologic diagnostics of SARS-CoV-2; these assays are in varying stages of development.
- Serologic assays for SARS-CoV-2, and especially neutralization assays, need to keep up with the emergence of viral variants, necessitating a high degree of vigilance among laboratory practitioners.

INTRODUCTION

The SARS-CoV-2 pandemic resulted in an intense demand for serologic diagnostics, leading to the development of hundreds of assays across the world, especially lateral flow assays (LFAs). The COVID-19 test directory on the FindDx Web site includes an exhaustive catalog of these assays.[1] By June 2021, the US Food Drug Administration (FDA) had issued Emergency Use Authorizations (EUA) for 80 serologic tests for SARS-CoV-2.[2] These range from rapid qualitative LFAs to semiquantitative enzyme-linked immunosorbent assays (ELISAs) and include assays that are performed on fully

[a] Micelio Labs, 58, 15th Cross Road, 2nd Phase, J P Nagar, Bengaluru 560078, India; [b] Ragon Institute of MGH, MIT and Harvard, 400 Technology Square, Cambridge, MA 02139, USA; [c] Department of Pathology, Brigham and Women's Hospital, 75 Francis St, Boston, MA 02215, USA

* Corresponding author. Ragon Institute of MGH, MIT and Harvard400 Technology Square, Cambridge, MA 02139, USA.

E-mail address: vinay.mahajan@mgh.harvard.edu

Clin Lab Med 42 (2022) 57–73
https://doi.org/10.1016/j.cll.2021.10.007
labmed.theclinics.com

automated laboratory analyzers. In most COVID-infected patients, antibodies are observed approximately 1 to 2 weeks following symptom onset or polymerase chain reaction positivity in symptomatic or asymptomatic individuals, respectively.[3,4] The observed timing of seroconversion also depends on the sensitivity of the assay. Seroconversion may be picked up as early as the day of the first positive nucleic acid test after symptom onset with ultrasensitive single-molecule approaches (Simoa, Quanterix Technologies, Billerica, MA).[5] Unlike typical seroconversion profiles in other infectious contexts, near-simultaneous production of IgM, IgG, and IgA has been observed in patients with confirmed SARS-CoV-2.[6,7] Although IgM titers may disappear within a month, IgG titers are detectable for much longer but also exhibit a gradual decline in the months following infection.[8–10] Higher antibody titers are seen following symptomatic or severe disease.[11]

The serologic assays that have been developed have focused on the structural proteins of the virus, that is, spike and nucleocapsid. Spike is a transmembrane glycoprotein comprising two parts, S1 and S2. The binding of the SARS-CoV-2 spike to its receptor, angiotensin-converting enzyme 2 (ACE2), is mediated by S1. S2 mediates fusion of the viral envelope to the cell membrane and cell entry during infection. The S1 receptor binding domain (RBD) binds ACE2 and is highly immunogenic. The spike protein contains sequences unique to SARS-CoV-2 and shared with other betacoronaviruses. Thus, assessing the serologic response to antigens from other human coronaviruses can help exclude cross-reactive responses. Determining the antibody isotype (eg, IgM, IgG, or IgA) may provide additional information for determining immune status. Unlike natural infection, most vaccines induce antispike but not antinucleocapsid responses and IgG rather than IgA. These characteristics may help distinguish between vaccine-induced responses and natural infection. Vaccines are designed to elicit antibodies against the S1 RBD because antibodies to this region can neutralize the virus. Thus, in addition to antibody specificity, a complete serologic characterization involves determining the isotype and neutralization potential.

There is a great interest in using serologic parameters to determine infection risk, vaccine efficacy, or vaccine prioritization. However, serologic assays do not detect the presence of memory B or T cells, and it is possible that memory lymphocytes may offer some immunity in subjects with declining antibody titers.[12] The relationship between seropositivity or neutralization titers and protection remains unknown and is being evaluated in clinical and epidemiologic studies. Given the rise of SARS-CoV-2 variants and the variable magnitude of serologic responses among convalescent individuals, the use of serology as a surrogate for protection is likely to remain a controversial and changing landscape. Because of the previously mentioned uncertainties, the Centers for Disease Control and Prevention and FDA have advised against the use of serologic assays for assessing protection, and interpretive guidance is imperative while reporting a serologic test result.[13] Given their utility in a point-of-care setting, LFAs for antibodies have been widely used in population serosurveys to estimate exposure rates and guide public health policy. Seroconversion indicates prior exposure but not active infection, and LFAs that detect viral antigens rather than antibodies should be used to determine infectious risk.[14]

Although manufacturers monitor performance using traceability to recognized standards, independent monitoring of assay performance by clinical laboratories was especially vital in ensuring that these assays were effectively used during a time that witnessed widespread supply chain disruptions with the potential to impact assay manufacturing and distribution. Indeed, when concerns were raised about the reliability of certain LFAs, the EUA for some assays were revoked by the FDA. Independent vigilance of serologic assays by clinical laboratory practitioners continues to be

important because the antigen formulation used in the serologic tests has remained unchanged despite the emergence of SARS-CoV-2 variants. In particular, the interpretation of neutralization assays may be significantly impacted by SARS-CoV-2 variants. Variants that may have an enhanced capacity to evade neutralizing antibodies are referred to as variants of concern.[15] Such variants are likely to keep emerging as the virus continues to evolve in the face of immune pressure when herd immunity builds up in a population.[16] Although the FDA has issued an EUA for one ELISA-based qualitative neutralization assay (cPass, GenScript, Piscataway, NJ), it is only intended to assess recent infection and its clinical applicability to determine the degree of immunity is not known. Most neutralization assays are high-complexity tests and are performed by specialized laboratories. They have primarily been used by vaccine developers and need nuanced interpretation in the light of concerns about variants of concern. The continued emergence of variants raises concerns about the need to update the assays to assess neutralization capacity against emerging variants. We have described neutralization assays in detail in a separate section.

There is a need for the development of reliable and clinically scalable multiplexed serologic assays, suitable for point-of-care use. Some of these challenges may be addressed by adopting emerging alternate technologies. This article highlights a few novel and alternative approaches that rely on electrochemical analysis, luminescent signals, or label-free optical detection. Ultrasensitive and quantitative approaches, such as Simoa (Quanterix Technologies), have now entered the market, which has opened a range of novel possibilities in serologic diagnostics, but these instruments are not widely available. However, they have also been used as a quantitative reference method to compare the performance of other serologic assays across a broad range of antibody dilutions.[17] We emphasize that many of the approaches described in this article are still in development and are yet to receive regulatory clearance. This article is also not meant to be an exhaustive review of alternative approaches to serology; it is divided into sections based on the physical principle used for assay signal generation.

GENERAL PRINCIPLES OF A SEROLOGIC ASSAY

Broadly, a serologic assay involves a mechanism for signal generation, signal amplification, and signal detection (Fig. 1). If the unbound label can generate a signal on

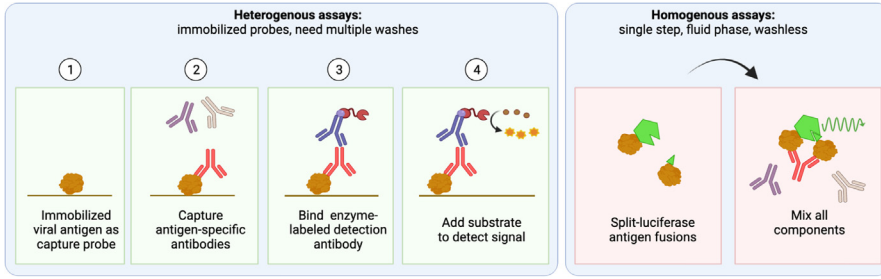

Fig. 1. General principles of an immunoassay. A heterogeneous immunoassay for antigen-specific antibodies relies on immobilized antigen as a capture probe and often uses enzyme labels for signal amplification; it requires stepwise reagent addition with multiple washes. A homogeneous immunoassay is washless and generates a signal only in the presence of the target analyte. An assay devised using antigens fused to split-luciferase domains is shown as an example.

its own, it needs to be separated by washing before measurement. Such an assay is called "heterogeneous." If the label generates a signal only in presence of the analyte, the immunoassay can be performed in a washless or "homogeneous" format. If the signal can be quantitatively measured across a sufficiently wide dynamic range, the assay can be calibrated with a range of accepted standards to generate a quantitative result. Some approaches are based on label-free or direct measurement of the physical or chemical changes induced by antibody binding, and do not require the use of a labeled secondary antibody as a detection probe.

After incubation of patient serum with the specimen and removal of unbound antibodies, the antigen-bound antibodies are measured using a labeled detection probe, such as anti-IgG or anti-IgM. Such a setup is called a sandwich immunoassay (see **Fig. 1**). When the detection probe is labeled with an enzyme to amplify a signal-generating chemical reaction, it is called a sandwich ELISA. ELISAs are typically coupled to an optical signal. Test sensitivity and specificity are established based on comparison against established positive and negative samples based on polymerase chain reaction positivity. Prepandemic specimens have been used as negative control subjects. For all assays that were issued an EUA, the FDA has published estimates of sensitivity and specificity on its Web site.[18] However, these numbers are estimates with 95% confidence intervals and positive and negative predictive values depend on the prevalence. The kinetics of seroconversion and the potential loss of titers on convalescence should be considered when interpreting negative results.

The design of each assay step influences the overall signal-to-noise ratio and consequently the sensitivity and specificity of the test. SARS-CoV-2 antigens, typically spike or nucleocapsid proteins, are used as capture probes for antibodies. Some assays include other human coronaviral antigens as specificity controls. Blocking of nonspecific binding is especially important for minimizing the background signal in assays where antibodies bound to an antigen-coated surface are measured. The choice and source of the antigens may also influence their antigenicity and contribute to differences in performance between assays that detect the same antigen. For instance, the spike protein may be used as a capture probe in its full-length form, or just the S1 domain or RBD alone. However, the full-length spike protein is less stable than the S1 domain or RBD. The approach used for surface coupling may also impact antigenic stability and access to antigenic epitopes.[19] The antigens need to be stabilized especially for use in a point-of-care setting without refrigerated storage. Antigenicity is also influenced by glycosylation, and thus fully glycosylated antigens expressed in a mammalian host are likely to best capture the full breadth of the serologic response.

Point-of-care LFAs and serologic assays performed on central laboratory analyzers have been described in concurrent articles in this issue. The rapid assay from NanoEnTek (Seoul, South Korea) that received an FDA EUA resembles an LFA but uses a microfluidic cartridge for precise control of fluid flow.[20] The fluorescent signal in the cartridge is read using a dedicated instrument with connectivity to the laboratory information system. ELISA kits, which can yield quantitative results, are typically manufactured as microparticle-based or 96-well microtiter-based immunoassays and require manual setup, washing, and a plate reader. This involves significant infrastructure and operator skill and is suitable for medium throughput applications. Several COVID-19 serologic assays for automated central laboratory analyzers have now become available and should be considered for high-throughput operations. In this article, we have focused on serologic assays that use alternate or unconventional detection methodologies. These assays may help bridge the gap between LFAs and central laboratory analyzers in terms of assay performance and throughput. Although many of the approaches described in this article are proof-of-principle demonstrations

and are yet to be commercialized or implemented in a clinical setting, a few have received an FDA EUA. The assays from Genaltye (San Diego, CA; Maverick Multi-Antigen Serology Panel) and Genscript (cPass) are two such examples and are among those described in this article.[21,22] Among other serologic assays that have received an EUA and rely on an unconventional detection approach, the assays from MosaicQ (Quotient Diagnostics Ltd, United Kingdom) and Luminex (Austin, TX) are noteworthy; they are solid-phase ELISAs that use light scattering or fluorescence properties of nanoparticles for sensitive or multiplexed detection.[23,24] Both platforms can be combined with antigen tests. However, a specialized instrument is required for analysis.

ELECTROCHEMICAL IMMUNOASSAYS

This approach relies on the functionalization of the surface of a working electrode in an electrochemical cell with desired antigens.[25] Antibody binding to the electrode surface results in quantitative changes in electrical properties, such as impedance, which is measured in a label-free and washless setup (**Fig. 2**). Alternatively, antibody binding is coupled to a reduction-oxidation (redox) reaction, such as one catalyzed by the widely used ELISA label, horseradish peroxidase (HRP), and the generated current is measured in a low-cost setup (see **Fig. 2**). A few such assays have been implemented in the context of COVID-19 serology as a proof-of-concept and are listed next.

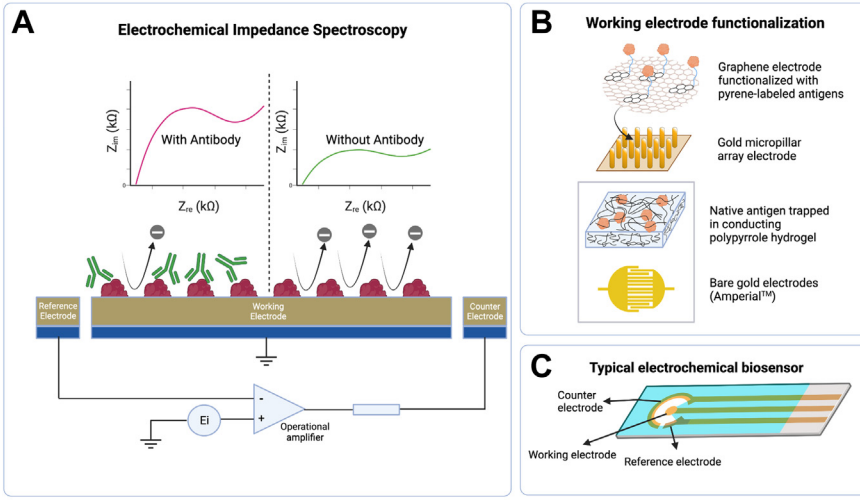

Fig. 2. Electrochemical assays. (*A*) The binding of antibodies to antigens coated on the surface of a working electrode impedes electron transport, and results in a quantitative change in impedance. This is measured using various electrochemical impedance spectroscopy techniques. For instance, as illustrated using Nyquist plots, the frequency dependence of the impedance is influenced by a change in the surface properties of the electrode when it is bound by an antibody. (*B*) Two approaches to functionalize a working electrode are shown. A graphene electrode is functionalized with noncovalently stacked pyrene-labeled antigens; such a graphene layer can also be coated on gold electrodes. Electrodes used in the Amperial platform are functionalized with native antigens entrapped in a conducting polypyrrole hydrogel using in situ electropolymerization. (*C*) A typical electrochemical electrode design comprising a working electrode, a counterelectrode, and a reference electrode.

Amperometric Enzyme-Linked Immunosorbent Assay in Microtiter Plates

The Amperial assay platform (Liquid Diagnostics, San Clemente, CA) is designed to implement an ELISA assay with an electrochemical readout.[26] It uses microtiter plates fabricated with gold electrodes at the bottom of each well. First, the surface of the electrode is functionalized with the target antigen by electropolymerizing a pyrrole solution containing native antigens. This entraps antigens in a native state on the surface of the electrode within a conductive polypyrrole hydrogel. Next a sandwich ELISA is performed with an HRP-labeled secondary antibody for detection (see **Fig. 2**). On addition of a redox substrate and the application of a voltage, the peroxidase reaction produces an electric current that serves as a quantitative assay readout. A sensitivity greater than 88% and specificity greater than 99.85% was observed with S1 antigen. The immobilization of native antigen in an electropolymerized polypyrrole hydrogel preserves its antigenicity and offers the flexibility to test various types of antigens. The assay is quantitative, and the current in nanoamperes is read for the whole plate within 3 minutes. However, it is a conventional heterogeneous sandwich immunoassay involving multiple wash steps.

Multiplexed Miniaturized Amperometric Enzyme-Linked Immunosorbent Assay

A scalable low-cost laser-engraving technique was used to fabricate a miniaturized arrangement of disposable printed graphene electrodes individually functionalized with S1 domain, nucleocapsid antigen, C-reactive protein, and an antinucleocapsid antibody for simultaneously assessing the serologic response, an inflammatory biomarker, and virus detection, respectively.[27] A graphene counterelectrode and an Ag/AgCl reference electrode was also included to complete the biosensor circuit (see **Fig. 2**). The low cost, high charge mobility, surface area, and ease of bioconjugation make graphene an ideal material for biosensor electrodes. The biosensors are subjected to a conventional ELISA and the signal is amplified by HRP-labeled detection antibodies that produce an amperometric readout in the presence of a redox substrate. The device is linked to a compact battery-powered circuit that transmits the signal to a cellphone via Bluetooth. Analytical sensitivity of 1 pm was achieved with only 10 minutes of specimen incubation. The electrode was manually rinsed with wash buffers and incubated with the redox substrate, but this is easily automated. Comparable sensitivity was seen with saliva and serum. The study showed the potential of this approach in a quantitative, multiplexed assay that is suitable for point-of-care settings.[26]

Repurposed Cellular Impedance Monitoring Platform

The xCELLigence system (Agilent, Santa Clara, CA) is an instrument designed to detect cellular impedance for real-time monitoring of cell cultures with label-free measurement of cellular function, such as cell growth, shape, or toxicity. The cells are grown and monitored in specially fabricated plates with an electrode at the base of each well. If, however, the same multiwell cell culture plates are used to perform an ELISA with coated antigens, the change in impedance as a result of antibody binding is measured in a washless format.[28] This was tested with S1 and RBD antigens. Antibody binding led to a sharp increase in impedance followed by a gradual decay over several minutes. Antibody binding is rapidly detected in a washless format, within minutes. This was an experimental demonstration of electrochemical measurement of SARS-CoV-2 serology by repurposing a device already in use in some research laboratories.

Label-Free Electrochemical Immunoassay Using a Gold Micropillar Array Electrode

A three-dimensional gold microelectrode array was fabricated and decorated with reduced graphene oxide, and then conjugated to the SARS-CoV-2 spike protein; the three-dimensional geometry of the electrode results in a stronger current and also permits immobilization of the antigen at a higher density, resulting in greater antibody capture.[29] S1 and RBD were tested and yielded a quantitative measurement range of 1 pm to 10 nM. A change in impedance was detected within 3 seconds of incubation. The signal is saturated over 10-nM concentration when all available binding sites on the sensor surface are occupied by the specific antibodies. A low-cost coin-sized portable electrochemical impedance spectroscopy analyzer (Sensit, PalmSens, Houten, the Netherlands) connected to a smartphone was used for measurements. This approach offers label-free detection with a few microliters of blood. The biosensor is regenerated and reused after removing the bound antibodies with pH 2.5 formic acid. The sensor has a high regeneration capability with a good signal output even after nine regeneration cycles. The electrodes require specialized techniques for fabrication, but the assay characteristics are suitable for point-of-care use.

A Paper-Based Electrochemical Biosensor

A simple label-free rapid paper-based electrochemical serology sensor was implemented with RBD antigen immobilized on printed graphene electrodes in a vertical flow assay format.[30] Antibody binding was detected using an electrochemical spectroscopy technique called square wave voltammetry using a portable PalmSens analyzer. In this technique, the signal-to-noise ratio increases by the square root of the scan rate. The improved signal is a function of the time between pulse application and the current measurement, and the change in the faradaic current is measured as peaks on the voltammogram. The test was performed with patient sera and compared with a homemade colorimetric LFA device, prepared using the same batch of RBD. It exhibited better sensitivity for detection of IgM compared with IgG because of its larger size. It is a low-cost, rapid qualitative test suitable for point-of-care use with greater sensitivity compared with conventional LFAs, but requires an electrochemical impedance spectroscopy analyzer for readout. Being a label-free approach, unlike conventional immunosandwich LFAs, it is not impacted by the risk of false-negatives because of the high-dose “hook effect” or prozone phenomenon.

BIOLUMINESCENT LABELS

Luminescent labels, which catalyze a light-emitting chemical reaction, are widely used for sensitive detection using ELISAs. Unlike colorimetric or fluorescence detection, a light source or excitation is not required, and this approach offers greater sensitivity, lower background, and a wide dynamic range using inexpensive instrumentation. The collected photons are amplified into a current signal that is reported as relative light units. Several luminescent substrates for the widely used immunoperoxidase or alkaline phosphatase labels have been developed and they form the basis of most modern ELISAs. Bioluminescence refers to analogous reactions observed in living organisms that are catalyzed by specialized enzymes called luciferases acting on substrates called luciferins. The luciferase-luciferin systems have been adapted to build highly sensitive immunoassays. These assays have been implemented in a fluid-phase ELISA and homogeneous washless formats using antigen-luciferase fusion proteins, split luciferases, or designer proteins. Their broad dynamic range allows measurement across decades of concentration

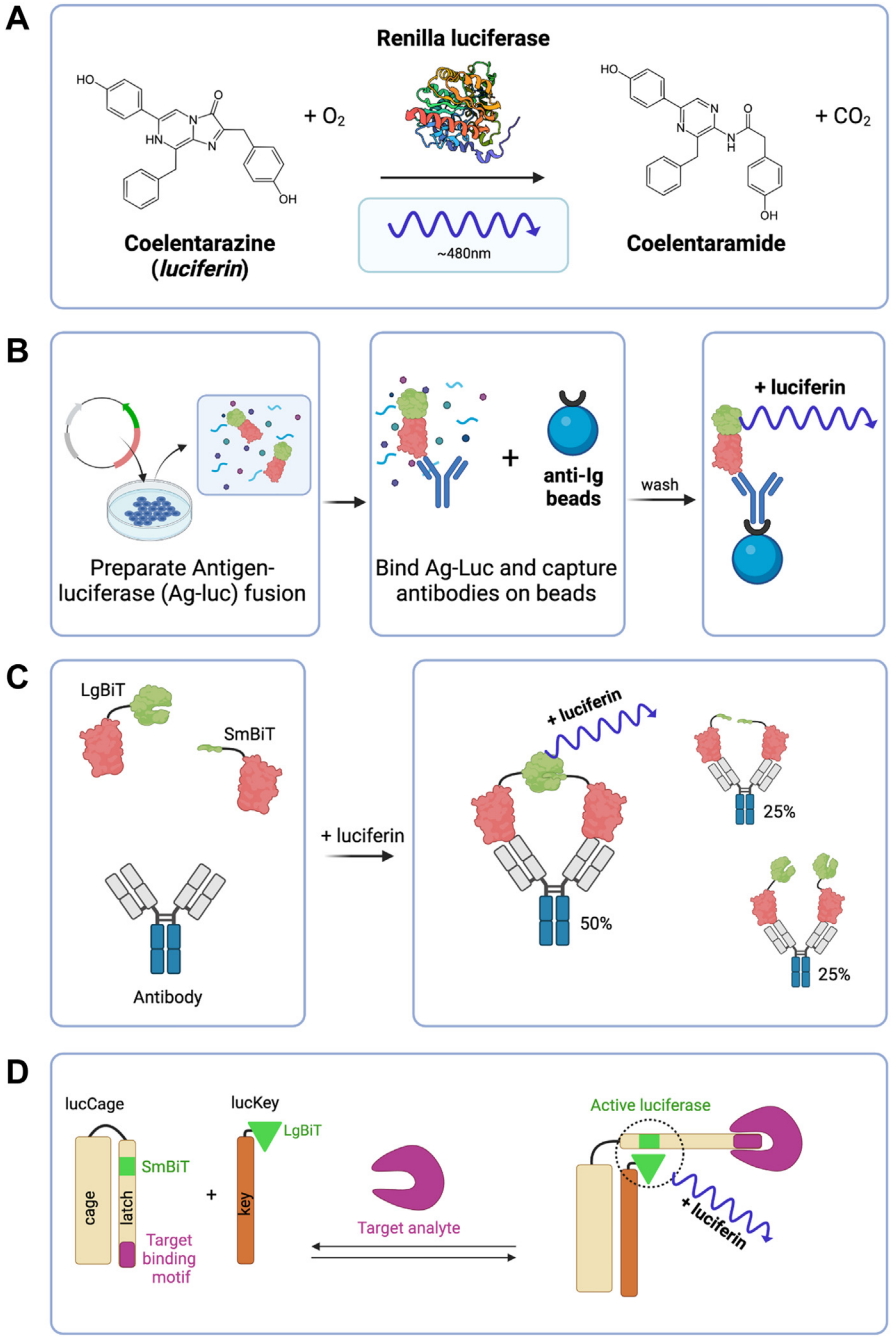

Fig. 3. Bioluminescence assays. (*A*) Luciferase is an enzyme that catalyzes a bioluminescent reaction by oxidizing a luciferin substrate. Luciferase systems that are well-characterized are widely used in bioassays (Renilla luciferase and coelenterazine substrate are shown). (*B*) LIPS. Crude lysate bearing recombinant antigen-luciferase fusion protein is incubated with serum and antibodies are pulled down with anti-Ig beads. Antigen-specific antibodies

without the need for sample dilutions. The reagent systems for luminescent reactions are more complex than those used for spectrophotometric and fluorometric analyses and require more careful control and low temperature storage. Furthermore, luminescence measurements must follow the kinetics of the reaction. Although rapid luciferin oxidation can produce a bright signal, signal detection may need to be timed with the injection of the luciferin substrate.

Luciferase Immunoprecipitation Assay

Luciferase immunoprecipitation (LIPS) assays are heterogeneous liquid-phase immunoassays (**Fig. 3**A and **3**B).[31,32] Unlike ELISA, which is typically a solid-phase assay, antibody-antigen binding takes place in the fluid phase in an LIPS assay, thus better maintaining the native antigen conformation. Luciferase-labeled antigens are expressed as recombinant fusion proteins. The ability to use crude cell lysates of mammalian cells transfected with an antigen-luciferase fusion protein expression vector without further purification greatly simplifies assay development. This was particularly helpful for rapid development of these assays in the early days of the pandemic when antigens were not readily available. It also allows the rapid development of serologic tests for antigens from variants of concern without the need for purified antigens. A single preparation of crude lysates containing antigen-luciferase fusion protein can be used for thousands of assays. However, various configurations of antigen-luciferase fusions need to be tested and optimized during assay development. Luminescence is measured on addition of the luciferase substrate using a microplate reader, yielding a quantitative result with high analytical sensitivity and a wide dynamic range without the need for sample dilution. LIPS assays have been implemented using luciferase fused to nucleocapsid and spike proteins.[31] For IgG against nucleocapsid, the sensitivity and specificity were 100% at 14 days after the onset of symptoms (n = 35). For IgG against spike, sensitivity of 94% and specificity of 100% was observed.

Engineering Luminescent Biosensors for Point-of-Care SARS-CoV-2 Antibody Detection

This approach uses rationally designed antibody biosensors based on split nanoluciferase fragments (SmBiT and LgBiT) fused to SARS-CoV-2 antigens (**Fig. 3**C).[33] Because an antibody has two fragment antigen-binding (Fab) arms, incubating the specimen with a 1:1 mix of SmBiT and LgBiT biosensors results in half of the antiviral antibodies binding LgBiT with one Fab arm and SmBiT with the other Fab arm. If the antigen binding orientation is such that it brings the LgBiT and

are measured using a luciferase signal. (*C*) Split nanoluciferase fragments (large BiT [LgBit] and small BiT [SmBiT]) are fused to RBD. An active nanoluciferase is assembled on bivalent antibody binding, which is sensitively detected with a luciferase substrate in a homogeneous assay format. (*D*) A de novo designed lucCage:lucKey protein biosensor. The lucCage protein is built with a cage domain and a latch domain, which contains a target-binding motif and a split luciferase fragment (SmBit). The lucKey contains a key peptide that binds the lucCage cage domain and the complementary split luciferase fragment (LgBit). Binding of the analyte to the lucCage latch stabilizes the open conformation of lucCage, interaction with the lucKey, and assembly of an intact luciferase. The thermodynamics of the system are designed such that the intact luciferase is reconstituted only in the presence of the target analyte.

SmBiT fragments into close proximity, it results in the reconstitution of an intact, enzymatically active nanoluciferase enzyme. This is used for luminescence-based detection of antigen-specific antibodies in a homogeneous assay format. Sensors for RBD and nucleocapsid were designed using this approach and shown to require less than 30 minutes to result, suitable for point-of-care applications.[33] It is sensitive enough for testing serum or plasma (>99% sensitivity) and to a lesser extent saliva (79% sensitive).

De Novo Designed Protein Biosensor with Miniprotein Sensing Domains

Although once considered impossible, the explosion in the knowledge of protein structure and folding has led to the realization of de novo designed proteins.[34] One such rationally designed modular protein biosensor (lucCage and lucKey) was built such that antibody binding to the biosensor is thermodynamically coupled to switching from a closed dark state and an open luminescent state (**Fig. 3**D).[35] This biosensor is used in a homogeneous assay format and was built with RBD as the antigen. Antibodies against RBD could be detected at a sensitivity of 15 pm with a signal over background of more than 50-fold. The lucCage biosensor is based on thermodynamic coupling between defined closed and open states of the system; thus, its sensitivity depends on the free energy change on the binding of the sensing domain to the target but not the specific binding geometry. This enables the incorporation of various binding modalities, including small peptides, globular miniproteins, antibody epitopes, and de novo designed binders, to generate sensitive sensors for a wide range of protein targets with little or no optimization. For point-of-care applications, the system has the advantages of being homogeneous, no-wash, and gives a nearly instantaneous readout; the quantification of luminescence is carried out with inexpensive and accessible devices, such as a cell phone camera. The ability to modularly design sensors with identical readouts for diverse antigens could enable multiplexed serologic assays using an array of different sensors. However, there is considerable variation between different sensors in the level of activation at saturating target concentrations.

LABEL-FREE OPTICAL APPROACHES

Nanoscale changes resulting from the binding of a protein to an optical surface is amplified and detected by specialized optical techniques or devices, such as surface plasmon resonance, biolayer interferometry, optical cavities, or resonators.[36,37] They have been commercialized into benchtop instruments and we highlight two approaches that have been applied to serologic diagnosis of SARS-CoV-2. Semiconductor-based photonic technologies are rapidly evolving, and such devices may soon become more commonplace.

Biolayer Interferometry

Biolayer interferometry is used to measure the binding of a protein in solution to an immobilized ligand on a biosensor tip (**Fig. 4**A). The biolayer interferometry instrument (Octet, Sartorius, Göttingen, Germany) is increasingly used in the research and biotechnology space. Protein binding produces an increase in optical thickness at the biosensor tip, altering the interference pattern of reflected light. This approach was used to build a proof-of-concept assay for the rapid and semiquantitative measurement of SARS-CoV-2 antibodies in saliva and plasma and is called biolayer interferometry immunosorbent assay.[36] Rapid real-time and quantitative antibody binding data are obtained in less than 20 minutes in a washless and

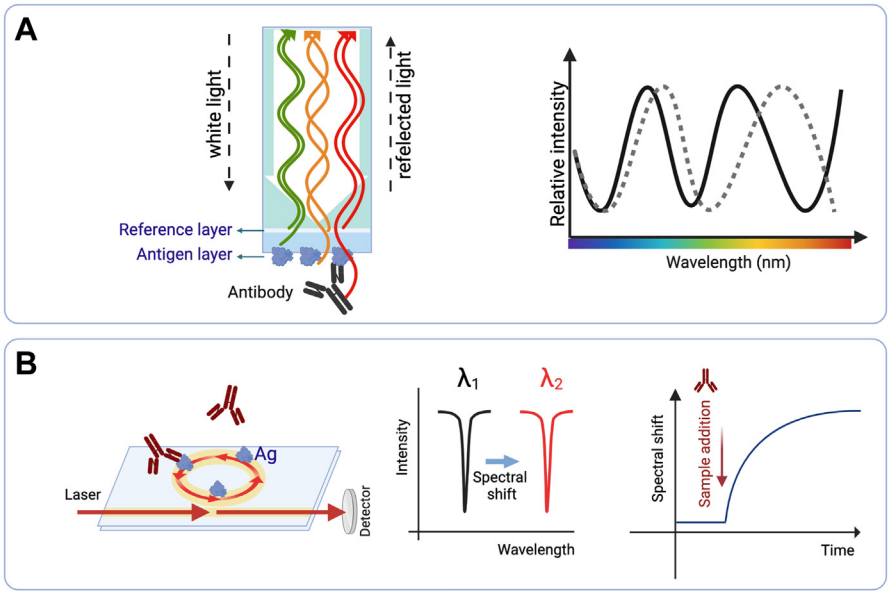

Fig. 4. Label-free optics. (*A*) Biolayer interferometry relies on measuring the interference pattern of white light reflected from an internal reference layer and the biosensor tip coated with antigen. Binding of antibodies to the test surface can shift the optical path by a few nanometers and this is detected by analyzing the interference pattern. This approach allows real-time monitoring of protein binding and dissociation and is compatible with crude samples because the surrounding medium does not influence these measurements. (*B*) An optical ring biosensor is an optical cavity that is functionalized with antigen and coupled to a tunable laser. A dip in the signal intensity of a tunable laser is used to determine the resonant wavelength of the optical ring. Binding of an analyte results in a shift in the resonant wavelength, which can be monitored in real time.

label-free fashion. Various sample types, such as saliva and serum, are tested on the same platform. The sensitivity and specificity were not assessed with clinical specimens.

Optical Ring Resonators

The diagnostic applications of optical ring resonators have been commercialized by Genalyte. An optical ring resonator is a type of optical wave-guide-based biosensor that traps light passing along an adjacent linear waveguide at its resonance frequency (**Fig. 4**B).[37] The light makes multiple passes in the resonator allowing for larger effective interaction length (several centimeters) and improving the sensitivity of detection. This also significantly reduces the physical size of the sensor. Antibody binding to antigen coated on the ring resonator results in a detectable shift in its resonant frequency proportional to the mass of bound biomolecules. Primary and secondary antibodies are flowed over the biosensor and detected in real-time with a tunable laser. The assay protocols take less than 15 minutes and are run on an automated platform (Maverick, Genalyte), which supports a large number of immunoassays in addition to Sars-CoV-2 serology.[38] A SARS-CoV-2 Multi-Antigen Serology Panel was developed by Genalyte on this platform and was issued an EUA by the FDA. The assay performance is similar to other fully automated platforms. It includes an array of antigens, including spike proteins from other human coronaviruses as specificity controls.[21,39]

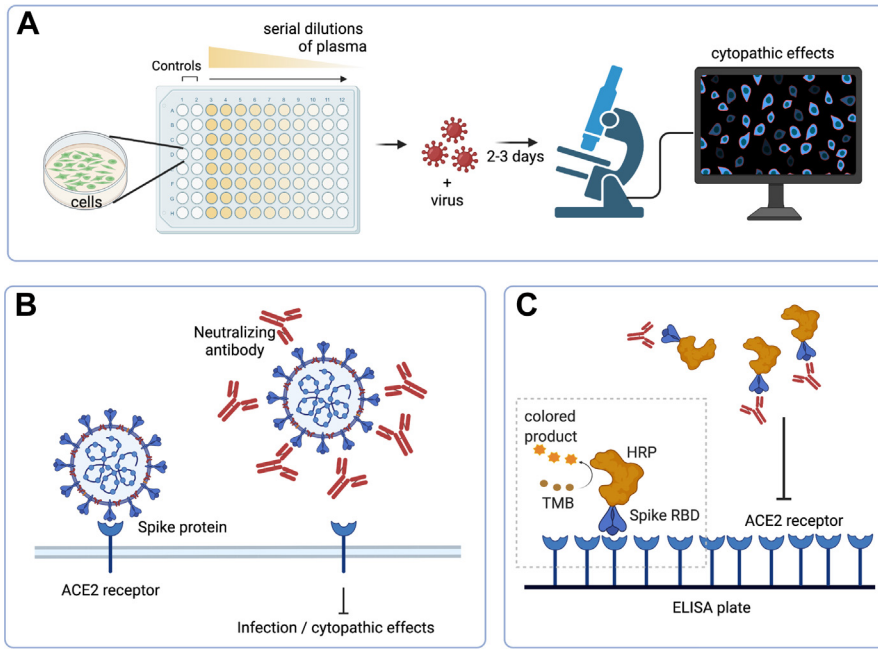

Fig. 5. Neutralization assays. (*A*) A neutralization assay is typically set up using cells cultured in a multiwell plate. The ability of serial dilutions of test serum to interfere with virus-induced cytopathic effects using a predetermined dose of infectious virus is measured. (*B*) Neutralization assay detects the presence of antibodies, which can prevent the infection or cell entry of infectious SARS-CoV-2. Alternatively, reporter viruses pseudotyped with the SARS-CoV-2 spike protein and that rely on spike protein for cell entry are used at a lower biosafety level. (*C*) Surrogate neutralization assay relies on measuring the ability of the specimen to interfere with the binding of surface-bound ACE2 receptor with spike protein or RBD in a competitive ELISA. (TMB - 3,3′,5,5′-Tetramethylbenzidine)

NEUTRALIZATION ASSAYS

A virus neutralization test (VNT) is a serologic test used to quantify the subset of antibodies that can prevent viral infection (Fig. 5). Such antibodies are called neutralizing antibodies, and in the case of SARS-CoV-2 infection or vaccination, they interfere with binding to its cellular receptor ACE2 and inhibit viral entry. Conventional VNTs are used alongside an infectivity assay (eg, plaque assay) to assess the ability of antibodies to inhibit viral replication or neutralize viral infection, which takes 2 to 4 days to complete. Surrogate VNTs that measure the ability of antibodies to block the interaction between the spike and ACE2 proteins have also been devised. Seropositivity against spike protein measured by commercial assays does correlate with neutralization activity.[40,41] The specimens that did not correlate with neutralization activity also exhibited greater discordance among serologic assays from different manufacturers, suggesting that a neutralization assay could also be used for improving the specificity of a conventional serologic assay because other common human betacoronaviruses do not use ACE2 as a receptor. Because of the dimeric nature of secreted IgA in saliva, it has been shown to be 15 times more potent at neutralization than its monomeric form in plasma.[42] This highlights the potential value of measuring isotype-specific neutralization, which is typically not done. Neutralization assays have primarily been

used in research, epidemiologic studies, or vaccine development; their role in routine clinical diagnostics is yet to be defined.

Cellular Virus Neutralization Assays

Conventional VNTs measure the infection of a susceptible cell line with a defined amount of a specific replication competent SARS-CoV-2 strain in the presence of varying dilutions of the plasma. Multiple viral strains may be used to assess neutralization breadth. The resulting infectious virions are quantified using a plaque assay that can take an additional 2 to 4 days. Alternatively, cytopathic effects are observed and the estimated dilution at which 50% of the wells show a cytopathic effects is reported as tissue-culture infectious dose (TCID50). The neutralizing titer is reported as the dilution required to produce a 50% reduction in infectious virions (PRNT50). Although this is the gold standard approach, it is a labor-intensive protocol that takes several days and needs to be performed in specialized biosafety facilities, because SARS-CoV-2 culture requires a higher biosafety level (BSL3). Recently, a high throughput label-free optical approach called laser force cytology that examines cellular deformability using optical tweezers in a microfluidic channel (Radiance, LumaCyte, Charlottesville, VA) has been adapted to count virally infected cells to automate the readout of VNTs.[43] To overcome the BSL3 requirements, pseudotyped retroviruses or replication-defective VSV particles have been engineered that use the SARS-CoV-2 spike for cell entry. The pseudotyped viruses are used in neutralization assays in a conventional BSL2 laboratory and show good agreement with assays using replication-competent SARS-CoV-2.[44] Furthermore, pseudotyped viruses have also been engineered to express a fluorescent or luciferase reporter for ease of measurement and scalability in a clinical setting. The IMMUNOCOV assay that uses pseudotyped VSV-G engineered with a luciferase reporter is commercially available; sufficient virus reagent has been banked to test 5 million clinical samples.[45] VNTs based on surrogate engineered viruses are readily adapted to study neutralization of variants by incorporating spike mutations from variants of interest.

Surrogate Virus Neutralization Assays

Surrogate VNTs that assess the ability of antiviral antibodies to inhibit the interaction between the viral receptor (ACE2) and the spike protein have been devised (see **Fig. 5**C). Surrogate VNT assays may miss neutralizing antibodies that interfere with downstream steps of cell entry following ACE2 receptor binding involving membrane fusion and cell entry. Thus, the full spectrum of neutralizing capacity is most reliably measured using neutralization assays that rely on a live virus. ELISA-format surrogate VNTs require the lowest biosafety level and yield a result within hours but may miss samples with lower neutralizing capacity. Once such assay, cPass (GenScript), has received an FDA EUA.[22] Updated ELISA assays that assess the neutralization of emerging variants are under development (Axim Biotechnologies, San Diego, CA). Surrogate VNTs can also be implemented using other rapid approaches including LFAs (eg, NeuCOVIX, Axim Biotechnologies).

FUTURE DIRECTIONS

We have highlighted selected alternative approaches to the serologic diagnosis of SARS-CoV-2. A broad variety of biosensors harnessing nanoscale phenomena, nanopore physics, oligonucleotide chemistry, or next-generation sequencing have been proposed in the literature, some of which have been applied to SARS-CoV-2 serology.[46–48] Nanomaterial phenomena have also been exploited to enhance the

performance of LFAs.[49] Although such approaches can be applied toward improving the characterization of the antibody response, a serologic assay does not provide complete information about humoral immunity. For instance, persistent antigen-specific memory B cells are not directly assessed by serologic tests. Furthermore, the cellular immune response consisting of $CD4^+$ and $CD8^+$ T cells is integral to the immune response, and a comprehensive assessment may be required to better assess infection risk. The T-Detect COVID test (Adaptive Biotechnologies, Seattle WA) based on the analysis of the T-cell repertoire by next-generation sequencing is a step in this direction.[50] Although the bulk of the serologic diagnostics have focused on blood specimens, it has been shown that the antibodies to SARS-CoV-2 found in the saliva do correlate well with levels in the blood.[51] Serologic monitoring of saliva may offer a noninvasive alternative to monitor seropositivity at a population scale.[52] Rapid advances have been made in SARS-CoV-2 serology, but concomitant measurement of cellular immunity is critical in obtaining a comprehensive picture of immunity in the context of natural infection, vaccine-induced protection, or population surveys of immunity. Use of novel or alternate technologies is required to develop assays for clinically scalable and multiplexed assessment of immune function in COVID-19.

CLINICS CARE POINTS

- The clinical utility of serological assays is currently limited in the context of SARS-CoV-2, but they have been most impactful in seroprevalence studies. Further studies are needed to determine the correlates of protection.
- The adoption of emerging technologies for comprehensive assessment of the immune response including assessment of the T cell response will be needed to provide better correlates of immunity and protection.

ACKNOWLEDGMENTS

All figures were created with BioRender.com

DISCLOSURE

R. Patel and S. Khare are affiliated with Micelio Labs, India, a technology incubator focused on artificial intelligence, health care, and diagnostics.

REFERENCES

1. FindDx test directory. Available at: https://www.finddx.org/test-directory/. Accessed May 23, 2021.
2. EUAs: serology and other adaptive immune response tests for SARS-CoV-2. Available at: https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-serology-and-other-adaptive-immune-response-tests-sars-cov-2#individual-serological. Accessed June 17, 2021.
3. Sethuraman N, Jeremiah SS, Ryo A. Interpreting diagnostic tests for SARS-CoV-2. JAMA 2020;323(22):2249–51.
4. Maine GN, Lao KM, Krishnan SM, et al. Longitudinal characterization of the IgM and IgG humoral response in symptomatic COVID-19 patients using the Abbott Architect. J Clin Virol 2020;133:104663.

5. Norman M, Gilboa T, Ogata AF, et al. Ultrasensitive high-resolution profiling of early seroconversion in patients with COVID-19. Nat Biomed Eng 2020;4(12):1180–7.
6. Orner EP, Rodgers MA, Hock K, et al. Comparison of SARS-CoV-2 IgM and IgG seroconversion profiles among hospitalized patients in two US cities. Diagn Microbiol Infect Dis 2021;99(4):115300.
7. Sterlin D, Mathian A, Miyara M, et al. IgA dominates the early neutralizing antibody response to SARS-CoV-2 n.d. Sci Transl Med 2021. https://doi.org/10.1101/2020.06.10.20126532.
8. Van Elslande J, Oyaert M, Ailliet S, et al. Longitudinal follow-up of IgG anti-nucleocapsid antibodies in SARS-CoV-2 infected patients up to eight months after infection. J Clin Virol 2021;136:104765.
9. Lumley SF, Wei J, O'Donnell D, et al. The duration, dynamics and determinants of SARS-CoV-2 antibody responses in individual healthcare workers. Clin Infect Dis 2021. https://doi.org/10.1093/cid/ciab004.
10. Crawford KHD, Dingens AS, Eguia R, et al. Dynamics of neutralizing antibody titers in the months after SARS-CoV-2 infection. J Infect Dis 2020. https://doi.org/10.1093/infdis/jiaa618.
11. Röltgen K, Powell AE, Wirz OF, et al. Defining the features and duration of antibody responses to SARS-CoV-2 infection associated with disease severity and outcome. Sci Immunol 2020;5(54). https://doi.org/10.1126/sciimmunol.abe0240.
12. Turner JS, Kim W, Kalaidina E, et al. SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans. Nature 2021. https://doi.org/10.1038/s41586-021-03647-4.
13. Bermingham WH, Wilding T, Beck S, et al. SARS-CoV-2 serology: test, test, test, but interpret with caution. Clin Med 2020;20(4):365–8.
14. Mina MJ, Parker R, Larremore DB. Rethinking Covid-19 test sensitivity: a strategy for containment. N Engl J Med 2020;383(22):e120.
15. Geers D, Shamier MC, Bogers S, et al. SARS-CoV-2 variants of concern partially escape humoral but not T-cell responses in COVID-19 convalescent donors and vaccinees. Sci Immunol 2021;6(59). https://doi.org/10.1126/sciimmunol.abj1750.
16. Harvey WT, Carabelli AM, Jackson B, et al. SARS-CoV-2 variants, spike mutations and immune escape. Nat Rev Microbiol 2021;19(7):409–24.
17. Trombetta BA, Kandigian SE, Kitchen RR, et al. Evaluation of serological lateral flow assays for severe acute respiratory syndrome coronavirus-2. BMC Infect Dis 2021;21(1):1–14.
18. EUA authorized serology test performance. Available at: https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/eua-authorized-serology-test-performance. Accessed June 21, 2021.
19. Liu G, Rusling JF. COVID-19 antibody tests and their limitations. ACS Sens 2021;6(3):593–612.
20. Orsi A, Pennati BM, Bruzzone B, et al. On-field evaluation of a ultra-rapid fluorescence immunoassay as a frontline test for SARS-CoV-2 diagnostic. J Virol Methods 2021;295:114201.
21. Ikegami S, Benirschke RC, Fakhrai-Rad H, et al. Target specific serologic analysis of COVID-19 convalescent plasma. PLoS One 2021;16(4):e0249938.
22. Murray MJ, McIntosh M, Atkinson C, et al. Validation of a commercially available indirect assay for SARS-CoV-2 neutralising antibodies using a pseudotyped virus assay. J Infect 2021;82(5):170–7.

23. Santano R, Barrios D, Crispi F, et al. Agreement between commercially available ELISA and in-house Luminex SARS-CoV-2 antibody immunoassays. bioRxiv. Sci Rep 2021. https://doi.org/10.1101/2021.03.09.21252401.
24. Martinaud C, Hejl C, Igert A, et al. Evaluation of the Quotient® MosaiQ™ COVID-19 antibody microarray for the detection of IgG and IgM antibodies to SARS-CoV-2 virus in humans. J Clin Virol 2020;130:104571.
25. Zhu C, Yang G, Li H, et al. Electrochemical sensors and biosensors based on nanomaterials and nanostructures. Anal Chem 2015;87(1):230–49.
26. Chiang SH, Tu M, Cheng J, et al. Development and validation of a highly sensitive and specific electrochemical assay to quantify anti-SARS-CoV-2 IgG antibodies to facilitate pandemic surveillance and monitoring of vaccine response. medRxiv 2020. https://doi.org/10.1101/2020.11.12.20230656.
27. Torrente-Rodríguez RM, Lukas H, Tu J, et al. SARS-CoV-2 RapidPlex: a graphene-based multiplexed telemedicine platform for rapid and low-cost COVID-19 diagnosis and monitoring. Matter 2020;3(6):1981–98.
28. Rashed MZ, Kopechek JA, Priddy MC, et al. Rapid detection of SARS-CoV-2 antibodies using electrochemical impedance-based detector. bioRxiv 2020. https://doi.org/10.1101/2020.08.10.20171652.
29. Ali MA, Hu C, Jahan S, et al. Sensing of COVID-19 antibodies in seconds via aerosol jet nanoprinted reduced-graphene-oxide-coated 3D electrodes. Adv Mater 2021;33(7):e2006647.
30. Yakoh A, Pimpitak U, Rengpipat S, et al. Paper-based electrochemical biosensor for diagnosing COVID-19: detection of SARS-CoV-2 antibodies and antigen. Biosens Bioelectron 2020;176:112912.
31. Burbelo PD, Riedo FX, Morishima C, et al. Sensitivity in detection of antibodies to nucleocapsid and spike proteins of severe acute respiratory syndrome coronavirus 2 in patients with coronavirus disease 2019. J Infect Dis 2020;222(2):206–13.
32. Burbelo PD, Ching KH, Klimavicz CM, et al. Antibody profiling by luciferase immunoprecipitation systems (LIPS). J Vis Exp 2009;(32). https://doi.org/10.3791/1549.
33. Elledge SK, Zhou XX, Byrnes JR, et al. Engineering luminescent biosensors for point-of-care SARS-CoV-2 antibody detection. Nat Biotechnol 2021. https://doi.org/10.1038/s41587-021-00878-8.
34. Huang P-S, Boyken SE, Baker D. The coming of age of de novo protein design. Nature 2016;320–7. https://doi.org/10.1038/nature19946.
35. Quijano-Rubio A, Yeh H-W, Park J, et al. De novo design of modular and tunable protein biosensors. Nature 2021;591(7850):482–7.
36. Dzimianski JV, Lorig-Roach N, O'Rourke SM, et al. Rapid and sensitive detection of SARS-CoV-2 antibodies by biolayer interferometry. Sci Rep 2020;10(1):21738.
37. Sun Y, Fan X. Optical ring resonators for biochemical and chemical sensing. Anal Bioanal Chem 2011;399(1):205–11.
38. Miyara M, Charuel J-L, Mudumba S, et al. Detection in whole blood of autoantibodies for the diagnosis of connective tissue diseases in near patient testing condition. PLoS One 2018;13(8):e0202736.
39. Donato LJ, Theel ES, Baumann NA, et al. Evaluation of the Genalyte Maverick SARS-CoV-2 multi-antigen serology panel. J Clin Virol Plus 2021;100030. https://doi.org/10.1016/j.jcvp.2021.100030.
40. Suhandynata RT, Hoffman MA, Huang D, et al. Commercial serology assays predict neutralization activity against SARS-CoV-2. Clin Chem 2021;67(2):404–14.

41. Luchsinger LL, Ransegnola BP, Jin DK, et al. Serological assays estimate highly variable SARS-CoV-2 neutralizing antibody activity in recovered COVID-19 patients. J Clin Microbiol 2020;58(12). https://doi.org/10.1128/JCM.02005-20.
42. Wang Z, Lorenzi JCC, Muecksch F, et al. Enhanced SARS-CoV-2 neutralization by dimeric IgA. Sci Transl Med 2021;13(577). https://doi.org/10.1126/scitranslmed.abf1555.
43. Hebert CG, Rodrigues KL, DiNardo N, et al. Viral infectivity quantification and neutralization assays using laser force cytology. Methods Mol Biol 2021;2183:575–85.
44. Cantoni D, Mayora-Neto M, Temperton N. The role of pseudotype neutralization assays in understanding SARS CoV-2. Oxf Open Immunol 2021;2(1):iqab005.
45. Vandergaast R, Carey T, Reiter S, et al. Development and validation of IMMUNOCOVTM: a high-throughput clinical assay for detecting antibodies that neutralize SARS-CoV-2. bioRxiv 2020. https://doi.org/10.1101/2020.05.26.117549.
46. Zhang Z, Wang X, Wei X, et al. Multiplex quantitative detection of SARS-CoV-2 specific IgG and IgM antibodies based on DNA-assisted nanopore sensing. Biosens Bioelectron 2021;181:113134.
47. Bhalla N, Pan Y, Yang Z, et al. Opportunities and challenges for biosensors and nanoscale analytical tools for pandemics: COVID-19. ACS Nano 2020;14(7):7783–807.
48. Xu GJ, Kula T, Xu Q, et al. Viral immunology. Comprehensive serological profiling of human populations using a synthetic human virome. Science 2015;348(6239):aaa0698.
49. Soh JH, Chan H-M, Ying JY. Strategies for developing sensitive and specific nanoparticle-based lateral flow assays as point-of-care diagnostic device. Nano Today 2020;30:100831.
50. Dalai SC, Dines JN, Snyder TM, et al. Clinical validation of a novel T-cell receptor sequencing assay for identification of recent or prior SARS-CoV-2 infection. medRxiv 2021. https://doi.org/10.1101/2021.01.06.21249345.
51. Isho B, Abe KT, Zuo M, et al. Persistence of serum and saliva antibody responses to SARS-CoV-2 spike antigens in COVID-19 patients. Sci Immunol 2020;5(52). https://doi.org/10.1126/sciimmunol.abe5511.
52. Pisanic N, Randad PR, Kruczynski K, et al. COVID-19 serology at population scale: SARS-CoV-2-Specific antibody responses in saliva. J Clin Microbiol 2020;59(1). https://doi.org/10.1128/JCM.02204-20.