Luminex

xMAP® Technology:
A Benchmark for Serological Testing

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Introduction

In December 2019, a series of pneumonia cases of unknown etiology were reported in the Wuhan province of China which evolved into a global outbreak, affecting more than 200 countries and territories within just a few weeks.[1] A novel coronavirus strain of zoonotic origin—SARS-CoV-2—which has affected more than 7.9 million people globally and caused more than 434,000 deaths as of June 16, 2020, was identified as the cause of "coronavirus disease 2019" (COVID-19). With the increasing number of cases and the current lack of therapeutics or vaccines, it is essential to expand testing to enhance surveillance, monitor exposure rates within communities, detect stages of infection, and determine the longevity of protective immunity.

Nucleic acid tests based on viral genome sequences for SARS-CoV-2 are excellent tools for detecting current infection, and can aid in patient management, infection control, and the prevention of transmission. However, these tests cannot determine prior exposure to the pathogen or possible immunity, nor can they identify susceptible individuals. Therefore, serology (antibody) testing is essential to provide community-level immune response data that can identify exposure or past infection, potential donors of convalescent plasma for therapeutic purposes, and to assist public health officials in the implementation of safety policies, including social distancing. Serological testing will also be critical for determining the duration of protective immunity against the SARS-CoV-2 virus. From a therapeutic perspective, serological testing is a key component of vaccine and drug development because it assists with determining drug efficacy and the immune response to vaccines. Additionally, surveillance data obtained from serological testing can provide insights into the rate of community transmission and the efficacy of non-pharmaceutical interventions such as social distancing, quarantine, and travel restrictions.[2]

Multiplex serological testing can be particularly useful in a pandemic, as it can simultaneously analyze large numbers of antigens for large-scale screening and has the potential to replace traditional ELISA assays. Compared to ELISA, multiplex assays can shorten the time to results, minimize the volume of sample required, and eliminate excess labor by reducing the amount of testing that is needed. Among the various assay platforms that have been developed over the years, xMAP® Technology from Luminex® has emerged as the most common and well-established platform for multiplex analysis, with more than 50,000 peer-reviewed publications, and 65+ Luminex partners offering 1,300+ kits and custom assay solutions.

xMAP Technology

xMAP Technology uses a bead-based multiplexing assay platform that can rapidly detect and quantify multiple analytes in a single sample. xMAP Technology is based on the principles of flow cytometry, and uses polystyrene microspheres (beads) that are identical in size, physical properties, and surface composition, but have different amounts of internal classification dyes. The beads are internally dyed with precise amounts of two or three spectrally distinct fluorochromes, which are excited at the same wavelength, but have unique emission profiles that provide distinct spectral characteristics for each individual microsphere region (bead set), and allow each bead to be differentiated from all others in the multiplexed reaction (**Figure 1**). Each bead set can be covalently coupled with capture molecules that are specific to a target of interest. For a multiplex reaction, a mixture of coupled beads specific to different target molecules are added in a single reaction to simultaneously detect multiple analytes. A single reporter fluorochrome, R-phycoerythrin (PE), quantifies the binding events on the bead surface, and the fluorescence of the internal dyes allows for differential analysis of the multiplex data (**Figure 2**).

Figure 1.

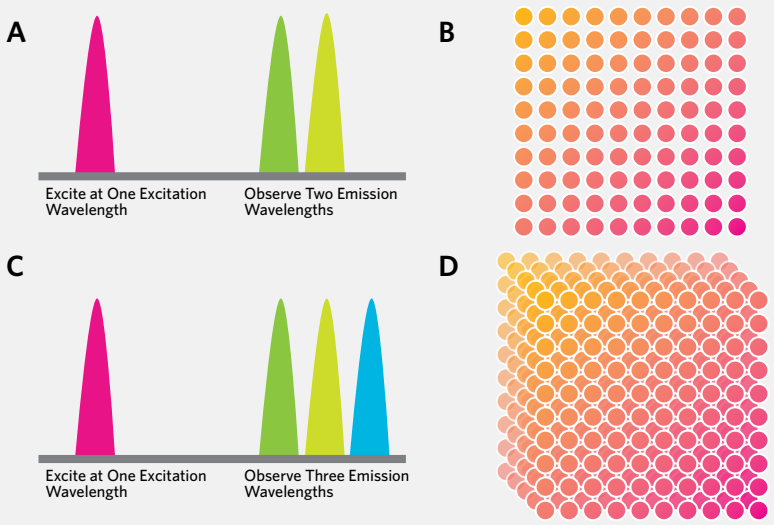

Figure 1. xMAP® Microspheres include two dyes, where one excitation wavelength allows for the observation of two separate fluorescence emission wavelengths (**A**), yielding 100 unique microsphere sets (10 x 10 dye matrix) (**B**), or three dyes, where one excitation wavelength allows for observation of three separate fluorescence wavelengths (**C**), yielding 500 unique microsphere sets (10 x 10 x 5 dye matrix) (**D**).

Figure 2.

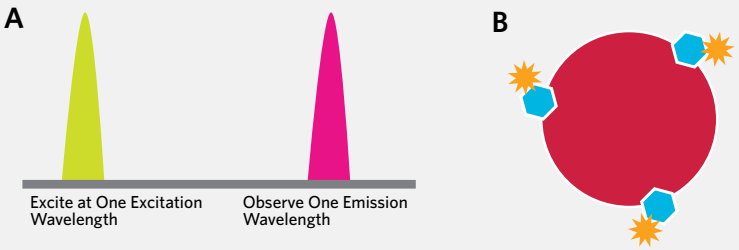

Figure 2. In addition to detection of internal bead dyes (shown in **Figure 1**), a second excitation wavelength allows for the observation of a separate fluorescent reporter molecule (**A**) that enables the detection of the analyte captured on the surface of the microsphere (**B**).

For an xMAP serology (indirect) immunoassay, antibodies of interest are captured using specific antigen-coupled beads. After the target antibody binds to the specific antigen, a secondary anti-species labeled antibody detects the antigen-antibody complex on the bead surface (**Figure 3A**). Competitive immunoassays are also commonly used for multiplex serological testing in vaccine research and development. In this particular competitive assay format, antigens for the pathogen of interest are covalently coupled to the microspheres. An antigen-specific reporter antibody and the antibody present in the sample compete for binding to the antigen-coupled bead. Thus, the signal decreases with increasing antibody titer in the sample (**Figure 3B**).

Figure 3.

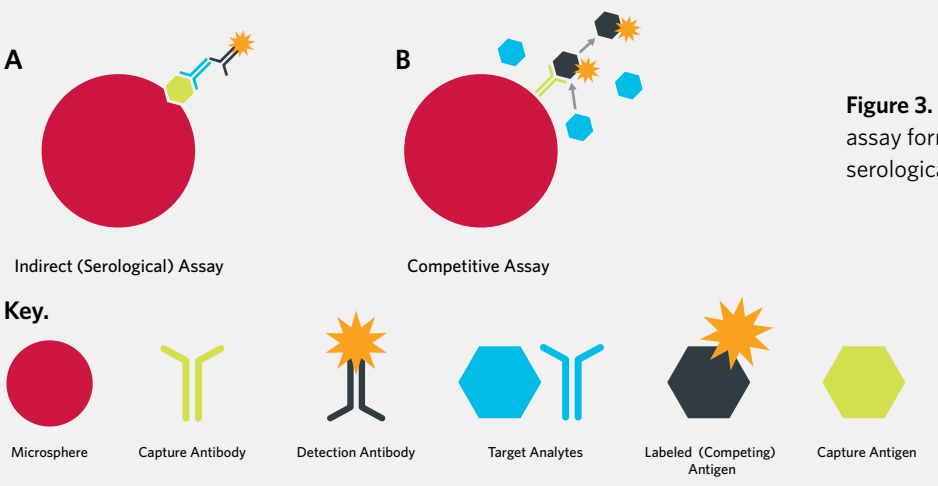

Figure 3. (**A**) Indirect serological assay format. (**B**) Competitive serological assay format.

xMAP Microspheres and Instruments

Depending on the application and the analytes of interest, there are several different types of beads that can be used to generate the best results (**Table 1**), and there are three different instruments for analysis of xMAP bead-based assays that cover a range of laboratory and budgetary needs (**Table 2**). MAGPIX® is the simplest, most affordable, and most compact system, and can perform up to 50 different tests in a single reaction volume using magnetic microspheres, delivering results on up to 96 samples in about an hour. The system can detect approximately 10^6 copies of DNA, or single-digit picogram levels of protein, and has a typical dynamic range of ≥3.5 logs. The Luminex® 100/200™ flow cytometry-based analyzer can measure up to 100 analytes in a single microplate well using just a few microliters of sample, and can read a 96-well plate in approximately 45 minutes. FLEXMAP 3D® is the most advanced and versatile multiplexing platform currently available, and is capable of simultaneously measuring up to 500 analytes in approximately 20 minutes for up to 96 samples and 75 minutes for up to 384 samples. FLEXMAP 3D also provides an extended dynamic range of ≥4.5 logs. All xMAP instruments can be easily automated by connecting to other front-end automation platforms, and can also interface with Laboratory Information Systems (LIS).

Table 1. Types of xMAP® Microspheres

Application	Microsphere	Type	Regions Available
All-Purpose	MicroPlex®	Non-magnetic	1–100
All-Purpose	MagPlex®	Magnetic	1–500
Nucleic Acid	MagPlex-TAG™	Magnetic	1–150
Protein	MagPlex®-Avidin	Magnetic	1–100
Protein	SeroMAP™	Non-magnetic	1–100

Table 2. xMAP® Instruments

Feature	MAGPIX®	Luminex® 100/200™	FLEXMAP 3D®
Cost	$	$$	$$$
Multiplex Capacity	50	100	500
Compatible Microspheres	MagPlex®, MagPlex-TAG™, and MagPlex®-Avidin	All xMAP® Microspheres	All xMAP® Microspheres
Technology	CCD camera with LEDs	Flow cytometry with lasers	Flow cytometry with lasers
Read Time	96-well plate in ~60 min (up to 4,800 tests/hour)	96-well plate in ~45 min (up to 12,800 tests/hour)	96-well plate in ~20 min (up to 144,000 tests/hour) 384-well plate in ~75 min (up to 153,600 tests/hour)

Applications of xMAP Technology for Serological Analysis

xMAP Serology in the Clinical Laboratory

xMAP-based serology tests have been widely implemented for the detection of pathogenic bacteria, viruses, and parasites. One of the earliest applications of a multiplexed xMAP serological assay involved the screening of HIV antibodies in newborn dried blood spot specimens for the early detection of HIV exposure in neonates.[3] The multiplexed assay developed by Bellisario et al. was able to simultaneously measure antibodies to three different purified recombinant HIV-1 antigens (p24, gp120, and gp160). The assay could distinguish 92 previously tested newborn specimens as either HIV-negative or HIV-positive, and was also able to clearly differentiate Centers for Disease Control and Prevention (CDC) Anti-HIV-1 blood-spot controls (zero, low, and high). Between run precision was determined using five independent assays, and the coefficient of variation (CV) was 6.5% and 5.6% for the low and high CDC controls, respectively. Similarly, Wu et al. used the xMAP platform to develop a rapid, sensitive, and high-throughput multiplexed serological assay for identifying specific IgG antibodies to nine hemorrhagic fever viruses.[4] Performance evaluation of the assay with clinical serum samples showed sensitivities of 90.7%–98.04% and specificities of >90% for all but two of the viruses.

Waterboer et al. developed a multiplexed serological immunoassay that could simultaneously detect antibodies against 100 *in situ* affinity–purified recombinant human papillomavirus (HPV) proteins.[5] The assay could measure antibodies at serum dilutions of >1:1,000,000, and demonstrated high reproducibility (R^2 = 0.97) and excellent concordance with single-plex ELISAs (κ = 0.846). Similar HPV serological assays were also developed to determine the seroprevalence of 34 HPV strains and to understand the natural history of cutaneous HPV infections.[6] The authors analyzed sera from 1,797 adults and children (aged 1-82 years), and observed three major seroprevalence patterns for HPV of phylogenetically distinct genera: antibodies to mu and nu skin gamma papillomaviruses appear early in life, those to mucosal alpha papillomaviruses appear in women after puberty, and antibodies to beta and gamma skin papillomaviruses accumulate later in life.

Pickering et al. developed an indirect immunofluorescent assay for antibodies to pneumococcal polysaccharides (PnPs) that could simultaneously determine serum IgG concentrations to 14 PnPs serotypes.[7] The xMAP assay demonstrated excellent correlation with single-plex ELISA for all serotypes, with correlation coefficients (R^2) ranging between 0.85–0.95. Compared to ELISA, the xMAP assay format was more efficient for pneumococcal antibody testing, as it eliminated the need for running multiple individual assays, which reduced time and labor, and conserved sample. The same investigators developed a similar multiplexed assay to detect antibodies to *Haemophilus influenzae* type b (Hib) polysaccharide, the toxoids of *Clostridium tetani* (Tet), and *Corynebacterium diphtheriae* (Dip).[8] A high correlation was observed when the xMAP assay was compared with single-plex ELISAs—0.96, 0.96, and 0.91 for Tet, Dip, and Hib, respectively. The above studies clearly demonstrate that xMAP bead-based serological assays are an attractive alternative to ELISAs for antibody testing in the routine clinical setting, and can greatly simplify immunogenicity testing for combination vaccines because they reduce the number of assays and sample volume required, hands-on time, and overall testing costs.

xMAP bead-based serological assays have also been implemented in outbreak situations. Ayouba et al. developed a multiplexed serological assay for the Zaire ebola virus (EV) using nine recombinant proteins (nucleoprotein [NP], 40-kDa viral protein [VP40], and glycoprotein [GP]) representing different viral regions from four EV lineages.[9] The performance of the assay was evaluated using 94 positive and 108 negative samples, and revealed high sensitivities of 95.7%, 96.8%, and 92.5%, and high specificities of 94.4%, 95.4%, and 96.3% for NP, GP, and VP40, respectively. Additionally, this high-throughput xMAP assay was extremely cost-effective when compared to single-plex ELISA ($4.09 vs. $54 for each sample), and therefore could be easily and economically implemented to screen large numbers of samples in an outbreak situation.

xMAP Serology in Vaccine Development and Research

In recent years, combination and polyvalent vaccines have become prominent from a public health perspective, as they allow consolidation of doses compared to single-component vaccines. Consolidation of doses not only provides protection against several different pathogens at the same time, but can also increase vaccine protection against pathogens that have closely related pathogenic strains or serotypes. For determining the efficacy of combination and polyvalent vaccines, multiplexing is the technology of choice, as it reduces the need for conducting multiple assays to confirm immune responses and cross-reactivity, uses less sample, and is faster, more reliable, and cost-effective.

An xMAP bead-based serological assay was developed by Opalka et al. for the detection of human IgG antibodies to nine anogenital HPV strains and virus-like particles (VLPs) in serum following natural infection or immunization with VLP-based vaccines.[10] The assay demonstrated an overall specificity of >99%, with an intra-assay precision of <10% relative standard deviation (RSD) and an inter-assay precision of <18% RSD across three different VLP-microsphere lots, two secondary antibody lots, and two different operators, over a period of three weeks. Opalka et al. also developed the first competitive Luminex serological assay (cLIA) that was used by Merck to simultaneously quantitate neutralizing antibodies to HPV types 6, 11, 16, and 18 to evaluate antibody responses in the GARDASIL® vaccine clinical trials.[11] To compare the accuracy of the cLIA with competitive radioimmunoassays (cRIAs), a panel of 45 human sera was tested using both assays. Comparison of serum titers revealed excellent concordance between the two assays (R^2 > 0.75). Additionally, the cLIA had 0% false positive and false negative rates, indicating that the xMAP assay is a sensitive and robust tool for quantitating antibody immune responses, and is well-suited for monitoring vaccine efficacy and studying natural history of infection.

Kelly et al. customized the Merck cLIA and automated the assay on a TECAN Genesis Workstation.[12] The automated program generated statistically similar data to the manual assay, with a 2–6.6% improvement in antibody detection levels. The TECAN platform was able to process 192 samples 13% faster than the manual assay. Dias et al. further optimized the Merck cLIA assay and improved its analytic sensitivity, specificity, accuracy, precision, and reproducibility for use in epidemiological studies and vaccine clinical trials.[13] To support Merck's nine-valent HPV (9vHPV) vaccine program, Roberts et al. developed a 9-plex cLIA to determine HPV-specific antibody titers in serum samples.[14] The assay demonstrated <1% cross-reactivity of the reporter monoclonal antibodies (mAbs), indicating that the relevant mAbs selected for the assay have high analytical specificity.

Serology assays have also been implemented for measuring antibody responses to pneumococcal vaccines. Biagini et al. developed a 25-plex fluorescent covalent microsphere immunoassay (FCMIA) for the detection of IgG antibodies to the 23 PnPS serotypes present in PNEUMOVAX23.[15] The assay also included two internal controls to assess preadsorption of pneumococcal cell wall polysaccharide (C-PS, a contaminant in PnPS vaccines) and inter-assay reproducibility. Tan et al. and Pavliakova et al. developed and validated a 13-plex xMAP-based competitive immunoassay to quantify antibodies to capsular PnPS (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) in human serum.[16,17] The lower limit of quantification ranged between 0.002–0.038 µg/mL serum IgG, and the difference between the lower limit and upper limit of the assay range (dynamic range) was >500-fold for all 13 serotypes. The assay variability was <20% RSD and was deemed a suitable replacement to the WHO pneumococcal ELISA platform.

Bandiera et al. developed and validated an xMAP-based tetraplex serological assay for the simultaneous quantitation of anti-DT, anti-TT, anti-PTxd, and anti-FHA antibodies in a guinea pig model following injection of a pediatric DTaP combination vaccine.[18] The assay could simultaneously measure antibodies for all four antigens in a single blood sample, reducing the sample volume required. Additionally, since the assay is a serological test, it eliminated the need for handling the toxins, thereby reducing operator risk. The authors concluded that the assay could be easily automated, since the operating conditions have been standardized, and it was estimated that it could reduce the testing time by one-third once implemented for both pediatric combination vaccines that are available.

Itell et al. developed an xMAP-based multiplex assay to determine whether an infant HIV vaccine interfered with the antibody responses to other commonly administered vaccines.[19] The seven-plex serological assay simultaneously measured antibodies against vaccines for hepatitis B, *Haemophilus influenzae* type B, diphtheria, tetanus, pertussis, rubella, and respiratory syncytial virus. The assay was highly sensitive, specific, and showed excellent concordance with single-plex ELISA. Furthermore, the multiplex assay required only half the time to run as compared to a 384-well ELISA, and it only used one-eighth of the sample volume, making the multiplex assay more suitable for vaccine interference studies of large cohorts.

A competitive serological assay has also been used in Pfizer's SA4Ag vaccine trials, which is a four-antigen *Staphylococcus aureus* investigational vaccine.[20] Begier et al. found through xMAP serological testing that the SA4Ag vaccine rapidly induced high levels of bacteria-killing antibodies in the 100 participants who were vaccinated.

xMAP Serology in Transplant and HLA Testing

Patel and Terasaki were the first to demonstrate that pre-formed donor-specific human leucocyte antigen (HLA) class I antibodies (DSA) in renal transplant recipients can cause hyperacute rejection.[21] Since then, it has been imperative to test all potential organ recipients for HLA antibodies to prevent transplantation of incompatible grafts. The complement-dependent cytotoxicity (CDC) assay had been the gold standard for detecting HLA antibodies, but this has been replaced by the multiplex xMAP serological assay.[22] xMAP-based serological assays are considered to be far more sensitive than the CDC assay, and allow for detection of multiple clinically relevant antibodies. Colombo et al. analyzed 1,421 sera to compare the results of an xMAP-based serological assay with the CDC assay and observed an 85% concordance between the two methods.[23] Using the xMAP assay, 18% of the patients on the waiting list were considered (and managed) as sensitized, as compared to only 7% when testing with the CDC assay alone. This indicated that the xMAP assay could detect a higher number of antibody specificities when compared to the CDC method.

xMAP serological assays are also used to determine the humoral response to donor alloantigens, which can play an essential role in acute vascular rejection and allograft failures. Zou et al. developed an xMAP serological assay to detect alloantibodies against MHC class I chain-related A (MICA) alleles, and analyzed sera from 85 kidney transplant recipients (waiting list) and 66 transplanted patients.[24] Anti-MICA antibodies were detected in 24.7% of the waiting list patients and 22.7% of the transplanted recipients, suggesting that anti-MICA antibodies can be involved in the pathogenesis of kidney allograft rejection.

xMAP Serology in Epidemiology and Surveillance

Epidemiological surveillance is key to infection control and monitoring, and can assist with disease elimination efforts in low-transmission areas. In addition to pathogen identification, detection of serological markers can provide additional information to estimate recent and past exposure to the pathogen. xMAP-based multiplex serological assays have been well-established in epidemiology for surveillance purposes. To monitor vaccine-preventable diseases, Caboré et al. developed a penataplex immunoassay for the simultaneous detection of IgG antibodies against diphtheria, tetanus, pertussis toxins, and other pertussis antigens.[25] The assay demonstrated good correlation with ELISA (coefficients between 0.89–0.98), a lower than 10% intra- and inter-assay variability, and was implemented successfully for performing large serosurveillance/seroprevalence studies. O'Hearn et al. developed a similar multiplexed assay to detect IgG against Lassa, Ebola, Marburg, Rift Valley fever, and Crimean-Congo

hemorrhagic fever viruses, as well as pan-assays for flaviviruses and alphaviruses.[26] Analysis of 675 serum samples revealed that 50.2% of samples were positive for Lassa virus, 52.9 % for flaviviruses, and 55.8 % for alphaviruses, but only 5.2% for Ebola virus, 10.7% for Marburg virus, 1.8% for Rift Valley fever virus, and 2.0 % for Crimean-Congo hemorrhagic fever virus. The study highlights the importance of surveillance, as the results revealed that in addition to Lassa virus (which is endemic in West Africa), other strains of viruses are also responsible for hemorrhagic fever and often go undetected and untreated.

Kerkhof et al. was the first to deploy a multiplexed malaria serological assay in Southeast Asia for field use.[27] They implemented a previously validated multiplexed xMAP assay for detection of antibodies against 14 *Plasmodium*-specific peptides and analyzed the assay reproducibility and the stability of the coupled beads over time. The assay was used to analyze 2,000 field blood spots and showed excellent reproducibility, with <15% RSD for inter-plate and intra-plate variability. The results indicated which antigens to use as serological markers for past and recent infections, and showed that multivariate analysis of antibody responses to various antigens could provide insights into the half-life of the antibodies.

Conclusion

In the 25 years that xMAP Technology has been available, thousands of peer-reviewed publications have described a multitude of applications for xMAP-based serological assays, establishing xMAP as a proven technology for serological testing. xMAP Technology combines the sensitivity and specificity of a traditional single-plex assay, while enabling the simultaneous detection of multiple analytes, providing more information with a faster turnaround time and less sample volume than ELISA. These features are advantageous in a variety of settings, but are particularly important for testing during an outbreak. The current COVID-19 pandemic demands rapid and accurate detection of the viral pathogen, as well as effective surveillance and monitoring to determine infection rates and status, to allow for the implementation of operational public health policies. Several scientists and Luminex partners are currently developing xMAP-based serological assays to aid in testing during this pandemic. Weiss et al. developed an xMAP serological assay and used it to assess the presence of antibodies for two proteins of SARS-CoV-2 in human sera from COVID-19-infected and uninfected individuals.[28] The assay could identify patients who have been infected with SARS-CoV-2 and have seroconverted. Randad et al. developed an xMAP-based ten-plex SARS-CoV-2 immunoassay for detecting salivary antibodies that had a significant correlation with serum sample results.[29] The assay demonstrated a 100% sensitivity and specificity for detecting prior SARS-CoV-2 infection. Dobaño et al. developed quantitative, xMAP-based multiplex assays for detecting IgM, IgA, and IgG against a panel of eight SARS-CoV-2 antigens.[30] For the best-performing combination of Ig isotypes and antigens, the assay demonstrated 100% specificity for SARS-CoV-2. Sensitivity was 94.94% for positive samples collected at ≥14 days following the onset of symptoms and 96.08% for those collected at ≥21 days. Additionally, the Wadsworth Center of the New York Department of Health developed an xMAP-based serological assay for the detection of total antibody (IgG, IgM, and IgA) to SARS-CoV-2 in human serum, which received Emergency Use Authorization (EUA) from the Food and Drug Administration (FDA).[31] The assay demonstrated a sensitivity of 88% and a specificity of 98.8%, and was implemented to detect reactive and nonreactive SARS-CoV-2 antibodies.

Luminex is currently developing and validating two xMAP-based antibody assays to aid in the identification of individuals who have been exposed, and developed an immune response, to SARS-CoV-2. These are qualitative assays that detect antibody responses against SARS-CoV-2 antigens. Both assays provide highly specific and sensitive results by targeting three different antigens—nucleocapsid (N), spike S1, and spike RBD—and using an algorithm to determine positivity. The first assay will detect the presence of IgG, and the second assay will detect and distinguish IgG, IgM, and IgA, with separate isotype calls for each. Both assays will be compatible with all existing xMAP platforms (MAGPIX, Luminex 200, and FLEXMAP 3D) and will be able to analyze 96 samples within a few hours. Additionally, Luminex is supporting numerous academic medical centers and research labs that are creating innovative multiplex antibody assays to better profile the immune response of patients and the public, as well as support vaccine and antiviral development. The SARS-CoV-2 pandemic continues with a daily emergence of new cases, so the implementation of serological testing, in addition to nucleic acid tests, is increasingly important to understand the pathology and impact of COVID-19. xMAP Technology has become the established benchmark for running these multiplex serology tests.

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Appendix A. Summary of Cited References

Application Area	References	Assay Focus	Plex
Clinical Laboratory/ Pathogen Detection	Bellisario R, et al. Early Human Development. 2001. 64(1): p. 21-25.	HIV-1 antibodies in newborn dried blood spots	3
	Wu W, et al. Virus Research. 2014. 187: p. 84-90.	Viral hemorrhagic fever	9
	Waterboer T, et al. Clinical Chemistry. 2005. 51(10): p. 1845-1853.	Human papillomaviruses	27
	Michael KM, et al. PLoS Pathogens. 2008. 4(6): p. e1000091-e1000091.	Human papillomaviruses	34
	Pickering JW, et al. American Journal of Clinical Pathology. 2002. 117(4): p. 589-596.	Pneumococcal capsular polysaccharides	14
	Pickering JW, et al. Clinical and Diagnostic Laboratory Immunology. 2002. 9(4): p. 872-876.	Tetanus, diphtheria, and *H. influenzae* Type b	3
	Ayouba A, et al. Journal of Clinical Microbiology. 2017. 55(1): p. 165-176.	Zaire Ebola virus	9
Vaccine Development and Research	Opalka D, et al. Clinical Vaccine Immunology. 2010. 17(5): p. 818-827.	Human papillomaviruses – VLP based vaccine	9
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