Introducing Multiplex and High Sensitivity Immunoassays for Characterization of SARS-CoV-2 Humoral Immunity

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Introduction

The coronavirus, SARS-CoV-2, is the pathogenic agent which causes COVID-19 in humans. This disease has become a global pandemic since it was first identified in Wuhan, China, in December 2019. SARS-CoV-2 is one of seven identified coronaviruses which infect humans, and along with SARS-CoV and MERS-CoV, cause severe illness. The remaining viral types (229E, NL63, OC43, HKU1) cause cold-like symptoms.

Multiple proteins make up the SARS-CoV-2 virus: the spike (S) proteins which form the "corona" of the virus and are composed of the subunit S1, which contains the receptor binding domain (RBD), and subunit S2. The spikes surround the membrane glycoprotein (M) and envelope protein (E) which contain the viral RNA encased by the nucleocapsid (N) protein (Figure 1). The SARS-CoV-2 viral RBD protein binds to the human angiotensin-converting enzyme 2 (ACE-2) receptors of cells found in multiple organs including lung, heart, arteries, gut, and kidney. Once bound, the virus enters the cell, replicates, and is released to continue the infection cycle. Each of these viral proteins are potential antigens against which the immune system can form antibodies to fight infection. The earliest antibodies to appear are immunoglobulin A (IgA) which forms in the mucosal tissues of the nasal passages and gut, and the humoral immunoglobulin M (IgM). The humoral immunoglobulin G (IgG) forms later and can confer lasting immunity to disease. All three immunoglobulins can be measured in blood serum and plasma samples.

By testing COVID-19 patient serum/plasma sample immunoglobulin response to SARS-CoV-2 antigens, researchers may identify individuals who have been exposed to the SARS-CoV-2 virus and have generated some level of immune response. Researchers may further understand the immune response to the virus over the course of infection and recovery from COVID-19.

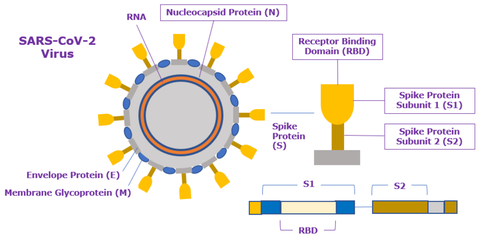

Figure 1. Antigenic proteins of the SARS-CoV-2 coronavirus.

Multiplex Assays

We have developed three MILLIPLEX® multiplex configurable assays for use with human serum and plasma samples, each to detect IgA, IgM, or IgG antibodies which recognize the selection of SARS-CoV-2 antigenic protein analytes: S1, S2, RBD, and N (Table 1). Each kit contains all reagents and the 96-well plate required to run the assay. MILLIPLEX® assays utilize Luminex® xMAP® technology and results in median fluorescent intensity (MFI) may be read on any Luminex® instrument system (Figure 2). The core of this technology consists of Luminex® MagPlex® magnetic carboxylated polystyrene microbeads, each dyed with a unique ratio (bead region) of two fluorophores which allow the instrument to discriminate each bead with its associated bound immunoassay sandwich. The fluorophore, phycoerythrin (PE), gives detection signal of the assay analyte.

MILLIPLEX® Assay Kits 96-well Plate Format	Cat. No.
SARS-CoV-2 Antigen Panel 1 IgM	**HC19SERM1-85K**
SARS-CoV-2 Antigen Panel 1 IgG	**HC19SERG1-85K**
SARS-CoV-2 Antigen Panel 1 IgA	**HC19SERA1-85K**
Available Analytes for Each Panel	
SARS-CoV-2 Spike Subunit 1 (S1)	
SARS-CoV-2 Spike Subunit 2 (S2)	
SARS-CoV-2 Receptor Binging Domain (RBD)	
SARS-CoV-2 Nucleocapsid Protein (N)	

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Table 1. Multiplex Assays.

Figure 2. Luminex® 200™, FLEXMAP 3D®, and MAGPIX® instruments.

The assay format (Figure 3) consists of the specific SARS-CoV-2 antigens conjugated to unique beads. These capture beads are incubated for 2 hours at room temperature (20-25°C), with shaking, with 25 µL human serum or plasma samples which have been diluted 1:100 in Assay Buffer. Antibodies in the sample which recognize each antigen will bind forming a bead-analyte sandwich. Sample is then washed away. A detection anti-human immunoglobulin type-specific antibody conjugated with the PE reporter, is then incubated to complete the sandwich. Excess detection antibody is washed away, and the sample MFI is read in the Luminex® instrument. Each panel is specific for detection of human immunoglobulin IgM, IgG, or IgA. These assays are qualitative and do not include standards for quantitation. It is recommended that researchers run non-infected control samples to establish an experimental MFI cutoff.

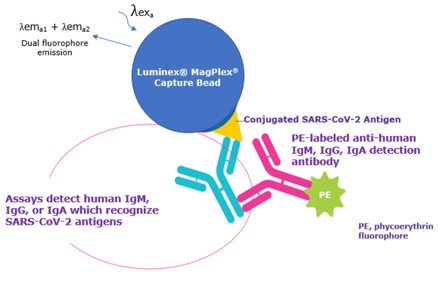

Figure 3. MILLIPLEX® assay format for these kits.

Each kit includes a set of control beads, which may be combined in the same assay with the analyte beads, to qualify assay performance. Three immunoglobulin-conjugated Positive Assay Control beads (IgM, IgG, or IgA, dependent on the assay kit) has been conjugated with a different amount of immunoglobulin and will show varying levels of relative MFI readings, covering the detectable range of the assay. One Negative Assay Control bead is included (no immunoglobulin conjugation).

Intra-assay precision results for all three panels were found to be <15% CV as calculated from the mean of the %CV's from eight reportable results in a single assay. Inter-assay precision for all three panels were <20% CV as calculated from the mean of the %CV's across four different assays. Example data from a reagent COVID-19 PCR positive patient is shown (Figure 4).

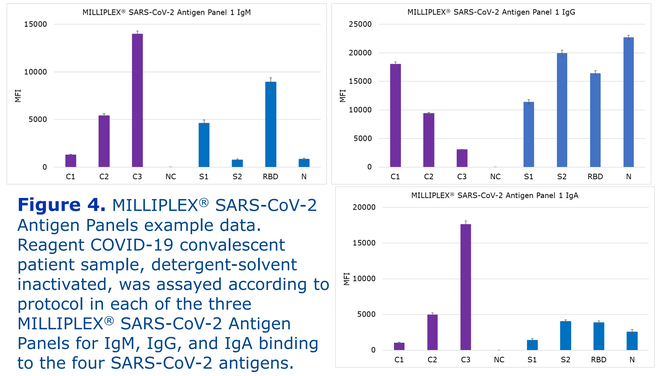

Figure 4. MILLIPLEX® SARS-CoV-2 Antigen Panels example data. Reagent COVID-19 convalescent patient sample, detergent-solvent inactivated, was assayed according to protocol in each of the three MILLIPLEX® SARS-CoV-2 Antigen Panels for IgM, IgG, and IgA binding to the four SARS-CoV-2 antigens. Assay control beads C1, C2, C3 and Negative Control are shown in purple. Note that the assay control beads for the IgG assay are shared components with our auto-antibody kits and are in reverse order of high, medium, and low IgG bound to bead. Antigen-beads for SARS-CoV-2 Spike S1, Spike S2, Receptor Binding Domain (RBD), and Nucleocapsid protein (N) are shown in blue. (Mean, n=4 separate assays, +/- SD.)

Single Molecule Counting Assays

In addition to the MILLIPLEX® kits for broadly profiling SARS-CoV-2 mediated humoral immune response, we also offer three focused assays on the Single Molecule Counting (SMC™) ultrasensitive immunoassay platform. This platform has already proven itself to be very effective in studying low abundance biomarkers in many other disease models. Similarly, SARS-CoV-2 researchers analyzing IgG, IgA, and IgM can harness the improved sensitivity and resolution of this platform over traditional serology-based assays (Figure 5) for COVID-19 research spanning many fields, including vaccine development, epidemiology, and public health (Figures 6). Example applications for our new SMC™ SARS-CoV-2 antibody detection kits (Table 2) include: **1)** thorough mapping SARS-CoV-2 immunity from early to late stages of infection (Figure 7), **2)** stratification of vaccine candidate efficacy among clinical trial participants based on heterogeneity within populations, **3)** improved real-time monitoring of COVID-19 transmission patterns to inform public policy decisions and predict emerging infection hotspots, and **4)** understanding the correlation between level of immune response and clinical outcome through the use of our comprehensive SMC™ cytokine assays. Above all, scientists can enjoy these benefits using the user-friendly, easy-to-maintain SMCxPRO™ instrument platform.

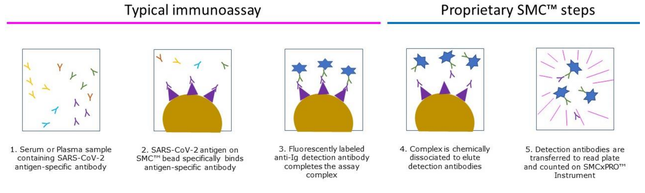

Figure 5. Mechanism of SMC™ SARS-CoV-2 antigen-specific antibody detection. Proprietary assay complex elution and digital counting of detection antibody confer improved sensitivity and dynamic range than offered by typical immunoassays

- **Sensitively and specifically detect low level SARS-CoV-2 antigen-specific response**
- **Thoroughly track humoral response beginning within 3 days of PCR positive result**
- **Characterize variation in immune response among individuals and subpopulations**
- **Requires as little as 2 µL of sample per assay**
- **Easy to use instrument and software platforms**
- **SMC™ cytokine assay also available for correlating humoral and cellular immunity**
- **Comprehensive user support from Merck**

Figure 6. SMCxPRO™ instrument and benefits of utilizing SMC™ technology to study SARS-CoV-2 mediated immunity.

SMC™ SARS-CoV-2 Antigen-Specific Ig Kits	Cat. No.
SARS-CoV-2 RBD IgG Kit	**03-0193-00**
SARS-CoV-2 S1 IgA Kit	**Coming Soon**
SARS-CoV-2 RBD IgM Kit	**Coming Soon**
SMC™ Cytokine-Based Kits for COVID-19 Research	**Cat. No.**
SMC™ Human IL-6 High Sensitivity Immunoassay Kit	03-0155-00
SMC™ Human TNFα High Sensitivity Immunoassay Kit	03-0163-00
SMC™ Human IFNα2 High Sensitivity Immunoassay Kit	03-0186-00
SMC™ Human IL-4 High Sensitivity Immunoassay Kit	03-0161-00

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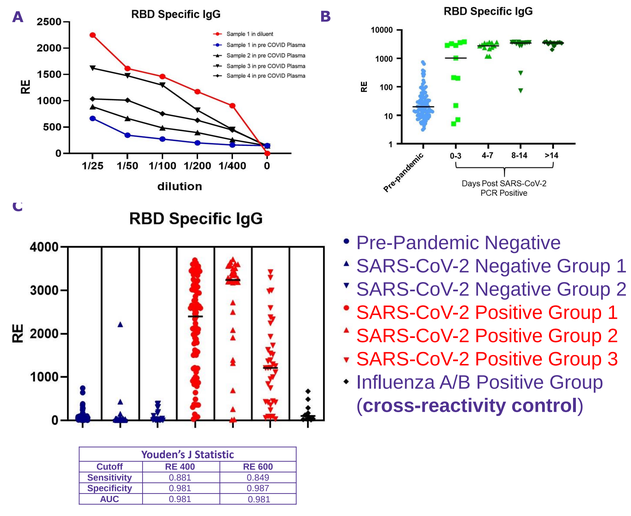
Table 2. Single Molecule Counting (SMC™) Assays.



Youden's J Statistic		
Cutoff	RE 400	RE 600
Sensitivity	0.881	0.849
Specificity	0.981	0.987
AUC	0.981	0.981

Figure 7. SMC™ SARS-CoV-2 RBD IgG Kit example data. **A)** SMCxPRO™ instrument response (RE) in five plasma samples diluted in kit standard diluent or pre-COVID plasma; "0" condition corresponds to diluent or pre-COVID-plasma only. **B)** SARS-CoV-2 RBD IgG detection on samples from pre-pandemic and SARS-CoV-2 infected patients, showing antibody detection within first three days after PCR SARS-CoV-2 positive result. **C)** Aggregate data from testing of >300 samples from pre-pandemic, SARS-CoV-2 positive, and Influenza A/B positive training cohorts, demonstrating high assay sensitivity and specificity when 400 and 600 are used as example cutoffs in Youden's J Statistic calculations.

Summary

Our scientists have developed several MILLIPLEX® and Single Molecule Counting (SMC™) based SARS-CoV-2-specific immunoassay kits in response to the COVID-19 research community's growing need for research tools used to further our understanding of the novel coronavirus. These qualitative Research Use Only (RUO) assays, which can detect antibodies against SARS-CoV-2 antigens in human serum or plasma samples, are intended to empower researchers performing vaccine efficacy, epidemiology, and population studies. Our new MILLIPLEX® SARS-CoV-2 panels can facilitate simultaneous detection of the SARS-CoV-2 Spike S1, Spike S2, Receptor Binding Domain (RBD), and Nucleocapsid (N) proteins and antigen-specific IgM, IgG, or IgA antibody responses on the Luminex® multiplex platform. They are well suited for examining the immune response to the virus over the course of infection and recovery from COVID-19. Meanwhile, our SMC™ SARS-CoV-2 kits offer the ability to characterize viral antigen specific humoral immunity with greater sensitivity, such as in cases involving acute infections and subclinical immune responses. For these new assays, data are presented from human patient samples sets confirmed positive or negative for COVID-19 by SARS-CoV-2 PCR tests. Overall, our new MILLIPLEX® and SMC™ assays can assist in COVID-19 research efforts to help society overcome the current global pandemic.

sigmaaldrich.com/immunoassays