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AACC Practical Recommendations for Implementing and Interpreting SARS-CoV-2 EUA and LDT Serologic Testing in Clinical Laboratories

Y. Victoria Zhang[1], Joseph Wiencek[2], Qing H. Meng[3], Elitza S. Theel[4], Nikolina Babic[5], Lusia Sepiashvili[6], Nicole D. Pecora[7], Patricia Slev[8], Andrew Cameron[9], David D. Koch[10], Danijela Konforte[11], Stacy Melanson[12], Hubert Vesper[13], Ted Schutzbank[14], Caitlin Ondracek[15], Gyorgy Abel[16], Khosrow Adeli[17]

[1]Y. Victoria Zhang, PhD, MBA, DABCC, FAACC
Chairperson of the Task Force for Serologic Testing
Department of Pathology and Lab Medicine
University of Rochester Medical Center
victoria_zhang@urmc.rochester.edu

[2]Joesph R. Wiencek, PhD
Department of Pathology, Microbiology, and Immunology
Vanderbilt University Medical Center
joe.wiencek@vumc.org

[3]Qing H. Meng, PhD
Department of Laboratory Medicine, Division of Pathology
and Laboratory Medicine
The University of Texas/MD Anderson Cancer Center
QHMeng@mdanderson.org

[4]Elitza S. Theel, PhD
Department of Laboratory Medicine and Pathology
Mayo Clinic
theel.elitza@mayo.edu

[5]Nikolina Babic, PhD
Department of Pathology and Laboratory Medicine
Medical University of South Carolina
babic@musc.edu

[6]Lusia Sepiashvili, PhD
Departments of Biochemistry and Laboratory Medicine & Pathobiology
The Hospital for Sick Children/University of Toronto
lusia.sepiashvili@sickkids.ca

[7]Nicole D. Pecora, MD, PhD
Department of Pathology and Laboratory Medicine
University of Rochester Medical Center
Nicole_Pecora@URMC.Rochester.edu

[8]Patricia R. Slev, PhD
Immunology Core Laboratory
ARUP Institute for Clinical and Experimental Pathology
patricia.slev@aruplab.com

[9]Andrew Cameron, PhD
Department of Clinical Microbiology
University of Rochester Medical Center
Andrew_Cameron@URMC.Rochester.edu

[10]David Koch, PhD
Department of Pathology and Laboratory Medicine
Emory University
ddkoch@emory.edu

[11]Danijela Konforte, PhD
LifeLabs Medical Laboratories
Danijela.Konforte@lifelabs.com

[12]Stacy E.F. Melanson, MD, PhD
Department of Pathology
Brigham and Women's Hospital/Harvard Medical School
semelanson@bwh.harvard.edu

[13]Hubert Vesper, PhD
Division of Laboratory Sciences, Clinical Chemistry Branch
Centers for Disease Control and Prevention
Hav2@cdc.gov

[14]Ted E. Schutzbank, PhD, ABMM
Meridian Bioscience
ted.schutzbank@meridianbioscience.com

[15]Caitlin R. Ondracek, PhD
AACCcondracek@aacc.org

[16]Gyorgy Abel, MD, PhD
Departments of Laboratory Medicine and Pathology
Lahey Hospital & Medical Center
gyorgy_abel@hms.harvard.edu

[17]Khosrow Adeli, PhD, FCACB, DABCC
Departments of Biochemistry and Laboratory Medicine & Pathobiology
The Hospital for Sick Children/University of Toronto
khosrow.adeli@sickkids.ca

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1. Executive Summary

The clinical laboratory continues to play a critical role in managing the coronavirus disease 2019 (COVID-19) pandemic. Currently, molecular testing is the method of choice for detection of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in respiratory specimens and diagnosis of active infection. Over the past several months, numerous serologic tests have become available. Notably, both the performance characteristics of these assays and their clinical utility continue to be defined and are evolving in real-time during this pandemic. Interim guidelines for appropriate serologic test utilization and interpretation have been published by multiple professional organizations. However, no guidelines to date provide comprehensive, literature-based recommendations for laboratory professionals who are directly involved in the selection, validation, implementation, and quality management of Food and Drug Administration (FDA) emergency use authorization (EUA) or laboratory developed tests (LDTs) in clinical laboratories. Building on the AACC position statement entitled "AACC Recommendations for SARS-CoV-2 Serology Testing", we convened a panel of experts from clinical chemistry, microbiology, and immunology laboratories. This group also includes experts from the *in vitro* diagnostics (IVD) industry and regulatory agencies. The outcome is these expert-based practical recommendations to support laboratory professionals in implementing serologic testing amidst the rapidly-evolving COVID-19 pandemic.

The currently available serologic tests and platforms with FDA EUA are presented, including a discussion of neutralizing antibody (nAb) assays. Additionally, information on serologic assay design, antibody classes (i.e., IgM, IgG, IgA, total immunoglobulins), and the kinetics of the humoral immune response are provided. Verification and validation of both EUA and LDTs are described, with a focus on the number and type of samples required, selection of patient populations, design of cross-reactivity studies and acceptability criteria, among other topics.

Four indications for serologic testing are discussed: 1) supporting the diagnosis of COVID-19 and related sequelae (i.e., multisystem inflammatory syndrome in children [MIS-C]), 2) identifying potential convalescent plasma donors and manufacturing of convalescent plasma, 3) epidemiologic and seroprevalence studies, and 4) vaccine efficacy studies. This document summarizes the utility of serologic testing for each of these indications and provides detailed information that may aid laboratorians when discussing serologic assay selection and implementation with clinical colleagues.

Recommendations for quality management, result interpretation, reporting comments, and the role of orthogonal testing are also outlined.

2. Introduction

As of November 7, 2020, over 50 million cases and 1 million deaths have been reported to the World Health Organization (WHO) worldwide as a result of COVID-19. In the United States, over 9.5 million confirmed cases and 234,000 deaths were reported to the WHO[1] . COVID-19 is caused by SARS-CoV-2, a member of the genus *Betacoronavirus* (Beta-CoV) and a close relative of severe acute respiratory syndrome (SARS) coronavirus (CoV) and Middle East Respiratory Syndrome MERS-CoV[2,3]. Other genus members include the seasonal human CoVs OC43 and HKU1.

While molecular testing, including real-time reverse transcription polymerase chain reaction (RT-PCR) performed on respiratory specimens is routinely used for diagnosis of active infection with SARS-CoV-2, serologic testing is used to determine recent or previous antibody immune response to the virus.

Rapid development and implementation of SARS-CoV-2 antibody assays has been ongoing during the pandemic. Typically, commercially-produced IVD assays undergo thorough evaluation by the FDA before distribution to clinical laboratories. However, during an emergent national or international outbreak, the

Secretary of Health and Human Services (HHS) may declare a public health emergency, which under section 564 of the Federal Food, Drug and Cosmetic Act[4] allows the FDA to grant EUA of unapproved medical products or devices for the purpose of diagnosing, treating, or preventing the disease **(Table 1)**.

The HHS Secretary issued a public health emergency declaration for SARS-CoV-2 on January 31st, 2020. Notably, while the FDA immediately required EUA for SARS-CoV-2 molecular tests, EUA was only recommended for serologic assays because such tests were not considered diagnostic, and per the FDA, were intended to only be used for seroprevalence and epidemiologic studies. This lenient regulatory environment for serologic assays led to the development and availability of over 200 antibody tests for SARS-CoV-2, of which 53 have obtained EUA as of October 20, 2020. Unfortunately, multiple reports began emerging of serologic assays with poor performance characteristics or lack of appropriate validation, prompting the FDA to update their policy on May 4th, 2020 to require EUA for all commercially available serologic assays[5] . The FDA also provided a list of serologic assays that should not be distributed or used due to either poor performance or lack of EUA submission. Importantly, the FDA reserved the right to revoke EUA status at any time should evidence of poor assay performance be documented. To date, EUA status has been revoked for 2 previously authorized serologic tests[6–8].

As a result of the limited FDA oversight of serologic tests, as well as due to incomplete understanding of kinetics, relevance, and durability of humoral immune response in COVID-19, questions have arisen in the clinical community on how to best utilize these assays as well as result interpretation in management of patients. In an effort to provide assistance for such scenarios, a panel of clinical diagnostic laboratory and industry experts from across North America reviewed the current literature to provide expert opinions on how to best implement these assays to support patient care needs.

This manuscript provides the most up-to-date understanding of host immune response to SARS-CoV-2, the associated antibody kinetics, and the currently available assays with EUA. Clinical utility and assay limitations are discussed to help laboratories select the appropriate test(s) for their purposes and targeted population needs. The process and considerations necessary to verify or validate either EUA or LDT serologic tests in a clinical setting are described. In addition, test interpretation, orthogonal testing strategies, and issues of laboratory biosafety are outlined. It is the intention of this document to provide a comprehensive reference for laboratory professionals and healthcare workers to appropriately implement these assays in the clinical laboratory and interpret the results to serve their patient needs during this pandemic. Additionally, given the more frequent occurrence of outbreaks associated with either vector-borne (e.g., Zika virus, dengue virus, etc.) or respiratory pathogens, this document will be a useful resource in planning for similar scenarios in the future.

3. SARS-CoV-2 and the Humoral Immune Response

3.1. Antigenic Targets

The approximately 30-Kb genome of SARS-CoV-2 encodes four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). The S protein is a 180-kD homotrimeric glycoprotein that extends from the viral surface, producing the namesake "corona" morphology of the virion. Structurally, the S protein is divided into S1 and S2 subunits. The former is the most distal from the viral surface and contains the receptor binding domain (RBD), which interacts with the human angiotensin-converting enzyme 2 (ACE2) receptor and mediates host cell entry[9]. The N protein is the most abundantly expressed immuno-dominant protein, which stabilizes viral RNA. Both S and N proteins have multiple conserved regions and are the most frequently used targets for serologic assays[9–11]. Notably, these proteins are also the main targets of antibody response in SARS-CoV-1 and seasonal human CoVs[10].

SARS-CoV-2 uses the S protein to bind and mediate entry into target cells through interactions with the

ACE2 receptor. Antibodies directed against the RBD of the S protein, and the S1 and S2 regions of the S protein are detected by Blockade-of-Binding (BoB) and neutralizing antibody assays[12], which are discussed below. Original studies with SARS-CoV-1 indicated that receptor binding and proteolytic cleavage at the S1/S2 junction triggers a conformational change in S2, which mediates host cell entry via a membrane fusion peptide sequence located within S2. The distal regions of the S protein (S1, RBD) are the least conserved among members of Beta-CoV (e.g., SARS-CoV-1, MERS-CoV) and are therefore more likely to induce a SARS-CoV-2 specific antibody response. Overall, the SARS-CoV-2 S protein shares 76% homology with SARS-CoV-1, whereas for the Beta-CoVs, including OC43 and HKU1, the SARS-CoV-2 S protein shares only about 30% homology[13].

The N protein functions in both the packaging and replication of viral nucleic acid and is the most abundant SARS-CoV-2 protein, which makes it a popular target of both serologic and viral antigen assays. The N protein is highly conserved between SARS-CoV-2 and related Beta-CoV, demonstrating 99% identity with related bat coronavirus RaTG13 and approximately 90% identity with SARS-CoV-1[14]. In contrast, the SARS-CoV-2 N protein shares approximately only 33% identity with seasonal Beta-CoVs, including OC43 and HKU1[13].

3.2. Antibody Classes

Commercial EUA SARS-CoV-2 serologic assays are available in many formats geared towards detecting either total antibodies or specific antibody subclasses (IgG, IgM, or IgA)[15–20]. A subset of antibodies to SARS-CoV-2 that have recently gained attention are neutralizing antibodies (nAbs). The detection of IgM antibodies may indicate a more recent infection; however, the dynamics of the IgM antibody response continue to be defined at present, and frequently IgG- and IgM-class antibodies develop simultaneously. Currently, there is insufficient data to support the clinical utility of standalone IgM testing[21]. IgA based assays have been reported to suffer from lower specificity as compared to IgG-based assays[13], and are currently not recommended for use by either the Centers for Disease Control and Prevention (CDC) or the Infectious Diseases Society of America (IDSA)[22,23]. Some studies indicated a total assay may be more sensitive for most infections[15–18]. Over time, it may be important to characterize and evaluate the performance of assays in samples that are IgM negative and IgG positive to ensure that assays remain useful for the purpose of population-level studies as the pandemic progresses, and more individuals are expected to have lower IgM levels.

Given that most clinical laboratories are not as familiar with nAbs as other antibody classes or how to specifically detect them using antibody neutralization assays, these tests are described in more detail below.

Generally, antibody response to a specific virus can be split into two broad categories – binding and neutralizing. While binding antibodies are able to inactivate the virus through complement activation or opsonization, nAbs are able to inhibit viral replication independent of other components of the immune system by binding to regions of the virus that directly interact with host cell receptors, effectively blocking viral entry and inhibiting replication. The reference standard for detection of viral nAbs are plaque reduction neutralization tests (PRNTs). These assays are technically challenging to perform, require live virus culture, and have a prolonged turnaround time, anywhere from days to weeks depending on the virus. Classically, live virus is incubated with serial dilutions of patient sample (i.e., serum or plasma), added to a monolayer of virus-susceptible cells, and subsequently overlaid with a semi-solid medium to limit virus diffusion. Following incubation (the duration of which depends on viral growth characteristics), the monolayer is stained and observed for the formation of plaques (i.e., areas in the monolayer that no longer have cells due to virus-induced apoptosis or death). The plaques are quantified and compared to the number of plaques in a virus-only control well. The end-point antibody titer is determined as the reciprocal of the last patient sample dilution that shows either 50% (PRNT50) or 90% (PRNT90) reduction

in plaques compared to the control (choice of percent plaque reduction endpoint of 50% or 90% varies depending on study/institution). An additional challenge associated with PRNTs is that, depending on the virus, enhanced biosafety facilities may be required for culture, including for SARS-CoV-2, which is a biosafety level (BSL) 3 pathogen. As a result, PRNTs are largely relegated to research and certain public health laboratories, including the CDC.

To overcome the challenges associated with PRNTs, alternative methods to detect nAbs have been and continue to be developed. These alternatives include the generation of pseudovirus-based live-cell neutralization assays able to be completed at lower biosafety levels (i.e., BSL2) or by using BoB immunoassays, among other methods. Pseudovirus neutralization assays utilize a chimeric, avirulent/non-pathogenic virus expressing the surface binding protein from the virus of interest (i.e., trimeric SARS-CoV-2 S glycoprotein), allowing for all steps of the assay to be performed at BSL2, even when assaying for antibodies to a BSL3 pathogen[24]. The viral backbone of these pseudoviruses is typically based on either retroviral or lentiviral vectors, or are recombinant rhabdoviruses, such as vesicular stomatitis virus for which the surface envelope glycoprotein has been deleted and replaced with the SARS-CoV-2 S protein. The read-out for pseudovirus-based neutralization assays is usually based on an incorporated reporter system within the chimeric virus, examples of which include the expression of different luciferase enzymes, green fluorescent protein, and Beta-galactosidase lacZ operon. These reporter systems allow for a shorter result turnaround time, as the outcome is not dependent on plaque formation. They also provide a more objective result as compared to classic PRNTs given that resulting reporter signal can be detected by luminometry, fluorescence microscopy, or fluorescence-activated cell sorting.

Although an improvement over classic PRNTs, pseudovirus-based neutralization assays are still complex methods associated with significant analytical variability and are challenging to support in most clinical laboratories given the need to maintain cell and viral cultures. As an alternative, high-throughput BoB immunoassays for detection of nAbs have become commercially available. Although designs may vary, BoB assays typically incorporate solid-phase immobilized human ACE2 receptor molecules and enzyme-labeled SARS-CoV-2 RBD antigens. Patient samples (serum or plasma) are first pre-incubated with labeled RBD antigens, and then added to the solid-phase ACE2 receptor. In the absence of nAbs, the enzyme-labeled RBD will bind ACE2 and a signal will be generated in the presence of substrate. In contrast, if nAbs are present, the interaction between RBD and ACE2 will be inhibited and an enzymatic signal will not be generated. These assays can be performed in a 96-well format and can be automated on a variety of different immunoassay processing platforms.

nAb assays will play a significant role in the development of and clinical trials for SARS-CoV-2 vaccines and have already been invaluable in research studies probing the host immune response to infection[25]. Their role in the clinical laboratory will likely be more limited, given the challenges associated with maintaining many of these methods, lack of standardization, currently unknown correlation of nAb titers with protective immunity, and the emergence of high-throughput, fully automated quantitative nAb assays.

3.3. Antibody Kinetics

Understanding the kinetics of the antibody response to SARS-CoV-2 relative to time post infection or post symptom onset is a pre-requisite for choosing the right serologic test for the laboratory and accurate interpretation of test results. Work in this area has gained significant attention from the scientific, medical, and public health communities, and novel findings enhancing our understanding of this topic are being published on a regular basis. Consensus has not yet been attained on many aspects of the SARS-CoV-2 antibody kinetics and long-term data remain incomplete. The overall kinetics of the antibody responses against SARS-CoV-2, as we currently understand them, are depicted in **Figure 1**.

Whereas viral RNA and antigens may be detectable in the incubation phase following infection, detection

of antibodies during this timeframe is unlikely. Serologic tests that are performed on samples collected too soon following infection will likely be negative. Several serologic studies published to date have demonstrated that many individuals develop an IgM/IgA response within 7-14 days of symptom onset, while the IgG response follows closely behind[26,27]. IgM/IgA levels may peak and decline earlier than IgG, often within weeks of symptom onset[28–31]. Serologic assay sensitivity is much better during the convalescent phase of disease when high IgM and IgG levels are produced in the blood (three to four weeks post symptom onset). The precise kinetics of the SARS-CoV-2 immune response remain unclear. Several studies have demonstrated that the IgG antibody response in symptomatic patients is more robust than in asymptomatic patients. Numerous studies have demonstrated that the antibody concentrations decline at varying rates and consequently the length of time that IgG Ab remains detectable is variable[32–40]. As experience with SARS-CoV-2 serologic testing begins to extend to long-term follow-up of patients, some studies have shown that up to 40% of individuals with molecular test-confirmed infections become IgG seronegative by the early convalescent phase[41]. In contrast, additional studies show that in most patients have demonstrated that anti-SARS-CoV-2 antibodies declined over time, but were still detectable for months post-infection[42]. Due to these inconsistencies, the precise kinetics of the SARS-CoV-2 antibody response remains unclear and requires further elucidation as the pandemic continues.

Currently, it remains unknown what implications that waning or subsequently undetectable antibodies levels have on the durability of an effective immune response for SARS-CoV-2[43–45]. Early work on the seasonal human CoV 229E demonstrated similar IgA and IgG kinetics to those observed for SARS-CoV-2[15], whereas the antibody response to SARS-CoV-1 peaks later (four months after infection) and remains detectable for 16 months, at which time many individuals cease to have detectable levels of antibodies[47]. Therefore, it appears that the immune response and the level of potential protective immunity provided by measurable antibodies against CoVs may be virus specific[36].

3.4. Antibody Kinetics in Special Populations

Differences in antibody kinetics may be observed in specific sub-populations and across the spectrum of disease severity and patient immunocompetence. Studies have shown that roughly 4-10% of the population with confirmed SARS-CoV-2 infection either have an undetectable or delayed antibody response as measured by current serologic assays[42]. It is becoming increasingly apparent that negative antibody results can be attributed to a number of factors, including time of sampling relative to days post symptom onset, assay sensitivity, assay design, and inter-individual variability[32,48]. There is growing evidence that, at least in the United States, individuals of different racial and ethnic backgrounds are affected to different degrees by SARS-CoV-2 infection[34,49,50].

Serologic testing for any pathogen in immunocompromised individuals has always presented unique challenges. The current literature indicates that the immunocompromised patient may be at increased risk for severe disease[51–54]. While the serologic response against SARS-CoV-2 in human immunodeficiency virus (HIV)-infected individuals has not been well described, one case report suggests that Immunocompromised patients (including cancer patients) may require 30 or more days or may never generate antibody levels that are detectable as positive by this assay, and therefore caution should be used when serologic testing is performed in this population[55]. Negative serology results should always be interpreted with caution in individuals who are immunocompromised. In addition, falsely negative serology results will lead to the wrong clinical decisions if used by themselves.

At least one study has demonstrated a significantly lower detection rate of SARS-CoV-2 antibodies in cancer patients compared to a control group of healthcare workers[56]. Benotmane and colleagues demonstrated that there is no impaired detection of the antibody response in kidney transplant recipients[57]. In contrast, a study from another group who investigated the SARS-CoV-2 antibody response in patients with chronic lymphocytic leukemia found that hypogammaglobulinemia was negatively

associated with SARS-CoV-2 IgG development[58].

In the case of pregnancy, women appear to be at increased risk of developing severe SARS-CoV-2 illness, but neonates born to mothers infected with SARS-CoV-2 do not appear to be similarly affected[59,60]. Gao and colleagues reported that of 24 infants born to SARS-CoV-2 infected mothers, 62.5% had detectable IgG and 25% had detectable IgM[61]. All of the babies born to infected mothers had detectable IgG at birth, and 45.5% had detectable IgM[61]. The presence of IgM suggests the possibility of vertical transmission. However, given that all neonates born to SARS-CoV-2 infected mothers tested negative for SARS-CoV-2 by molecular testing, it is not clear if vertical transmission of the virus occurs, and if it exists, it appears to be rare[62].

4. EUA Serologic Tests

4.1. Assay Designs

Several serologic methods for detection of SARS-CoV-2 antibodies have received FDA EUA, including lateral flow assays (LFA), enzyme-linked immunosorbent assays (ELISA), and chemiluminescent immunoassays/ chemiluminescent microparticle immunoassays (CIA/CMIA) and their fluorescent derivatives. Importantly, the aforementioned assays do not distinguish between binding antibodies and nAbs, the latter of which are known to be key components of viral protective immunity and are likely to play a similar role for SARS-CoV-2 infections[63,64].

In the initial months of the pandemic, most of the available antibody tests were rapid LFAs (akin to over-the-counter pregnancy tests) or ELISAs. LFAs utilize immunochromatographic chemistry to detect a target analyte within a liquid sample without the need of specialized instrumentation and can commonly be performed at the point-of-care (POC) (i.e., near-patient testing). Around the same time frame, several vendors and clinical laboratories designed manual or semi-automated 96-well plate ELISAs to detect SARS-CoV-2 antibodies. These two early methods were used to determine the preliminary antibody kinetics associated with SARS-CoV-2 infections[65,66]. The next SARS-CoV-2 antibody tests to become available were CIA/CMIA assays, performed on fully automated, high-throughput platforms[67,68]. The mechanisms of these methods are familiar to laboratory professionals and are illustrated in **Figure 2**. The EUA test characteristics are described in the following section.

4.2. Characteristics of EUA Serologic Tests

As of December 20, 2020, the FDA had granted EUA to 62 assays. Some key features of select assays are listed in **Table 2** (for additional information see Supplemental Table S1). Except for three EUA semi-quantitative assays, all the current assays are qualitative. The majority of serologic assays with EUA detect IgG-class antibodies, followed by IgM/IgG, total antibody, and IgM-only assays. All the EUA assays use serum, some accept plasma (with stated requirements for specific anticoagulants), and less frequently, whole-blood or dried blood spots. The most frequent antigen targeted by these assays is the S protein RBD, followed closely by S (including full S, S1, and S2), and N. Currently, only one assay uses all three antigens (Luminex xMAP SARS-CoV-2 Multi-Antigen IgG Assay), whereas most of the EUA assays utilize only one SARS-CoV-2 antigen.

The general characteristics of the selected assays with EUA, (as stated in the manufacturers' instructions for use [IFU]) are summarized in the supplemental **Table S1**. This table includes the positive and negative percent agreement (PPA and NPA), the extent of documented cross-reactivity, interference testing, and independent evaluations (as available). PPA and NPA are calculated tothe same as sensitivity and specificity but in this case convey that neither test being compared is a gold standard. For assays currently granted EUA, the manufacturers' stated PPA and NPA range from 89% - 100% and 96% – 100%, respectively. However, judging the relative performance of each EUA assay is complicated, as the approach and number of samples tested by each manufacturer varies widely and the patient populations upon which

these assays were characterized is not clear (e.g. disease severity). With respect to PPA, manufacturers have often stratified the performance of their assays according to day-from-symptom onset, or from the time post first positive molecular test result. The number of samples used in PPA determinations vary greatly (e.g. from <10 to >300) and the PPA often changes with respect to sample collection time. For NPA determination, EUAs have been based on the measured performance using a wide variation in the number of samples tested, from <50 to >5,000 unique specimens. These large variations in sample size and the different sample collection times contribute to challenges in selecting, implementing, and interpreting serologic testing by clinical laboratory professionals. Finally, as part of an FDA effort to provide independent evaluation of SARS-CoV-2 serologic assays, manufacturers of LFAs and ELISAs had the opportunity to submit their assays to the National Cancer Institute for evaluation using well-characterized specimens. These data were subsequently used as part of EUA submission data.

5. Clinical Utility and Limitations of SARS-CoV-2 Serologic Testing

Although SARS-CoV-2-specific serologic testing is not recommended as the primary approach for diagnosis of SARS-CoV-2 infection, four uses of this method have been suggested, including 1) supporting the diagnosis of COVID-19 and related sequelae (i.e., multisystem inflammatory syndrome in children [MIS-C]), 2) identifying potential convalescent plasma donors and manufacturing of convalescent plasma, 3) epidemiologic and seroprevalence studies, and 4) vaccine efficacy studies[21,22,69,70] **(Table 3)**.

5.1. Diagnosis

5.1.1. Utility

In general, serologic testing may be helpful to diagnose COVID-19 in patients presenting later in their disease course (e.g., >9-14 days post symptom onset), who repeatedly test negative or indeterminate by a molecular assay and have a clinical syndrome consistent with SARS-CoV-2 infection[23]. In such scenarios, it is likely that the viral load has decreased below the limit of detection by molecular testing, whereas antibody levels are increasing and ultimately detectable by serologic assays, with optimal sensitivity of serologic tests occurring at least 2-3 weeks post symptom onset[5,30,71,72]. It is important to note that sensitivity of an assay as evaluated in a severely ill patient population may not translate to an asymptomatic population which includes the translation of assay cutoffs indicated in the IFU in the laboratory.

Serologic testing has recently been recommended for diagnosis of MIS-C, a novel condition characterized by fever, inflammation, and multi-organ dysfunction exhibiting clinical features overlapping with Kawasaki disease[73]. Children infected with SARS-CoV-2 have been reported to have mild or no symptoms, therefore, MIS-C has emerged as a rare and potentially serious complication exhibiting temporal association with SARS-CoV-2 infection. MIS-C has been reported to manifest approximately 2-4 weeks after SARS-CoV-2 infection, often in children with no evidence of prior SARS-CoV-2 infection74–76.

Case definitions for MIS-C have been reported by the CDC, UK, and others[74,75,77–80]. Within the laboratory criteria, positive serology is included in the CDC case definition for MIS-C and is indicated in hospitalized individuals <21 years who present with fever, inflammation, and multi-system organ involvement following exclusion of other potential diagnoses[81]. In a recent systematic review, 75-100% of MIS-C cases had a positive SARS-CoV-2 serologic test, while only a minority were positive by molecular testing[82].

5.1.2. Limitations

Guidelines from the FDA, along with other international professional bodies, recommend against the use of serologic assays to diagnose an active SARS-CoV-2 infection[5,6,83–86]. Of particular concern is the use of antibody tests during the first 14 days post symptom onset, as a negative test result may occur due to variability in the time to seroconversion and the limited sensitivity of the assay used. Equally important is the awareness that different serologic assays detect different antibody classes and the result may not

be comparable between assays. Overall, total antibody or IgG testing maybe more useful for evaluating patients presenting late in disease course due to waning IgM and IgA, but this has not been extensively evaluated[72].

While serological testing has been incorporated into the MIS-C case definitions, additional studies elucidating antibody kinetics in children in relation to MIS-C onset, and validating the performance of SARS-CoV-2 antibody assays in suspected MIS-C are needed[73]. Although the above stated considerations apply to all serologic testing against SARS-CoV-2, studies have shown that LFAs specifically have more inconsistency in performance characteristics and more variability in sensitivity and specificity[87,88].

5.2. Convalescent Plasma Donation and Manufacturing

5.2.1. Utility

Currently, patients who have recovered from COVID-19 can donate their plasma to help others fight SARS-CoV-2 infection. The identification of such potential convalescent plasma donors is a recognized utility of serologic testing. This therapeutic intervention has received EUA, although the FDA continues to refine donor eligibility criteria, determine which serologic assays should be used in the manufacture of COVID-19 convalescent plasma units, and what thresholds should be met for the unit to be considered usable. Currently, the FDA recommends that the qualitative Ortho Clinical Diagnostics SARS-CoV-2 IgG CIA should be used in the manufacturing process of convalescent plasma, with signal/cutoff (S/CO) values equal to or greater than 12 considered to be indicative of "high titer" anti-SARS-CoV-2 plasma, whereas S/CO values less than 12 are considered "low titer". Importantly, use of this S/CO value is only to be used in the manufacturing of convalescent plasma as a biologic product; the S/CO value should not be reported on donor reports. The development and availability of semi-quantitative and quantitative serologic assays will offer an improvement over the currently utilized qualitative methods for the manufacturing of convalescent plasma[89].

5.2.2. Limitations

Majority of commercial EUA antibody assays do not distinguish between binding antibodies or nAbs, the latter of which are essential for virus inactivation and have been associated with protective anti-viral immunity. Laboratorians should pay attention to whether the assay detects a binding antibody, or nAb when implementing the assay in the lab. Correlation between commercial antibody assays and nAbs remains a topic of intense interest and research. However, given that most currently available serologic assays do not provide quantitative results, there are limited mechanisms for distinguishing between donors with high versus low neutralizing antibody titers[90].

5.3. Epidemiologic and Seroprevalence Studies

5.3.1. Utility

Serologic testing is widely used in epidemiologic and seroprevalence studies, as antibody detection can identify previously infected individuals regardless of disease manifestation. Determination of seroprevalence can provide an estimate of the percent of individuals in a population that have antibodies to SARS-CoV-2, which may help better characterize the epidemiology of COVID-19 in the community and provide additional information for public health efforts[42,91–93].

5.3.2. Limitations

While serologic testing can provide insight into the level of SARS-CoV-2 infection within a defined region, there are associated limitations that must be taken into consideration, which are impacted by test-specific performance characteristics including assay sensitivity, specificity, and the resulting positive and negative predictive values[31,86,94].

The time required for antibody production following infection with SARS-CoV-2 must be considered,

alongside the emerging understanding of waning antibody levels over time, which may fall below the assay detection limit and differ depending on the assay antigen and design[95]. Additionally, most commercial assays were optimized and validated using symptomatic patients with moderate to severe disease, and the assay cutoffs are set based on these populations. These cutoffs may be too high to detect lower level antibodies developed by individuals with prior asymptomatic or mild disease. Also, a small proportion of the population may never develop detectable antibodies following infection, and this group of people may be misclassified as not having been previously infected. Collectively, these limitations may lead to an underestimate of the true seroprevalence in the population. Lastly, this approach is challenged by prevalence of the disease in the community. Generally, the positive predictive value of test results is impacted by both assay specificity and disease prevalence. In regions with low disease, the positive predictive value of a positive serologic test result will be low, even if using a highly specific assay.

5.4. Vaccine Development and Assessment

5.4.1. Utility

There are several vaccines at various stages of development, including some already in phase 3 clinical trials, and currently the majority of these vaccines target the S protein specifically RBD in addition to S, which is a key target of many of the vaccines given its role in host cell binding and entry[96–99]. Vaccines in the pipeline have been designed differently, ranging from inactivated or live virus platforms, to more novel DNA or RNA based vaccines[100]. Vaccine trials have used a number of different approaches to assess vaccine efficacy. These include ELISA assays that measure binding antibodies and both classic PRNT and pseudovirus-based neutralization assays that specifically detect nAbs[99,101–104].

5.4.2. Limitations

To date, only one assay received FDA EUA that specifically detect and measure nAbs, and similarly, there are no identified thresholds to determine protective immunity. As vaccines become available, it is likely that development of high-throughput, quantitative assays that either measure nAbs directly or that correlate well with nAbs will become useful. In addition, because the S/RBD protein is primarily used for vaccines, it has been speculated that having antibody assays that detect antibodies to the N and the S/RBD proteins specifically, may be useful to distinguish between individuals who have been naturally infected and those who have been immunized. SARS-CoV-2 vaccines are designed to stimulate development of neutralizing antibodies against SARS-CoV-2 spike protein and RBD specifically, in vaccinated individuals. These SARS-CoV-2 neutralizing antibodies can then prevent infection of cells. Therefore, it has been hypothesized that individuals who are only positive by serology assays that detect spike and RBD specific antibodies indicates probable vaccination. In contrast, assays that detect antibodies against nucleocapsid can be used to determine natural infection, as individuals would only have nucleocapsid antibodies if they have been infected with SARS-CoV-2. Therefore, testing with a nucleocapsid assay may be very useful in seroprevalence studies to distinguish between naturally infected and vaccinated individuals.

6. Performance Verification of EUA Assays

Verification studies for waived and non-waived (e.g., CLIA moderate complexity) EUA assays must be performed before releasing patient test results. The requirements for moderately complex EUA verification are the same as those for FDA approved or FDA cleared assays in the same test complexity status. In addition, tests that receive waived EUA status should go through a similar thorough verification process as seen with moderately complex, non-waived tests. Therefore, in this section, regulatory and accreditation requirements will be reviewed, and expert-based guidance will be provided on how to perform basic precision, accuracy, and reportable range verification studies for waived and non-waived (moderately complex) EUA assays. In contrast, for serologic LDTs (CLIA high complexity), further evaluation studies are required, including carryover, cross reactivity, interference, and clinical sensitivity and specificity studies; details for this are provided in Section 7. Due to the expedited development and evaluation of EUA assays,

the onus is on each laboratory to determine the extent of studies needed to verify the selected assay. While a general overview is provided here, numerous Clinical & Laboratory Standards Institute (CLSI) guideline documents (summarized in Table 4) can be further used as resources for detailed protocols of method verification.

6.1. Regulatory and Accreditation Requirements

Laboratories must follow the guidance from the FDA, CDC, and CLIA (CMS) for conducting SARS-CoV-2 serologic testing. Pursuant to the CLIA regulation §493.1253(b)(1), clinical laboratories in the United States are required to verify assay performance of unmodified, FDA-cleared, FDA-approved, and EUA assays before patient testing can commence. Laboratories performing these tests must adhere to manufacturer instructions. Labs need to refer to the requirements from their own accrediting agency and we use the College of American Pathologists (CAP) as an example to discuss the regulations and requirements. The CAP considers assays with EUA similar to FDA approved/cleared tests, and as such, there are specific requirements provided by the CAP for verification and implementation of such assays:

1. Ensure testing personnel are properly trained and qualified based on test complexity authorized by the FDA;

2. Perform testing as outlined in the FDA-authorized EUA **without** modification;

    a. Any deviation from the FDA-authorized instructions for use will render this assay an LDT which needs to be validated accordingly (see Section 7).

3. Verify test method performance following the CAP's All Common Checklist requirements, including:

    a. COM.40300 - Verification of Test Performance Specifications – FDA cleared/approved Tests: The laboratory must assess analytical accuracy, analytical precision, and reportable range (as appropriate).

    b. COM.40475 - Method Validation and Verification Approval – Non-waived Tests: Laboratory director must have signed the laboratory's written assay assessment.

    c. COM.40500 – Analytical Interferences: Laboratory understands the analytical interferences for each test and has a plan of action when present.

4. Update the laboratory's CAP activity menu.

When preparing to verify EUA assays, the Centers for Medicare and Medicaid Services (CMS) and some professional organizations have provided brief guidance for clinical laboratories[105–108]. At the outset of an emergency declaration, CMS recognizes that there is likely going to be a limited number of positive samples available to verify the performance of the test. The verification plan is then left to the discretion of the clinical laboratory director who must determine the number of positive and negative samples that are required to adequately verify performance specifications. As the situation evolves and more positive patient samples become available, CMS encourages further performance verification assessments to be completed by the laboratory. Here, we build on prior recommendations for assay verification and provide additional details and factors to consider during the planning and implementation process for FDA EUA tests.

6.2. Sample Collection

Sample procurement is one of the most critical steps in verifying EUA assay performance. However, early

on in a public health emergency, it may be difficult to determine the sample type or the target population to collect samples from (e.g., ambulatory, hospitalized, pediatric, pregnant patients). In addition, the laboratory will need to select which sample types will be verified for the assay (i.e., serum, lithium heparin plasma, etc.) and determine whether the EUA assay will be used for symptomatic or asymptomatic cases or for both. Timely understanding of the purpose of and target population for the test will produce correct strategic planning for potential serial sample collection that could be helpful in performing the verification of the assay under evaluation. Below are recommended sample selection strategies to assist in designing assay verification and validation (See Section 7) studies.

6.2.1. Positive Samples

1. Ideally residual, unmodified patient samples collected after testing positive on a comparative EUA assay are the preferred sample type for accuracy and precision studies. In addition, comparator assays should be matched for sample matrix, antibody class(es), and antigenic targets for optimal evaluation. If a comparator EUA serologic assay is unavailable, samples collected from RT-PCR-confirmed patients can be used, with knowledge of known days post symptom onset or first RT-PCR-positive result.

2. If obtaining positive patient samples is difficult, a known positive sample with an increased index value or S/CO may be mixed, at different ratios, with one or more confirmed residual, negative patient samples to generate several positive samples with a range of S/CO levels.

3. Samples from a third-party vendor (e.g., positive QC material, patient samples, or pooled patient samples) may be used in an emergent situation if residual, positive patient samples are not available. However, laboratories need to be judicious in the selection of third-party materials and be aware of possible matrix effects.

6.2.2. Negative Samples

1. Residual, unmodified patient samples collected and properly stored before local spread of SARS-CoV-2.

2. Residual, unmodified patient samples collected after testing negative on a comparative EUA assay is the preferred sample type for accuracy and precision studies. In addition, comparator assays should be matched to sample matrix, antibody class(es), and antigenic targets for optimal evaluation.

3. Commercially verified, negative QC, or other commercial matrix may be used if the other samples are limited or not available.

6.3. Accuracy

For qualitative and semi-quantitative tests, accuracy is verified by assessing the result concordance with either another EUA test or clinical correlate (i.e. positive and negative agreement), reflecting clinical sensitivity and specificity of these tests[4]. A strategic approach using both known positive and negative samples must be used to verify the accuracy of each type of test. Below are selected suggestions for how to approach assay verification.

6.3.1. Single Analyte or Total Analyte

These are assays designed to detect one antibody class (e.g., IgM or IgG) or total antibodies against SARS-CoV-2 and provide one single reportable result. A minimum of 20 samples (10 negative and 10 positive) per sample type should be used to verify the assay. For total antibody tests, it is optimal to use known positive patient samples from each of the antibody classes (e.g., IgG or IgM) to verify the assay's performance.

6.3.2. Multiple Differentiated Analytes

These are assays designed to detect two classes of antibodies (e.g., IgM/IgG) against SARS-CoV-2 and provide two reportable results. Accuracy verification must be demonstrated with known positive samples for both anti-SARS-CoV-2 IgG and anti-SARS-CoV-2 IgM. Varying combinations of a minimum of 20 samples (i.e., IgM-/IgG+, IgM+/IgG-, IgM+/IgG+, IgM-/IgG-) should be used to assess class specific positive and negative agreement as well as to verify specificity of the assay. Of note, identification of IgM+/IgG- samples may be challenging due to the fairly concurrent seroconversion of these two analytes.

6.4. Precision

Assay manufacturers provide precision data used to determine accuracy around the cutoff for a positive or negative EUA result. Clinical laboratories must verify the reproducibility and repeatability of the assay around this cutoff through intra- and inter-day precision studies, respectively. For qualitative assays, one positive and one negative sample can be used, with the positive sample near the cutoff threshold. Semi-quantitative assays should be evaluated as quantitative assays, and samples should span low, mid, and high S/CO, with at least one sample near the positive cutoff threshold. Residual patient samples are the preferred sample type for precision studies. However, pooled residual samples and commercially verified QC or other commercial material may be used. The intra-day precision experiments should test both positive and negative samples over 10 replicates on the same day. The inter-day experiments should test both positive and negative samples over 10 runs on a minimum of 5 days and ideally over multiple shifts. Precision experiments for single use LFAs should be assessed for inter-day only over a period of 5 days with at least two or three separate testing operators.

6.5. Reportable Range

The reportable range must be verified for semi-quantitative assays, which can be performed in a manner similar to verification of the reportable range for an FDA approved/cleared quantitative test. Verification of the reportable range should be done by using a known standard of anti-SARS-CoV-2 antibodies (as available), or if unavailable, an alternate lot number of calibrator or patient samples that span the analytical measuring range, which have not been modified by dilution.

7. Validation of Laboratory Developed Tests

An *in vitro* diagnostic test that is designed and used in a clinical setting by a single laboratory is considered an LDT. All SARS-CoV-2 antigens are commercially available as either full proteins or domains (e.g., S1, RBD) for use in LDTs. Laboratories are encouraged to carefully research and review the performance characteristics of the antigens of interest prior to implementing. Clinical laboratories play a critical role in the evaluation of serologic tests to safeguard against these limitations and minimize false positive results that may undermine disease prevention strategies. Clinical laboratories authorized to perform high-complexity testing under CLIA must perform thorough validation studies of an LDT before patient testing. This section will discuss minimum validation requirements of LDTs that go beyond those necessary for EUA assay verification, specifically with respect to sensitivity and specificity, cross-reactivity, carryover, class specificity, and the establishment of assay result cutoffs.

7.1. Regulatory and Accreditation Requirements

Typically, following HHS declaration of a public health emergency of national and/or international threat, any clinical test used to diagnose or detect the pathogen, regardless of type (i.e., molecular or serologic or antigen), requires FDA EUA. While this requirement was also put in place for COVID-19, due to limited reagent availability of molecular assays and delays in EUA review at the FDA, on August 19th, 2020, the EUA requirement for laboratory assays was removed by HHS in order to ease regulatory burdens placed on high-complexity CLIA laboratories capable of developing LDTs[109]. However, acquisition of EUA is still required for assays developed by commercial manufacturers.

The CAP and other accreditation organizations provides specific requirements for clinical laboratories that develop LDTs, which can be found in the associated checklists provided by the organization[110].

7.2. Antibody Class Specificity

If a specific claim about antibody class specificity is made for an LDT, it must be validated. Multiple different methods to establish this can be used, including using a well-characterized detection or capture antibody with a claimed class specificity or class-specific antibody depletion of the sample. As an alternative, for IgG-class specific assays, the FDA recommends treating samples with dithiothreitol (DTT), a small-molecule sulfhydryl redox reagent which specifically inactivates IgM-class antibodies[111]. Treatment of a sample with DTT would effectively remove IgM-class antibodies and users should be able to show reactivity by the IgG-specific assay both with and without DTT, whereas IgM reactivity should be null in the DTT-treated sample. For additional details, readers are referred to the FDA website[6].

7.3. Sample Collection

Similar to verifying EUA assay performance, sample procurement is an essential step for LDT validations. Furthermore, for laboratories validating an LDT, it may be equally difficult to obtain adequate samples and to determine overall sample selection strategies. In general, LDTs require additional samples to establish assay performance characteristics in comparison to EUA assays that require verification only. The FDA recommends at least 30 antibody positive and 75 antibody negative (or pre-COVID-19) specimens in their guidance for laboratories who wish to file for an EUA[6]. In situations where an assay using 75 negative specimens does not demonstrate greater than 95% specificity, or if 75 specimens are not available, the FDA additionally recommends specific cross-reactivity studies with samples known to be positive for a variety of potentially cross-reactive antibodies such as antinuclear antibody or those directed against other respiratory pathogens such as influenza and the seasonal human CoVs. It is our recommendation to collect at least 75 negative samples, and ideally 100-200 to provide a more accurate assessment of assay specificity. See Section 6.2 for sample selection strategies that can be used in LDT validation planning.

7.4. Establishing Assay Cutoffs

Cutoff values for a qualitative assay can be established by performing limit of blank studies using known negative samples tested repeatedly over several runs (e.g., 20 known negative samples tested by multiple operators on 5 separate runs). The mean optical density (OD) and standard deviations from the mean should be calculated, with the assay threshold determined as the mean OD plus 3 to 5 SD. Further refinement of cutoff values can be performed using Receiver-Operating Characteristic (ROC) analysis to optimize sensitivity and specificity. Alternatively, if risk assessment dictates an overriding concern, then cutoffs can be set accordingly. Early on in a pandemic, there may be limited positive samples and materials to establish an assay cutoff. For SARS-CoV-2, assay specificity is of high importance, particularly in low prevalence areas. While there is uncertainty about the interpretation of a true positive result in terms of potential immunity, there is a major concern that a false positive result may lead to decreased physical distancing and other safety measures due to a belief that an individual is "immune." To address these concerns, cutoffs may need to be set to achieve 100% specificity, based on samples that predate the pandemic and potentially cross-reacting samples. Assays that report quantitative results, as well as those that indicate neutralization levels, are less commonly used in clinical laboratories, and require additional layers of validation. Once a cutoff is established, it is also recommended to verify the cutoff as required by the CAP.

7.5. Analytical Specificity

7.5.1 Cross-Reactivity

Cross-reactivity is an essential evaluation used to determine analytical specificity of a clinical laboratory assay. Cross-reactivity information is typically included in manufacturers' instructions for use for each individual assay or available from the literature. Although not required for EUA assays, cross-reactivity

experiments are recommended to be performed similar to LDT validations. Ideally, serologic tests should only detect antibodies against the particular virus being tested. Cross-reactivity can occur when there is direct competition between the analyte of interest and other analytes that bind the antibody. With respect to SARS-CoV-2 assay specificity, a large percentage of the population has been infected with other seasonal CoVs, including OC43, 229E, NL63, and HKU1, and there was significant concern early in the pandemic that antibodies developed against these viruses may lead to false positive results on SARS-CoV-2 antibody assays. From multiple studies evaluating this however, it appears that prior infection with seasonal CoVs does not lead to significant cross-reactivity on SARS-CoV-2 serologic tests[5,112].

Despite this finding, samples from patients with known acute respiratory infections should be included, at a minimum, for any LDT or EUA assay assessing the serologic response to SARS-CoV-2. Ideally, these would include samples from patients infected with one of the circulating seasonal human CoVs (NL63, OC43, HKU1, 229E) and those diagnosed with syphilis, Lyme disease, Epstein-Barr virus, cytomegalovirus, or rheumatoid arthritis. Notably, respiratory infections are routinely diagnosed using either individual or multiplex molecular respiratory panels performed from respiratory samples. Therefore, serum samples from such patients collected far enough post symptom onset for antibodies to have developed may not be available for cross-reactivity studies. While it does not appear that the seasonal CoVs are a significant cause of false positive results in SARS-CoV-2 serologic assays[112,113], a disclaimer to the effect that cross-reactivity can't be ruled out should be included if such samples were not evaluated in the validation[112,113]. Serum from individuals infected with SARS-CoV-1 and MERS-CoV may also be included in a validation set if available. These are obviously an extremely rare cause of infection, particularly in the United States, and it is unlikely that such samples can be acquired. The very fact of their rarity, however, removes them as much of a concern for cross-reactivity. Interestingly, one-way cross-reactivity was demonstrated in one study in patients with SARS-CoV-1 against the CoVs 229E and OC43[114].

Finally, strategies have been proposed to achieve higher specificity. For instance, it has been shown that deleting conserved amino acid sequences that were found in both N and S proteins among other human coronaviruses such as 229E, NL63, OC43, and HKU1[114,115] may increase specificity without affecting the sensitivity[116].

7.5.2 Interferences

Investigation of common and unknown interferences is another core component to determine an assay's analytical specificity. Interferences can be method- and analyte-dependent. The most common analytical interferences include hemoglobin, lipids, and bilirubin. Most large, automated platforms have specialty function checks that can detect these substances in a patient sample. However, many interfering substances are not readily identifiable until a patient's result is questioned, and further investigation is performed. For laboratories validating an LDT, it is required to research and investigate potential interferences based on assay design and devise interference validation studies prior to patient testing.

7.6. Carryover

Carryover can be a concern for instrumentation that uses non-disposable probes. Clinical laboratories should perform an assessment to verify that positive samples will not contaminate the next patient's sample, or that a positive result was not due to positivity from a nearby high-titer positive sample. This assessment is commonly performed for semi-automated ELISA and fully automated CIA/CMIA methods by performing alternating testing of a negative sample before and after a positive sample with a high index or S/CO value. If carryover cannot be eliminated from the assay, it is recommended to assess the impact on accuracy of a positive and negative result. Carryover should not exceed 20% of the lower limit of quantitation as recommended by the FDA[117]. The process for evaluating carryover are well-established for quantitative assays, which also applies to EUA and LDT serologic assays; additional details are available through CLSI EP10-A3-AMD (Table 4) and the CAP.

8. Other EUA Assay Implementation Considerations

8.1. Quality Management

As part of the quality management system for any assay, QC must be identified, verified, and implemented as part of routine testing for any SARS-CoV-2 serologic assay. A minimum of two levels of QC, positive, and negative materials, should be included with each run of the specified assay. For SARS-CoV-2 serologic assays, these types of controls may be provided as part of the assay kit, as is the case for most ELISAs, or may need to be sourced separately for many of the CIA/CMIA- and LFA-based assays. For assays without kit-provided controls, laboratories may have the option to purchase separate controls provided by the specific assay manufacturer, may purchase third-party vendor controls, or may choose to use pooled patient samples. Use of assay calibrator material to create assay controls is discouraged. However, if no other options are available, the calibrator material used should be from a different kit lot. Other general considerations for QC material would be to ensure that it matches the analyte detected by the specific assay and that the matrix is as similar to patient samples as possible. Ideally, the control batches would have a long shelf life and be large enough to allow for trending across kit lots, and of course, be cost-effective for the laboratory.

For non-waived tests, CAP and CLSI indicate that two levels of QC should be run at least daily, although the frequency is somewhat method dependent. Batch testing typically runs QC material with every batch, whereas for continuous load assays, a bracketed QC approach is usually implemented. For bracketed QC, QC is run at least daily, and immediately before and after any anticipated major assay changes, including but not limited to re-calibration events, reagent lot changes, major instrument repairs, and software updates. For qualitative and semi-quantitative assays, ideally among the two levels of QC, one would be below and the other above the assay cutoff threshold, with the positive control level near the threshold. The nearness of the positive control to the threshold is defined by the laboratory, considering assay imprecision to minimize risk of unnecessary run rejection. Utilization of such low-level positive controls is likely to detect analytical performance issues near the cutoff point. The target control mean and range is typically established by collecting at least 20 data points on separate days and determining the standard deviation (SD); however, if the laboratory has historical QC data available with these controls, the previously established SD may be applied to the new QC material lots after establishing a new mean using 10 data points. Importantly, while some vendor material may provide assigned QC values, these should be used as a guide, and not as a replacement for laboratory verification of the product. QC performance should be monitored in real time, for example using Levey-Jennings charts and Westgard Rules to identify shifts and trends overtime.

Laboratories should also participate in proficiency testing (PT) for SARS-CoV-2 serologic assays, either using vendor products or if necessary, through an alternative assessment program. Vendor PT is currently available through CAP.

Finally, given that EUA assays did not undergo extensive characterization and evaluation, laboratories may choose to implement a more rigorous quality management system. This may include analysis of additional QC material, performing additional lot-to-lot comparisons, and identifying a partner laboratory for more frequent sample exchanges than the required bi-annual PT evaluations. Once the reliability of the assay is established within the laboratory, this additional level of QC and extra precautions may be eliminated.

8.2. Pre-analytical Considerations

Basic pre-analytical recommendations should be noted for SARS-CoV-2 antibody tests. Every clinical laboratory assay should be thoroughly reviewed to determine possible limitations (e.g., interferences, sample stability). Hemolysis, icterus, and lipemia (HIL) indices should be verified and set within automated instrumentation, if applicable.

Time of sample collection for serologic testing is another key point to consider. When selecting positive samples for verification studies, it is important to consult the IFU and collect specimens at the disease course time point corresponding to the desired clinical sensitivity of the test. For example, most current EUA assays report optimal sensitivity at 14 days or later post-symptom onset while some may use days post PCR positivity. Thus, in order to verify the performance of the test at optimal sensitivity, the samples collected on and after 14 days post symptoms onset/post PCR positivity should be used for assay verification to ensure full seroconversion[5]. The limitation of the test in patients tested <14 days prior to symptom onset should be clearly stated. For LDTs, clinical sensitivity relative to days from symptom onset needs to be determined during the validation process described in Section 7. It is worth mentioning that asymptomatic patients may not have been included in the cohorts for the manufactures, which should be considered in sample selection during assay verification.

Lastly, depending on the category of serologic assays, there may be several different sample types being used in SARS-CoV-2 antibody testing. For instance, LFAs may be performed with whole blood, plasma, or serum depending on the EUA. It is important for the laboratories to define pre-analytical requirements such as sample types, collection devices, and transportation requirements prior to patient testing since there have been reports of varying accuracy. In addition, dried blood spots present another unique sample type that must be verified prior to widespread patient testing.

9. Interpretation of Serologic Test Results

The majority of EUA assays for SARS-CoV-2 antibody detection are qualitative in nature, and results should be reported according to the manufacturer instructions as either positive, negative, or indeterminate/equivocal (as applicable). Generally speaking, positive results indicate that individuals are either currently or were previously infected with SARS-CoV-2 and mounted a detectable antibody response. Negative serologic results indicate that the SARS-CoV-2 antibodies were not detected or are below the limit of detection, but an active or prior SARS-CoV-2 infection cannot be ruled out.

The interpretation of serologic test results is not necessarily straightforward. False-positive and -negative results may occur for various reasons such as the nature of the humoral immune response against the virus and assay performance characteristics, as has been discussed above.

It is essential to interpret results in the context of targeted antibodies and the antigenic targets used for the selected assay, sample collection relative to the time of symptom onset, disease severity, analytical performance of the assay (e.g., analytical sensitivity and specificity, limit of detection), disease prevalence, and the targeted patient populations[118–120]. Details were discussed in previous sections for antibody classes (Section 5.1.), antigenic targets and sample collection time (Section 3.3.) and cross-reactivity (Section 7.3.). Additional specific factors that should be considered but were not discussed in prior sections of this document are discussed below.

9.1. Sensitivity and Specificity

The sensitivity of a test refers to how frequently a test correctly identifies the presence of antibodies in an infected individual (i.e. does the test result as positive when antibodies are present). Specificity indicates the frequency with which a test correctly identifies the absence of antibodies in a person who has not been infected (i.e. does the test result as negative when antibodies are not present). These two parameters indicate the rates of false positive and false negative results.

The sensitivity and specificity of the current FDA EUA tests are somewhat variable. Generally speaking, during a pandemic, high specificity is preferred over high sensitivity. While a sensitivity of at least 95% has been recommended, alternative minimum thresholds can be set by the laboratory director and/or

the clinical team. To minimize potential false positives, the CDC has suggested using tests with specificity of 99.5% or greater. Additionally, the IDSA and CDC have stated that in order to be of value, SARS-CoV-2 antibody tests should have high clinical sensitivity and specificity (≥99.5%)[22,69].

9.2. Disease Prevalence

Appropriate interpretation of serologic test results requires an understanding of the disease prevalence in the target population, alongside knowledge of assay sensitivity and specificity, which when combined, affect the positive and negative predictive values (PPV and NPV, respectively). PPV and NPV are two essential indicators that provide insight into the accuracy of positive or negative test results within the population tested. PPV indicates the percent probability that a positive test result will correctly identify individuals with antibodies in a given population, while NPV is the percent probability that a negative test result will correctly identify cases without antibodies in a given population.

Figure 3A illustrates the effect of sensitivity, specificity, and disease prevalence on PPV and NPV. For illustration purposes, we chose a sensitivity of 95% and specificity ranges from 90% to 99%. Given a population with fixed prevalence, the PPV increases as specificity increases. For an assay with a fixed sensitivity and specificity, the PPV increases more rapidly with increased disease prevalence until it plateaus at ≥ 20% prevalence. Given a population with a fixed prevalence, although sometimes not known, the PPV is essentially determined by the specificity of the assay. The NPV, however, changes minimally with different levels of specificity and drops rapidly when disease prevalence increases.

To illustrate the impact of the disease prevalence further, we can examine a test that has a purported 95% sensitivity and 95% specificity within a population of 10,000 people with an antibody prevalence of 20% (2000 individuals have antibodies) (Figure 3B). In this situation, the test would correctly identify antibodies in 1,900 of those 2000 individuals, and incorrectly identify as positive 400 out of the remaining 8000 (i.e. false positive results). Overall, this results in PPV and NPV values of 82.6% (1900/[1900+400]) and 98.7% (7600/[7600+100]), respectively, which results in a 17.4% false positive rate and 1.3% false negative rate.

If that same test is used in a population with a prevalence of 5% (500 individuals have antibodies in the population of 10,000), the test would correctly identify antibodies in 475 of those 500 and incorrectly identify as positive 475 people among the 9,500 individuals who do not have the antibodies. In the first situation, 82% of the 2300 people who tested positive would actually have antibodies, whereas in the second situation, only half of the 950 who tested positive, would have antibodies. Until a clear picture emerges regarding prevalence, serologic test results should not be used as the sole basis for clinical or public health policy decisions.

Tests with higher PPV are preferred and three approaches can be considered to improve the PPV for serologic tests used in low prevalence settings[69]. Among these options is to choose a test with sufficient specificity (e.g., ≥ 99.5%). Alternatively, the laboratory may decide to only test individuals with a high pre-test probability of having antibodies, such as persons with a history of symptoms compatible with COVID-19 or who are exposed to areas or institutions experiencing outbreaks. Finally, an Orthogonal Testing Algorithm (OTA) may be implemented; additional information on orthogonal testing is provided in Section 10.

9.3. Comments for Test Results

Laboratories should include interpretive comments for SARS-CoV-2 serologic test results. These comments should include what the result means, what they do not mean, and what the key limitations of the assay are. For example, comments for positive results should include that neither the timing of infection, nor whether the individual is protected from re-infection can be determined based on the result, and that false positive results may occur due to pre-existing antibodies or other causes. For negative results, laboratories may consider including commentary on the risk of false negative results relative to timing of sample

collection post infection or in significantly immunosuppressed individuals.

Additionally, although it may be desirable from a clinician's standpoint for laboratories to report the actual S/CO values on patient reports as an indication of the antibody response level, the EUA status of qualitative tests prohibits this action. For CAP-accredited laboratories, reporting the S/CO value for any sample would be a violation of their guidance to "perform testing as outlined in the FDA-authorized EUA without modification" and therefore must be avoided. The reasons for this point-of-view are multi-fold. First, the assay-specific values are not standardized and do not indicate an actual concentration of antibody levels. Second, there is currently no correlation of S/CO or index values to protective immunity. Third, currently available platforms have different dynamic ranges. When tested by two different platforms with the same positivity cutoffs, the same patient may have S/CO or index value results that are vastly different. Such differences may be due to multiple factors, including assay design, dilution at which samples are tested, the antibody class detected, and the recombinant antigen used (i.e., full protein vs. specific protein domain), among others.

For all results, it is advisable to include a statement that diagnosis of COVID-19 should be performed using molecular tests. Finally, per FDA EUA requirements, reports should include what assay was used to perform the testing, and clinicians and patients should have access to the respective assay Fact Sheets. This may be provided in the comments directly, or in the laboratory test catalogue. Some examples of result comments are provided in Table 5.

10. Orthogonal Testing

If a desired PPV cannot be achieved using a single serological assay, the CDC recommends the use of an OTA as one of the strategies to improve the PPV of serological testing, particularly in populations with low disease prevalence[69]. This is a sequential testing strategy where all initially positive results are tested with a second independent serologic test. While an OTA is expected to minimize false positives, studies on the effectiveness of this approach are scarce[38,121]. Furthermore, it could be complicated by logistical challenges and high costs associated with multiple tests as well as their respective analytical platforms.

10.1. OTA Test Selection

In the context of SARS-CoV-2 serological testing, the appropriate OTA may include tests that use different methodologies with the same antigenic target (e.g., ELISA vs CMIA detecting N protein antibodies), targeting different domains of the same protein (e.g., 2-step ELISA detecting RBD and S2 protein antibodies) or tests that detect antibodies against different viral antigens (e.g., N vs S proteins)[38]. The approach is equally amenable to automated, high volume testing and POCT, as long as the anticipated volumes, disease prevalence and individual test performance characteristics are carefully considered as described below. OTAs that include IgM or IgA serologic tests may not be appropriate for this approach as the temporal dynamics of virus-specific IgM/IgA and IgG immune responses may differ and there is a high likelihood of discordant results. Important aspects that should be considered when setting up OTA are discussed in the example below using tests with sensitivity and specificity, respectively, of 95% and 98% (Test 1) and 99% and 95% (Test 2).

Orthogonal testing is best suited for testing populations with low disease prevalence (<2%), whereby even a test with specificity as high as 98% (Test 1) is expected to yield up to 50% false positive results. In such populations, adding a sequential test (Test 2) with specificity of 95% is expected to result in PPV >90% **(Table 6)**. An online calculator from the FDA (https://www.fda.gov/media/137612/download) serves as a helpful tool to assess the combined performance of individual tests in the context of disease prevalence.

While orthogonal testing has traditionally been employed in screen/reflex testing model, where the first test

has higher sensitivity and lower specificity, while opposite is the case with the reflex test, it may not always be advantageous to follow this model. This is particularly important if testing costs are a concern, which is the case for SARS-CoV-2 serologic testing, where reimbursement is not available for the reflex test. In this situation, both tests should ideally have high sensitivities (>95% or the lowest sensitivity threshold of a single test deemed acceptable by laboratory director and/or clinical team). If two tests of acceptable sensitivity are selected, the test with higher specificity should be chosen as the first-line test. This should minimize the number of discordant results while still retaining desirable test sensitivity and resulting in optimal PPV. The relationship of PPV/NPV and discordant rate with Test 1 and 2 in different OTA designs given a disease prevalence of 2% is illustrated in Table 6. Both OTA1 and OTA2 designs have the same combined PPV of 95%. However, using OTA1, which uses the highest specificity test first, results in a discordant rate of 1.9%, which is lower than that from OTA2 (4.9%).

10.2. OTA Result Reporting and Interpretation

In an OTA, there are three potential outcomes: (1) initial test result is negative/non-reactive, (2) initial test result is presumptive positive and confirmed with a second serologic test, or (3) initial test result is presumptive positive but the second test is negative/non-reactive **(Table 7)**. If the initial tests are negative, there is no need for the second test and a negative report will be issued. If both tests are positive, a positive result will be issued and the interpretation for the OTA is straight forward. The interpretive challenge arises when the second assay result is negative, giving rise to discordant or indeterminate results between the two serologic assays. If different antigenic targets are used in each assay, a discordant result may be attributed to: (1) a false-positive initial test, (2) early recovery and differences in antibody kinetics, (3) skewed immune response towards the positive antigen with undetectable antibodies to the negative antigen, or (4) waning immunity. To rule out the contribution of differences in antibody kinetics, retesting in 2-4 weeks may be attempted. It is imperative that the test with higher specificity is selected as Test 1, as described above. Otherwise discordance between Test 1 and 2 results may reflect a difference in sensitivities rather than identifying a potential false positive result. In summary, for optimal interpretation, OTA results should be interpreted in the context of disease prevalence, sensitivity and specificity of each test, assay methodology, and antigenic targets.

11. Laboratory Safety

In order to combat the COVID-19 pandemic, laboratories should be prepared to address issues of lab biosafety, sample handling, operation, prioritization of important tests, and workforce planning to minimize occupational SARS-CoV-2 infections. There is sufficient evidence to support that face masks, combined with other preventive measures, such as frequent hand-washing and physical distancing, help to prevent individuals from contracting SARS-CoV-2 from infected individuals[122]. Many international and national health agencies recommend that laboratory staff adhere to standard BSL2 precautions while handling specimens that are suspected or confirmed for SARS-CoV-2, which includes hand-washing and wearing personal protective equipment (PPE), such as facemasks, face shields, eye protection, gloves, and liquid impermeable lab coats or gowns[123,124].

All blood samples should be handled with standard precautions. Laboratories should follow the guidelines for specimen collection, handling, storage, and transportation to minimize the risk of infection[125]. Laboratories should minimize aerosol-generating procedures by centrifuging specimens in closed centrifuges or utilizing caps on centrifuge carriers to prevent specimen release. Routine blood testing can be performed manually or on automated-analytical systems with closed, automated uncapping and recapping capabilities[85,124].

Work benches, instruments, and frequently touched surfaces in the laboratory should be disinfected frequently to minimize the risk of contamination and transmission of SARS-CoV-2.

In addition to biosafety, the clinical laboratory should develop an institutional strategy for emergency preparedness. Laboratories can recognize that resources are limited and prioritize tests related to the pandemic and those for urgent and critical care. Laboratory leadership should acknowledge the substantial shortage of laboratory staff and make appropriate workforce planning during this outbreak. Overall, it is essential that laboratories are prepared with sufficient supplies of all PPE, reagents, and consumable materials, and carry out prioritization of important tests, and workforce planning during this pandemic.

12. Conclusions and Perspectives

COVID-19 is an imminent threat to people all over the world. The virus is known to be highly contagious, and the disease spreads rapidly from person to person. Though not recommended as the first line testing for diagnosis of SARS-CoV-2 infection, serologic testing can play some supportive functions in the management of the pandemic. Clinical laboratory professionals have played and will continue to play an indispensable role in the management of SARS-CoV-2 infection by developing, validating, performing, and providing guidance in the proper use and interpretation of serologic testing. It is the AACC's position that clinical laboratories should only use assays that have received FDA EUA or LDTs that have been developed and properly validated in the clinical laboratory. Laboratorians should recognize the utility and limitations of serologic tests, and carefully choose and interpret test results, by taking into account considerations such as targeted antibody type, specimen collection time, analytic and clinical sensitivity and specificity, cross reactivity, and disease prevalence in the targeted population. Assay standardization, properly designed quality management systems, and well-characterized QC and PT materials for the tests are also important to ensure the quality of these assays and result interpretation. The authors have provided expert opinions based on the current research on these topics in this document.

Many studies are underway to gain deeper and broader understanding of this novel virus and the tests used to detect and manage the infection will continue to improve. The scientific community will implement testing in an evidence-based approach as the pandemic continues to unfold and clinical laboratories in collaboration with their clinical colleagues, will continue to play an essential role in reviewing the evolving scientific literature and adjusting testing strategies to best serve patient and public health needs. ■

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Tables

Table 1: Definition of Common Regulatory Terms	
FDA Clearance	When a medical device is cleared, this means it has undergone a 510(k) submission, which FDA has reviewed and provided clearance.[1]
FDA Approval	For Class III medical devices to be legally marketed they must undergo a rigorous review and approval process. Following a successful submission of a premarket approval (PMA) or a Humanitarian Device Exemption (HDE), the device is given Approval by FDA.[1]
FDA Emergency Use Authorization	Unapproved medical products or unapproved uses of approved medical products to be used in an emergency to diagnose, treat, or prevent serious or life-threatening diseases or conditions.[2]
Laboratory Developed Test	A type of in vitro diagnostic test that is designed, manufactured, and used within a single laboratory.[3]
CLIA Waived	As defined by CLIA, waived tests are simple tests with a low risk for an incorrect result.[4]
CLIA Non-waived	As defined by CLIA, non-waived testing is the term used to refer collectively to moderate and high complexity testing.[4]
CLIA Test Complexity Categorization	Clinical laboratory test systems are assigned a moderate or high complexity category on the basis of seven criteria given in the CLIA regulations. For commercially available FDA-cleared or approved tests, FDA scores the tests using these criteria during the pre-market approval process. The final score determines whether the test system is categorized as moderate or high complexity. Tests developed by the laboratory or that have been modified from the approved manufacturer's instructions default to high complexity according to the CLIA regulations.[4]

FDA, Food and Drug Administration; CLIA, Clinical Laboratory Improvement Amendments

Table legend: This table provides a list of common definitions regarding regulations in the United States.

Reference:

[1]https://www.greenlight.guru/blog/fda-clearance-approval-granted;
[2]https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization;
[3]https://www.fda.gov/medical-devices/vitro-diagnostics/laboratory-developed-tests#:~:text=A%20laboratory%20developed%20test%20(LDT,used%20within%20a%20single%20laboratory;
[4]https://www.cdc.gov/clia/test-complexities.html#:~:text=As%20defined%20by%20CLIA%2C%20waived,the%20FDA%20for%20home%20use.

Table 2: Summary of EUAs Issued for SARS-CoV-2 Serologic Assays (as of 10/20/2020).

Date EUA[a] First Issued	Manufacturer	Diagnostic Test	Method[b]	Class	Specimen Type[c]	Antigen Target[d]
04/01/2020	Cellex Inc.	qSARS-CoV-2 IgG/IgM Rapid Test	LFA	IgM and IgG	S, P, WB	S, N
04/14/2020	Ortho-Clinical Diagnostics, Inc.	VITROS Immunodiagnostic Products Anti-SARS-CoV-2 Total Reagent Pack	CIA	Total antibody	S, P	S1
04/15/2020	Mount Sinai Laboratory	COVID-19 ELISA IgG Antibody Test	ELISA	IgG	S, P	RBD
04/24/2020	DiaSorin Inc.	LIAISON SARS-CoV-2 S1/S2 IgG	CIA	IgG	S, P	S1, S2
04/24/2020	Ortho-Clinical Diagnostics, Inc.	VITROS Immunodiagnostic Products Anti-SARS-CoV-2 IgG Reagent Pack	CIA	IgG	S	S
04/26/2020	Abbott Laboratories Inc.	SARS-CoV-2 IgG assay	CMIA	IgG	S, P	N
04/29/2020	Bio-Rad Laboratories	Platelia SARS-CoV-2 Total Ab assay	ELISA	Total antibody	S, P	N
04/30/2020	Wadsworth Center, New York State Department of Health	New York SARS-CoV Microsphere Immunoassay for Antibody Detection	FMIA	Total antibody	S	N
05/02/2020	Roche Diagnostics	Elecsys Anti-SARS-CoV-2	CIA	Total antibody	S, P	N
05/04/2020	EUROIMMUN US Inc.	Anti-SARS-CoV-2 ELISA (IgG)	ELISA	IgG	S, P	S1
05/29/2020	Siemens Healthcare Diagnostics Inc.	ADVIA Centaur SARS-CoV-2 Total (COV2T)	CIA	Total antibody	S, P	RBD

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Date EUA[a] First Issued	Manufacturer	Diagnostic Test	Method[b]	Class	Specimen Type[c]	Antigen Target[d]
05/29/2020	Siemens Healthcare Diagnostics Inc.	Atellica IM SARS-CoV-2 Total (COV2T)	CIA	Total antibody	S, P	RBD
05/29/2020	Healgen Scientific LLC	COVID-19 IgG/IgM Rapid Test Cassette	LFA	IgM and IgG	S, P, WB	S1
06/04/2020	Vibrant America Clinical Labs	Vibrant COVID-19 Ab Assay	CIA	IgM and IgG	S, DBS	S1
06/04/2020	Hangzhou Biotest Biotech Co., Ltd.	RightSign COVID-19 IgG/IgM Rapid Test Cassette	LFA	IgM and IgG	S, P, WB	RBD
06/08/2020	Siemens Healthcare Diagnostics Inc.	Dimension EXL SARS-CoV-2 Total antibody assay (CV2T)	CIA	Total antibody	S, P	RBD
06/08/2020	Siemens Healthcare Diagnostics Inc.	Dimension Vista SARS-CoV-2 Total antibody assay (COV2T)	CIA	Total antibody	S, P	RBD
06/10/2020	InBios International, Inc.	SCoV-2 Detect IgG ELISA	ELISA	IgG	S	
06/15/2020	Emory Medical Laboratories	SARS-CoV-2 RBD IgG test	ELISA	IgG	S	RBD
06/18/2020	Biohit Healthcare (Hefei) Co. Ltd.	Biohit SARS-CoV-2 IgM/IgG Antibody Test Kit	LFA	IgM and IgG	S, P, WB	N
06/19/2020	Hangzhou Laihe Biotech Co., Ltd.	LYHER Novel Coronavirus (2019-nCoV) IgM/IgG Antibody Combo Test Kit (Colloidal Gold)	LFA	IgM and IgG	S, P	S1
06/23/2020	Babson Diagnostics, Inc.	Babson Diagnostics aC19G1	CIA	IgG	S, P	S1

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Date EUA[a] First Issued	Manufacturer	Diagnostic Test	Method[b]	Class	Specimen Type[c]	Antigen Target[d]
06/26/2020	Beckman Coulter, Inc.	Access SARS-CoV-2 IgG	CIA	IgG	S, P	RBD
06/30/2020	InBios International, Inc.	SCoV-2 Detect IgM ELISA	ELISA	IgM	S	
07/06/2020	Assure Tech. (Hangzhou Co., Ltd)	Assure COVID-19 IgG/IgM Rapid Test Device	LFA	IgM and IgG	S, P, WB	S1, N
07/08/2020	Diazyme Laboratories, Inc.	Diazyme DZ-Lite SARS-CoV-2 IgG CLIA Kit	CIA	IgG	S, P	
07/10/2020	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.	WANTAI SARS-CoV-2 Ab Rapid Test	LFA	Total antibody	S, P, WB	RBD
07/13/2020	Salofa Oy	Sienna-Clarity COVIBLOCK COVID-19 IgG/IgM Rapid Test Cassette	LFA	IgM and IgG	S, P, WB	RBD
07/16/2020	Luminex Corporation	xMAP SARS-CoV-2 Multi-Antigen IgG Assay	FMIA	IgG	S, P	S1, RBD, N
07/17/2020	Megna Health, Inc.	Rapid COVID-19 IgM/IgG Combo Test Kit	LFA	IgM and IgG	S, P	N
07/24/2020	Xiamen Biotime Biotechnology Co., Ltd.	BIOTIME SARS-CoV-2 IgG/IgM Rapid Qualitative Test	LFA	IgM and IgG	S, P, WB	
07/24/2020	Access Bio, Inc.	CareStart COVID-19 IgM/IgG	LFA	IgM and IgG	S, P, WB	RBD
07/31/2020	Siemens Healthcare Diagnostics Inc.	Atellica IM SARS-CoV-2 IgG (COV2G)	CIA	IgG	S, P	RBD

Table 2: Summary of EUAs Issued for SARS-CoV-2 Serologic Assays (as of 10/20/2020).

Date EUA[a] First Issued	Manufacturer	Diagnostic Test	Method[b]	Class	Specimen Type[c]	Antigen Target[d]
07/31/2020	Siemens Healthcare Diagnostics Inc.	ADVIA Centaur SARS-CoV-2 IgG (COV2G)	CIA	IgG	S, P	RBD
08/05/2020	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.	WANTAI SARS-CoV-2 Ab ELISA	ELISA	Total antibody	S, P	RBD
08/06/2020	bioMérieux SA	VIDAS SARS-CoV-2 IgG	ELFA	IgG	S, P	RBD
08/06/2020	bioMérieux SA	VIDAS SARS-CoV-2 IgM	ELFA	IgM	S, P	RBD
08/17/2020	BioCheck, Inc.	BioCheck SARS-CoV-2 IgG and IgM Combo Test	CIA	IgM and IgG	S	S1
08/17/2020	Diazyme Laboratories, Inc.	Diazyme DZ-Lite SARS-CoV-2 IgM CLIA Kit	CIA	IgM	S, P	
08/25/2020	Biocan Diagnostics Inc.	Tell Me Fast Novel Coronavirus (COVID-19) IgG/IgM Antibody Test	LFA	IgM and IgG	S, P, WB	S, N
08/31/2020	University of Arizona Genetics Core for Clinical Services	COVID-19 ELISA pan-Ig Antibody Test	ELISA	Total antibody	S	RBD>S2
08/31/2020	TBG Biotechnology Corp.	TBG SARS-CoV-2 IgG / IgM Rapid Test Kit	LFA	IgM and IgG	S, P	S, N
09/03/2020	Sugentech, Inc.	SGTi-flex COVID-19 IgG	LFA	IgG	S, P, WB	RBD, N
09/09/2020	BioCheck, Inc.	BioCheck SARS-CoV-2 IgM Antibody Test Kit	CIA	IgM	S	S1

Table 2: Summary of EUAs Issued for SARS-CoV-2 Serologic Assays (as of 10/20/2020).

Date EUA[a] First Issued	Manufacturer	Diagnostic Test	Method[b]	Class	Specimen Type[c]	Antigen Target[d]
09/09/2020	BioCheck, Inc.	BioCheck SARS-CoV-2 IgG Antibody Test Kit	CIA	IgG	S	S1

Abbreviations:

[a] EUA, emergency use authorization.

[b] Assay type: CIA, chemiluminescent Immunoassay; CMIA, chemiluminescent microparticle immunoassay; ELFA, enzyme-linked fluorescence assay; ELISA, enzyme-linked immunosorbent assay; FMIA, fluorescent microparticle immunoassay; LFA, lateral flow assay.

[c] Specimen: S, serum; P, plasma; WB, whole blood; DBS, dried blood spot.

[d] Antigen: S, spike; S1, spike S1; S2, spike S2; RBD, spike receptor binding domain; N, nucleocapsid.

Table 3. Recommended Use of Serologic Testing and Limitations

Recommended use

Serologic testing may be offered as an approach to support diagnosis of COVID-19 illness in symptomatic patients and late phase negative molecular testing or for patients presenting with late complications such as multisystem inflammatory syndrome in children (MIC-C).

Serologic testing can help identify people who may have been infected with or have recovered from the SARS-CoV-2 infection.

Serologic testing can be used to screen potential convalescent plasma donors and in the manufacture of convalescent plasma.

Serologic testing can be used for epidemiology and seroprevalence studies.

Serologic testing can be of help to evaluate vaccine efficacy.

Limitations

False positive results may occur.

Negative results do not preclude acute SARS-CoV-2 infection or viral shedding.

Serologic tests may not differentiate between natural infection and vaccine response.

The durability and kinetics of the humoral immune response continue to be elucidated.

Serologic results should not be used for:

- Determining individual protective immunity
- Return to work decisions
- Cohorting individuals in congregate settings
- Assessment of convalescent plasma recipients
- Use of PPE
- Placement of high-risk job functions

Table 4. CLSI Documents for SARS-CoV-2 Serologic Assay Implementation	
EP05	Evaluation of Precision of Quantitative Measurement Procedures, 3rd Edition
EP06	Evaluation of Linearity of Quantitative Measurement Procedures: A Statistical Approach, 1st Edition
EP07	Interference Testing in Clinical Chemistry, 3rd Edition
EP10	Preliminary Evaluation of Quantitative Clinical Laboratory measurement Procedures, 3rd Edition
EP12	User Protocol for Evaluation of Qualitative Test Performance, 2nd Edition
EP17	Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures, 2nd Edition
EP18	Rick Management Techniques to Identify and Control laboratory Error Sources, 2nd Edition
EP19	A Framework for Using CLSI Documents to Evaluate Clinical Laboratory Measurement Procedures, 2nd Edition
EP23	Laboratory Quality Control Based on Risk Management, 1st Edition
EP25	Evaluation of Stability of In Vitro Diagnostic Reagents, 1st Edition
QSRLDT	Quality System Regulation for Laboratory-Developed Tests: A Practical Guide for the Laboratory
POCT04	Essential Tools for Implementation and Management of a Point-of-Care Testing Program, 3rd Edition
POCT07	Quality Management: Approaches to Educing Errors at the Point of Care, 1st Edition
POC15	Point-of-Care Testing for Infectious Diseases, 1st Edition
GP17	Clinical Laboratory Safety, 3rd Edition
GP36	Planning for Laboratory Operations During a Disaster, 1st Edition

CLSI: Clinical and Laboratory Standards Institute

Table legend: The table provides a list of CLIS documents to consider when implementing a SARS-CoV-2 serologic assay.

Reference: https://clsi.org/standards-development/helpful-documents-for-covid-19-testing/

Table 5. Result Comment Examples for SARS-CoV-2 Serologic Testing

FDA required statement for all manufacturers' claims

"The serology test is intended for use as an aid in identifying individuals with an adaptive immune response to SARS-CoV-2, indicating recent or prior infection. At this time, it is unknown for how long antibodies persist following infection and if the presence of antibodies confers protective immunity. The serology (SARS-CoV-2 Ig) assay should not be used to diagnose acute SARS-CoV-2 infection. Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C 263a, that meet requirements to perform moderate or high complexity tests."

Example 1

Negative:

No IgG antibodies to SARS-CoV-2 detected. Negative results may occur in serum collected too soon following infection or in immunosuppressed patients. Follow-up testing with a molecular test is recommended in symptomatic patients. This test should not be used to exclude active/recent COVID-19 infection.

ADDITIONAL INFORMATION: Testing was performed using the VITROS Immunodiagnostic Product Anti-SARS-CoV-2 IgG Reagent Pack assay (Ortho-Clinical Diagnostics, Inc.), which has received Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration.
Fact sheets for this Emergency Use Authorization (EUA) assay can be found at the following links:

- For Healthcare Providers: https://www.fda.gov/media/137361/download
- For Patients: https://www.fda.gov/media/137362/download

Positive:

Positive SARS-CoV-2 IgG antibodies detected. Results suggest recent or prior infection with SARS-CoV-2. Correlation with epidemiologic risk factors and other clinical and laboratory findings is recommended. Serologic results should not be used to diagnose recent SARS-CoV-2 infection. Protective immunity cannot be inferred based on these results alone. False positive results for IgG antibodies may occur due to cross-reactivity from pre-existing antibodies or other possible causes.

ADDITIONAL INFORMATION Testing was performed using the VITROS Immunodiagnostic Product Anti-SARS-CoV-2 IgG Reagent Pack assay (Ortho Clinical Diagnostics, Inc.), which has received Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration.
Fact sheets for this Emergency Use Authorization (EUA) assay can be found at the following links:

- For Healthcare Providers: https://www.fda.gov/media/137361/download
- For Patients: https://www.fda.gov/media/137362/download

Table 5. Result Comment Examples for SARS-CoV-2 Serologic Testing

Example 2

NEGATIVE Result
First-line test NEGATIVE
The final result is reported as: NEGATIVE.
No further testing is performed.

POSITIVE Result
First-line test POSITIVE
Supplemental test POSITIVE
The final result is reported as: POSITIVE
SARS CoV-2 antibodies to two antigenic targets, N and S proteins, detected.

DISCORDANT Result
First-line test POSITIVE
Supplemental test NEGATIVE
The final result is reported as: DISCORDANT
SARS CoV-2 antibodies to N protein detected and antibodies to S protein not detected.
This result may be due to either specimen collection too soon following infection or an initial false-positive result. To rule out latter, patient history should be considered and, if indicated, patient should be re-tested in 7-14 days.

Example 3

Negative results do not rule out past or present infection with SARS-CoV-2.
Positive results may be due to past or present infection with non-SARS-CoV-2 coronavirus strains, such as coronavirus HKU1, NL63, OC43, or 229E.
Detectable antibody response (seroconversion) may require 14 or more days post-symptom onset in the general population. Immunocompromised patients (including cancer patients) may require 30 or more days or may never generate antibody levels that are detectable as positive by this assay.
Results from antibody testing should not be used as the sole basis to diagnose or exclude SARS-CoV-2 infection or to inform infection status. Follow-up testing utilizing molecular diagnostics should be considered to rule out infection.
This test has not been reviewed by the FDA. The FDA has made this test available under an emergency access mechanism called an Emergency Use Authorization (EUA) and its performance characteristics have been evaluated within the Special Chemistry section of the MD Anderson Department of Laboratory Medicine and deemed acceptable for patient testing in conjunction with clinical findings.
Not for the screening of donated blood.

Table 6. Relationship of PPV/NPV with Sensitivity and Specificity in OTA in Population with 2% Disease Prevalence

	TEST 1 Sensitivity	TEST 1 Specificity	TEST 2 Sensitivity	TEST 2 Specificity	% Initial Positive (TEST 1)	% Discordant	TEST 1 PPV	T1 + T2 PPV	T1 + T2 NPV
OTA 1	95.0%	98.0%	99.0%	95.0%	3.9%	1.9%	49.2%	95.0%	99.9%
OTA 2	99.0%	95.0%	95.0%	98.0%	6.9%	4.9%	28.8%	95.0%	100.0%

PPV/NPV, Positive/Negative Predictive Value; OTA, Orthogonal Testing Algorithm

Table 7: Reports of SARS-CoV-2 Serologic Testing Based on Orthogonal Testing Algorithm

Test 1 Result	Interim Report	Test 2 Result	Final Report
Negative	N/A	N/A	Negative
Positive	Presumptive positive	Negative	Negative/indeterminant/discordant *
Positive	Presumptive positive	Positive	Positive

* The final result is based on selection of tests for:

a. both tests employ the same methodology but target two different antigenic domains, report as Negative (https://www.fda.gov/media/137029/download);

b. tests employ different methods or different antibody classes but same antigenic target, report as Negative or Indeterminant (e.g. two CIA/CMIA tests; one detecting IgG antibodies against N-protein and the other detecting pan-Ig antibodies against N-protein);

c. tests employ different methods, different antigenic targets, report as Discordant.

Figure Legends

Figure 1. Generalized kinetic and dynamic model of antibody responses to SARS-CoV-2 with the expected positivity of qualitative and semi-quantitative assays.

Figure 2. General strategies for the serologic detection of SARS-CoV-2 antibodies. Common assay formats include lateral flow assays (LFAs), fluorescent microsphere immunoassays (FMIA), chemiluminescent immunoassays (CIA), enzyme-linked fluorescent assays (ELFA), and enzyme-linked immunoassays (ELISA). LFAs: Specific antibodies (e.g., IgG) to SARS-CoV-2 in the added sample flow through the membrane, bind antigen-gold conjugated nanoparticles, and are captured by antibodies immobilized in the 'test line'. This leads to the generation of a colored band that the user visually interprets. ELISA: Specific antibodies (e.g., IgG) to SARS-CoV-2 are captured by antigens immobilized on coated wells or other solid phase surfaces. Detection is the result of enzymatic production of a chromogen, chemiluminescence, or fluorescence, which may be measured using spectrophotometry, luminometry, or fluorometry, respectively. FMIA and CIA/CMIA: Specific antibodies (e.g., IgG) to SARS-CoV-2 are captured by antigens immobilized on paramagnetic microparticles or similar technology. Detection strategies often involve fluorescent or chemiluminescent antibody conjugates.

Figure 3. The relationship between assay sensitivity, specificity, disease prevalence, and positive and negative predictive values (PPV/NPV). (A) PPV, the proportion of true positives, is strongly influenced by the specificity of an assay when a disease is low prevalence. NPV, the proportion of true negatives, declines as disease prevalence increases. (B) A visual comparison of PPV for the same assay (sensitivity, 95%; specificity, 95%) performed in two test populations of 10,000 people with high (20%, PPV = 82.6%) and low (5%, PPV = 50.0%) disease prevalence (upper and lower panels).

Figure 4. Effect of an Orthogonal Testing Algorithm (OTA) on Positive Predictive Value (PPV). Shown is the performance of two tests with different characteristics (Test 1: sensitivity, 95%; specificity, 98%. Test 2: sensitivity, 99%; specificity, 95%) and their combined performance. Regardless of the order in which the tests are performed, sequential testing can increase PPV in testing populations with low disease prevalence.

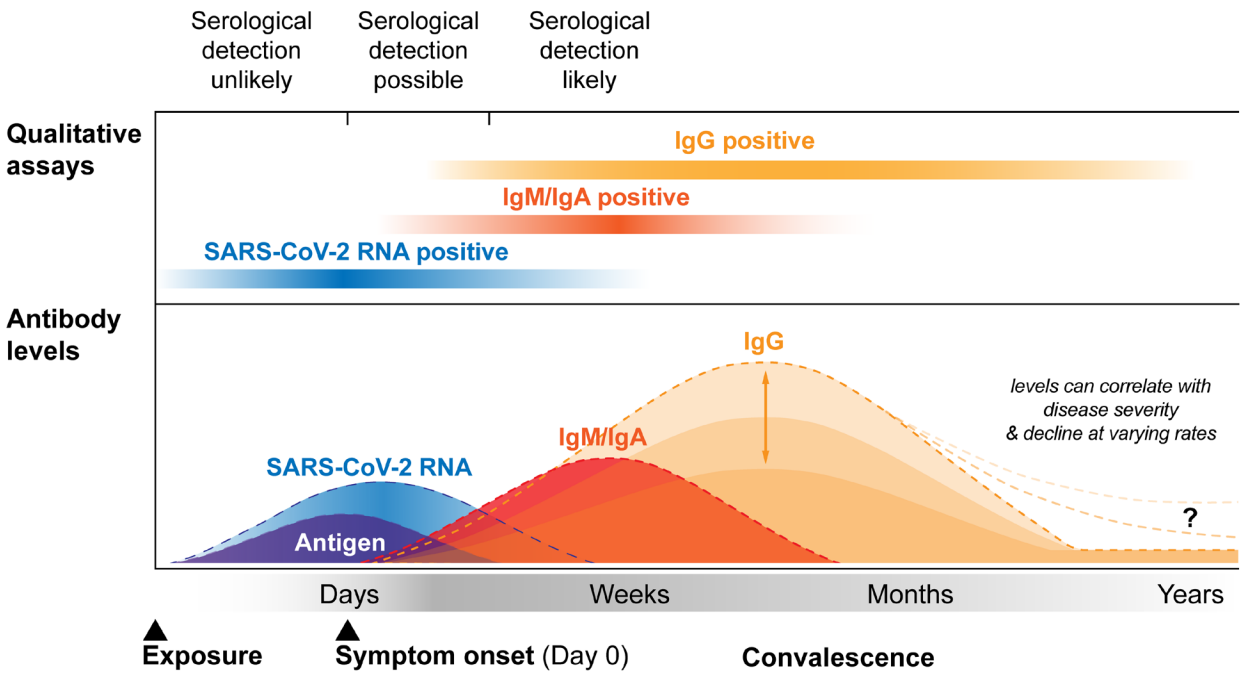

Figure 1. Generalized kinetic and dynamic model of antibody responses to SARS-CoV-2 with the expected positivity of qualitative and semi-quantitative assays.

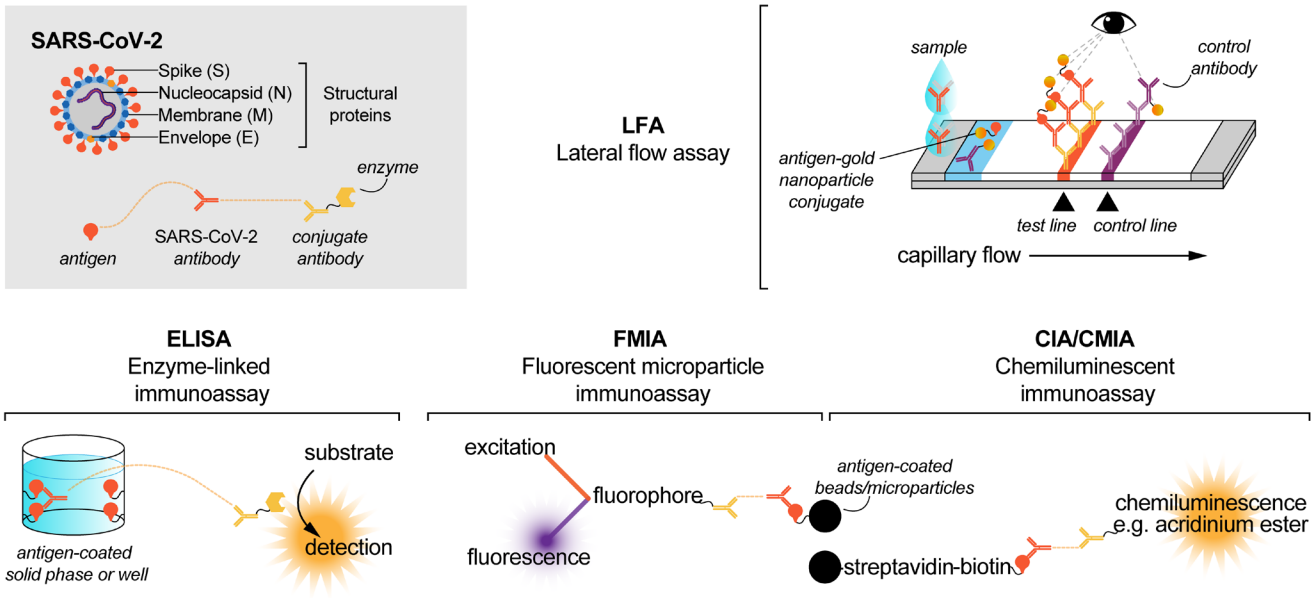

Figure 2. General strategies for the serologic detection of SARS-CoV-2 antibodies. Common assay formats include lateral flow assays (LFAs), fluorescent microsphere immunoassays (FMIA), chemiluminescent immunoassays (CIA), enzyme-linked fluorescent assays (ELFA), and enzyme-linked immunoassays (ELISA). **LFAs:** Specific antibodies (e.g., IgG) to SARS-CoV-2 in the added sample flow through the membrane, bind antigen-gold conjugated nanoparticles, and are captured by antibodies immobilized in the 'test line'. This leads to the generation of a colored band that the user visually interprets. **ELISA:** Specific antibodies (e.g., IgG) to SARS-CoV-2 are captured by antigens immobilized on coated wells or other solid phase surfaces. Detection is the result of enzymatic production of a chromogen, chemiluminescence, or fluorescence, which may be measured using spectrophotometry, luminometry, or fluorometry, respectively. **FMIA and CIA/CMIA:** Specific antibodies (e.g., IgG) to SARS-CoV-2 are captured by antigens immobilized on paramagnetic microparticles or similar technology. Detection strategies often involve fluorescent or chemiluminescent antibody conjugates.

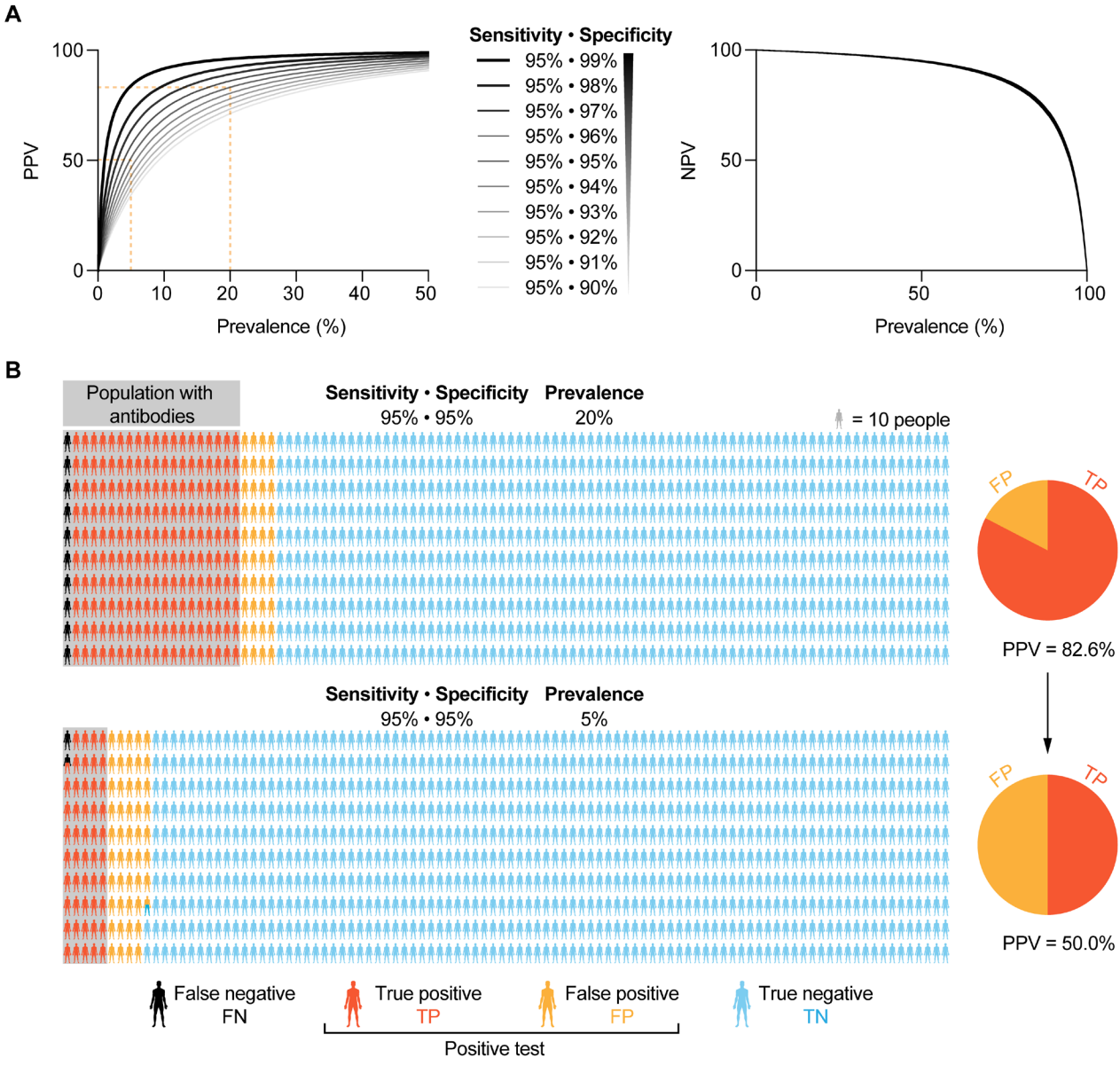

Figure 3. The relationship between assay sensitivity, specificity, disease prevalence, and positive and negative predictive values (PPV/NPV). **(A)** PPV, the proportion of true positives, is strongly influenced by the specificity of an assay when a disease is low prevalence. NPV, the proportion of true negatives, declines as disease prevalence increases. **(B)** A visual comparison of PPV for the same assay (sensitivity, 95%; specificity, 95%) performed in two test populations of 10,000 people with high (20%, PPV = 82.6%) and low (5%, PPV = 50.0%) disease prevalence (upper and lower panels).

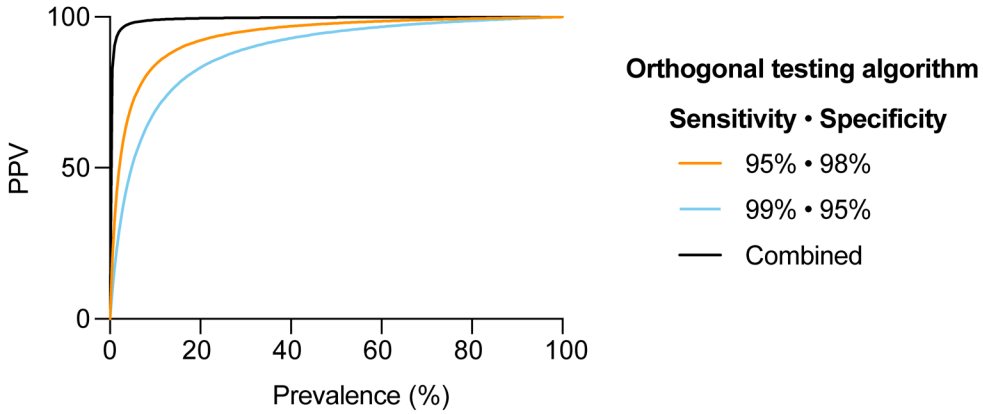

Figure 4. Effect of an Orthogonal Testing Algorithm (OTA) on Positive Predictive Value (PPV). Shown is the performance of two tests with different characteristics (Test 1: sensitivity, 95%; specificity, 98%. Test 2: sensitivity, 99%; specificity, 95%) and their combined performance. Regardless of the order in which the tests are performed, sequential testing can increase PPV in testing populations with low disease prevalence.