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Application Note

# Multiplex Assays to Detect Antibodies which Recognize SARS-CoV-2 Antigenic Proteins in Human Serum and Plasma

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## Introduction

The coronavirus, SARS-CoV-2, is the pathogenic agent that 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, can cause serious disease. The remaining viral types of coronavirus (229E, NL63, OC43, HKU1) cause cold-like symptoms.

Multiple proteins make up the SARS-CoV-2 virus. The spike (S) proteins that form the "corona" of the virus are composed of the subunit S1, which contains the 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 receptor binding domain (RBD) protein binds to the human angiotensin-converting enzyme 2 (ACE-2) receptors of cells found in multiple organs including the lungs, heart, arteries, gut, and kidneys. 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.

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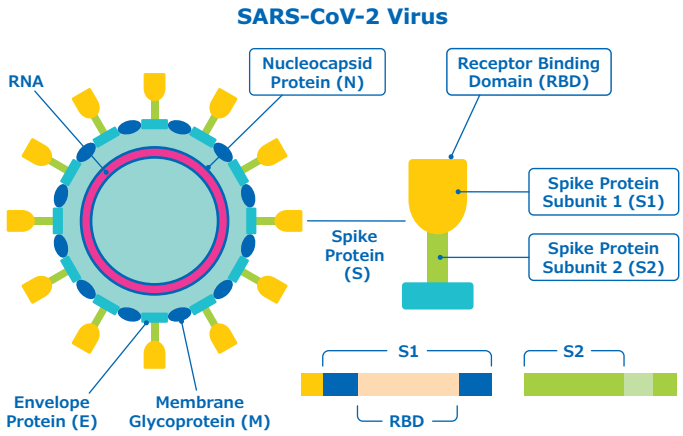


**Figure 1**. Antigenic proteins of the SARS-CoV-2 coronavirus.

## Multiplex Assays

We have developed three MILLIPLEX® multiplex kits for research use only (RUO), not for use in diagnostic procedures. Our MILLIPLEX® multiplex assays have been tested for use with human serum and plasma samples. Each kit can detect IgA, IgM, or IgG antibodies which recognize the selection of SARS-CoV-2 antigenic protein analytes: S1, S2, RBD, and N (**Table 1**). Users may select all of the analytes or just a subset of them for use. Each kit contains all reagents and the 96-well plate required to run the assay.

| 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 Binding Domain (RBD) | |
| SARS-CoV-2 Nucleocapsid Protein (N) | |

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**Table 1**. MILLIPLEX® Assay Kits for COVID-19 Research.



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MILLIPLEX® assays utilize Luminex® xMAP® technology and the 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 of two fluorophores which allow the instrument to discriminate each bead with its associated bound immunoassay sandwich. The fluorophore, phycoerythrin (PE), gives the detection signal of the assay analyte.

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

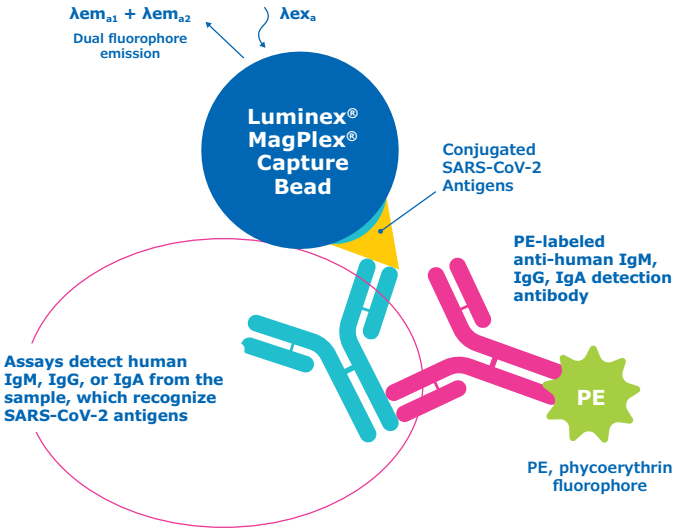


**Figure 3**. MILLIPLEX® assay format for these kits.

The assay format (**Figure 3**) for each of the three MILLIPLEX® SARS-CoV-2 panels consists of the specific SARS-CoV-2 antigen conjugated to a unique bead region of a MagPlex® bead. These capture beads are then incubated with appropriately diluted human serum or plasma samples. Antibodies in the sample which recognize each antigen will bind, forming a bead-analyte sandwich. The sample is then washed away. A detection anti-human immunoglobulin type-specific antibody conjugated to the PE reporter is then incubated to complete the sandwich, as illustrated. Excess detection antibody is removed, and the sample MFI is read on the Luminex® instrument. Each panel is specific for the 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.

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 kit) have 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) (**Figure 4**).

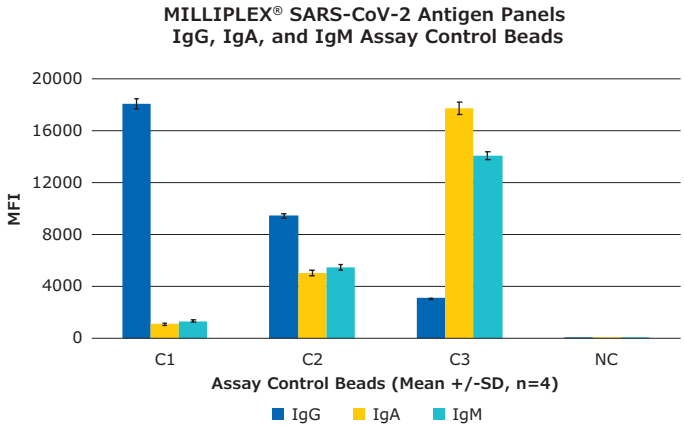


**Figure 4**. Assay Control Bead MFIs. Note that the IgG beads' relative MFIs read in high, medium, and low order, whereas the IgA and IgM beads' MFIs are in order of low, medium, and high relative MFI.

Commercially available or in-house recombinant IgG antibodies to SARS-CoV-2 subunits S1, S2, RBD, and N were diluted into human serum/plasma collected prior to 2020 and tested in the MILLIPLEX® SARS-CoV-2 Antigen Panel 1 IgG 4-plex assay to confirm assay performance. Due to a lack of commercially available IgA and IgM antibodies to the SARS-CoV-2 analyte subunits, similar tests could not be performed on the IgA or IgM panels.

Intra-assay precision results for all three panels were found to be <15% CV as calculated from the mean of the %CVs from 8 reportable results in a single assay. Inter-assay precision for all three panels were <20% CV as calculated from the mean of the %CVs across 4 different assays.

These MILLIPLEX® kits are manufactured in facilities which are ISO 9001:2015 compliant, and are for research use only, not for use in diagnostic procedures.

## Materials and Methods

### Samples

Patient samples were obtained, assayed, and analyzed as described.[1] The samples were from patient groups testing positive or negative by PCR for SARS-CoV-2 infection: COVID-19 positive "Ventilated" (n=68), COVID-19 positive "Not Ventilated" (n=115), and COVID-19 negative "COVID-" (n=41).

### Kit Protocol

EDTA plasma samples were assayed in duplicate for all four analytes, S1, S2, RBD, and N, according to protocol in each of the MILLIPLEX® SARS-CoV-2 kits, IgG, IgA, and IgM. All three kit protocols are identical, save for the immunoglobulin detected and the order of assay controls' MFI. Kit reagents were prepared and warmed to room temperature (RT) prior to use. Samples were diluted 1:100 in Assay Buffer.† 96-well plate wells were pre-wetted with 200 µL Wash Buffer, covered with adhesive plate sealer, and incubated for 10 minutes at RT with shaking, then decanted. To all wells, 25 µL of Assay Buffer was added, with an additional 25 µL of Assay Buffer added to background wells. 25 µL of each diluted sample was added to the appropriate sample wells. 60 µL of each sonicated (30 seconds) and vortexed (1 minute) analyte and control bead was combined and brought to a final volume of 3 mL with the addition of Assay Buffer, vortexed, and 25 µL of bead mixture was dispensed into each well. The plate was sealed and incubated for 2 hours at RT with constant shaking to maintain bead suspension. A handheld magnetic plate washer (Cat. No. 40-285) was used to retain magnetic beads while liquid contents were decanted and plate wells washed 3 times with 200 µL per well Wash Buffer. 50 µL of PE-anti-human immunoglobulin (IgG, IgA, or IgM per kit in use) detection antibody was added to each well, the plate was sealed, and incubated 90 minutes at RT with constant shaking. The magnetic plate washer was used to wash the plates 3 times with 200 µL per well Wash Buffer. 150 µL Sheath Fluid (Cat. No. 40-50015) was added to each well, the plate was then sealed and shaken at RT for 5 minutes. The plate was then read on a Luminex® 200™ instrument.

† Higher dilutions, such as 1:200, may be required for samples with very high antibody titer.

## Results

Graphed assay results (**Figure 5a, 5b, 5c**) show individual MFI for each sample in each group with group means, +/- standard deviation (SD) and p-value significance tests between groups: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

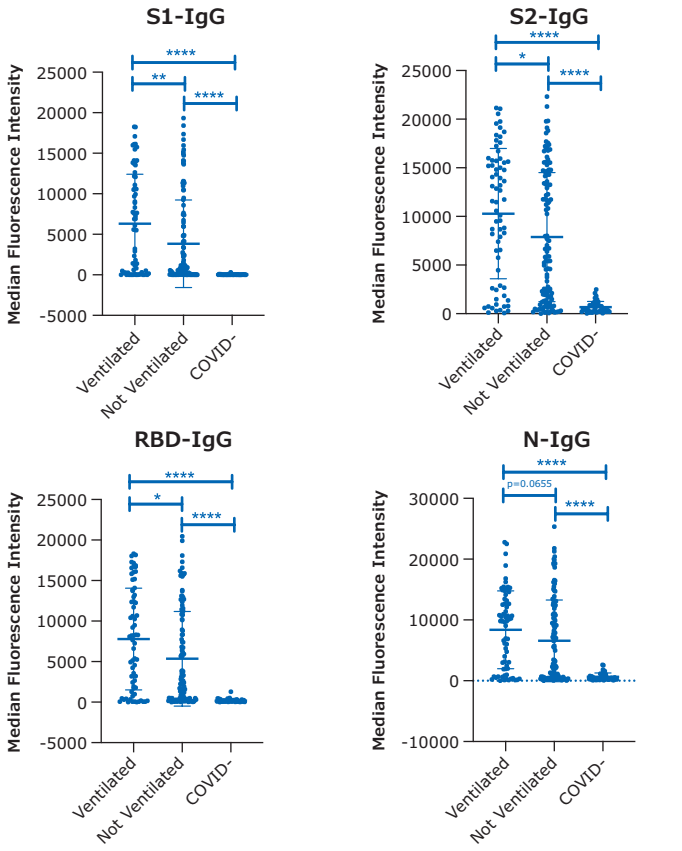


**Figure 5a**. Results of human patient samples run in MILLIPLEX® SARS-CoV-2 Antigen Panel 1 IgG, 4-plex analytes: S1, S2, RBD, N.

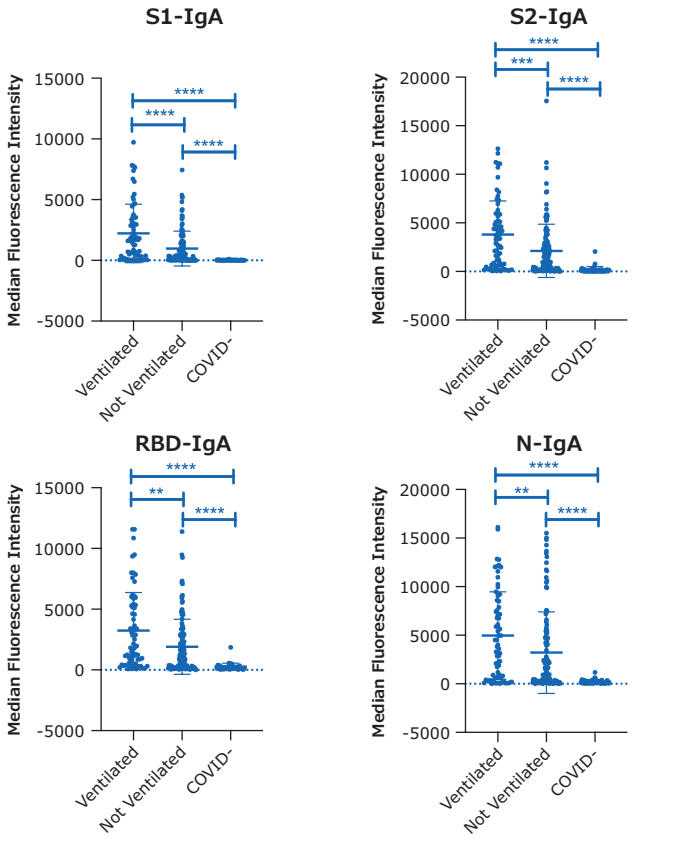


**Figure 5b**. Results of human patient samples run in MILLIPLEX® SARS-CoV-2 Antigen Panel 1 IgA, 4-plex analytes: S1, S2, RBD, N.

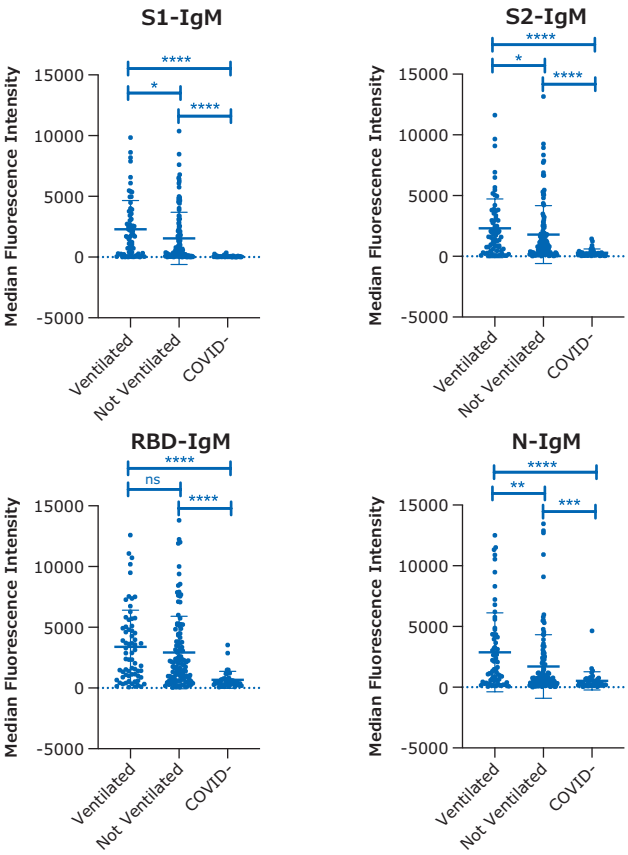


**Figure 5c**. Results of human patient samples run in MILLIPLEX® SARS-CoV-2 Antigen Panel 1 IgM, 4-plex analytes: S1, S2, RBD, N.

## Discussion

We have developed three new MILLIPLEX® kits which allow researchers to test human serum or plasma samples for IgG, IgA, and IgM antibodies which recognize the SARS-CoV-2 protein subunits S1, S2, RBD, and N.

These RUO assays have been verified by testing with human serum and plasma samples from a cohort of SARS-CoV-2 PCR positive and negative COVID-19 patients. These kits require minimal sample volume to run, at 25 µL of 1:100 dilution per well in a 96-well format. The assay duration is approximately one half-day, with capture-bead and sample incubation of 2 hours followed by a 90-minute incubation with detection antibody prior to reading on the required Luminex® instrument.

These kits will be of use to researchers who are studying COVID-19 in humans. Performing IgM, IgG, and IgA blood-based testing may help identify people who have been exposed to SARS-CoV-2, and who have generated some level of immune response. By testing the response of each immunoglobulin type against specific antigenic regions of the SARS-CoV-2 virus, researchers may further understand the immune response to the virus over the course of infection and recovery from COVID-19.

Now that vaccines against SARS-CoV-2 are available, these kits may provide researchers with tests to determine whether a person has immunity due to natural infection or immunity from vaccination. Vaccinated individuals would potentially have titers against spike proteins but not the nucleocapsid protein. Of course, should vaccines containing the nucleocapsid protein as well as spike proteins come into use, these tests would no longer apply.

**For additional information on these assays, contact our Technical Support team at SigmaAldrich.com/milliplex**

**For related panels, visit SigmaAldrich.com/milliplex-covid-19**

## Acknowledgments

We wish to thank our collaborators Mary K. Young, Michael D. Solga, Rebecca M. Carpenter, Jennifer M. Sasson, and William A. Petri Jr. of the University of Virginia Health System, Charlottesville, VA, 22908, USA.

## Reference

1. IgG Antibodies against SARS-CoV-2 Correlate with Days from Symptom Onset, Viral Load and IL-10, Mary K. Young, Christine Kornmeier, Rebecca M. Carpenter, Nick R. Natale, Jennifer M. Sasson, Michael D. Solga, Amy J. Mathers, Melinda D. Poulter, Xiao Qiang, William A. Petri Jr. https://doi.org/10.1101/2020.12.05.20244541 medRxiv.org preprint dated Dec. 7, 2020.

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