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APPLICATION NOTE

A CUSTOM, MULTIPLEX HUMAN CORONAVIRUS SEROLOGICAL ASSAY

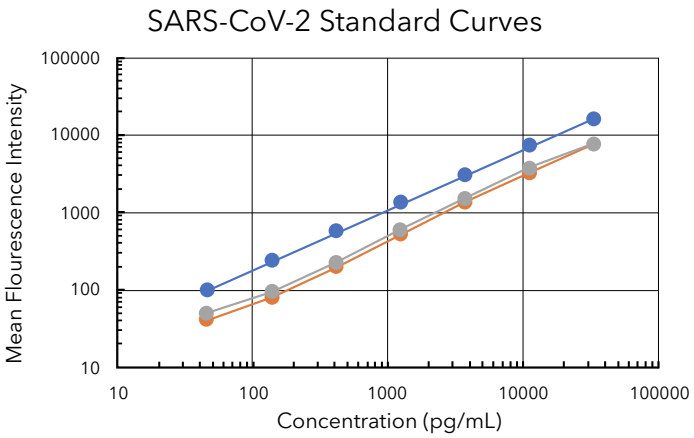
In less than a year, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) swept globally, infecting over 30 million people. Of those infected, more than 1 million people have been killed by Coronavirus 2019 (COVID-19) disease. As such, assessing the prevalence of SARS-CoV-2, along with SARS-CoV-2 vaccine development have been top global priorities. COVID-19 screening assays can be broadly classified into diagnostic/antigen tests[1] that detect viral RNA or viral protein in the host during an active infection, and serological assays that detect the antibody response to the virus. Screening for active infections has been challenging for two interrelated reasons. Although SARS-CoV-2 has a lower mortality rate than the related SARS-CoV and middle east respiratory syndrome coronavirus (MERS-CoV), it has a higher transmission rate[2-4]. Furthermore, it has been estimated that ~45% of those infected with the virus are asymptomatic[5]. In the absence of symptoms to inspire diagnostic testing, it is apparent that the percentage of COVID-19 infected people may be severely underestimated. Given the possibility of such a large percentage of asymptomatic carriers, serological assays play a crucial role in the Center for Disease Control (CDC) COVID-19 serology surveillance strategy[6].

SARS-CoV2 is a member of the *coronaviridae* family, specifically the *coronavirinae* subfamily that includes 4 genera: α-coronavirus, β-coronavirus, γ-coronavirus and δ-coronavirus[2]. SARS-CoV-2, SARS-CoV and MERS-CoV are all RNA β coronaviruses. SARS-CoV-2 shares 80% sequence identity with SARS-CoV and 50% sequence identity with MERS-CoV[3,7]. It has been estimated that members of the α-coronavirus and β-coronavirus family are also responsible for 10-30% of common colds[8,9]. Given the promiscuous nature of coronavirus, the need to evaluate the immune response to coronavirus subtypes becomes apparent. To what degree does exposure to the common cold virus, SARS-CoV or MERS-CoV enhance or diminish immunity to SARS-CoV-2? To answer this question, investigators need to be able to evaluate the specificity and cross-reactivity of the immune response to coronaviruses. R&D Systems, a Bio-Techne brand, has developed a novel custom Luminex serological assay that distinguishes between coronavirus subtypes. Using our high-quality recombinant proteins, this antigen-down assay saves time and precious sample by multiplexing many coronavirus subtypes simultaneously in each sample.

CORONAVIRUS SPIKE PROTEINS USED IN THE LUMINEX ASSAY		
CATALOG #	PROTEIN	LUMINEX ASSAY STANDARD CURVE RANGE
10522-CV	SARS-CoV-2 Spike S1	41-7,478 pg/mL
10523-CV	SARS-CoV-2 Spike RBD	49-7,608 pg/mL
10474-CV	SARS-CoV-2 Nucleocapsid	98-16,026 pg/mL
10521-CV	MERS-CoV Nucleocapsid	61-9,853 pg/mL
Coming Soon!	MERS-CoV Spike S1	964-21,912 pg/mL
10621-CV	MERS-CoV Spike RBD	58-8,380 pg/mL
10600-CV	HCoV-HKU1 Spike RBD	143.5-18,685.5 pg/mL
Coming Soon!	HCoV-OC43 Spike RBD	35-3,153 pg/mL
10605-CV	HCoV-NL63 Spike RBD Protein	22.5-2,286.3 pg/mL
10612-CV	HCoV-229E Spike RBD Protein	55.5-7,315.3 pg/mL

TABLE 1. Coronavirus Spike Proteins Used in the Luminex Assay

A



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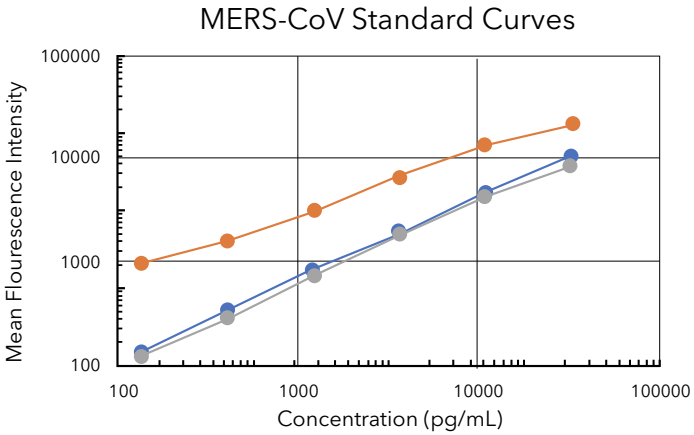

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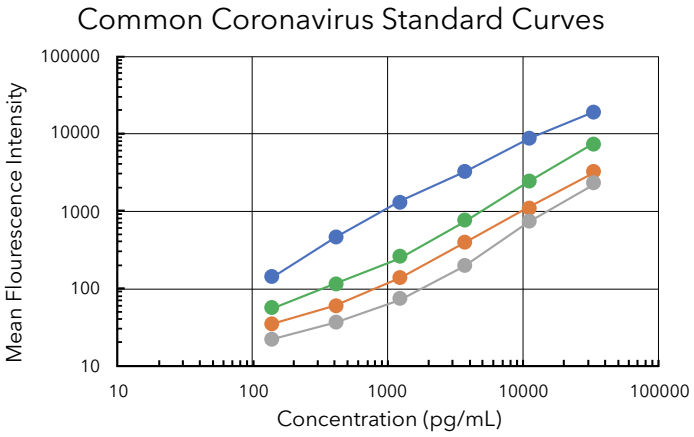

FIGURE 1. Bio-Techne Human Coronavirus Proteins in a Serological Immunoassay. A multiplex serological assay using Luminex MagPlex® Microspheres was assembled using human coronavirus proteins offered by R&D Systems, a Bio-Techne brand. Using anti-coronavirus and anti-His-Tag antibodies, standard curves were generated for: (**A**) SARS-CoV-2 proteins (Nucleocapsid-Blue, Spike S1-Orange, Spike RBD-Gray), (**B**) MERS-CoV proteins (Nucleocapsid-Blue, Spike S1-Orange, Spike RBD-Gray), and (**C**) common human coronavirus proteins (HKU1 RBD-Blue, OC43 RBD-Orange, NL63 RBD-Gray, 229E RBD-Green).

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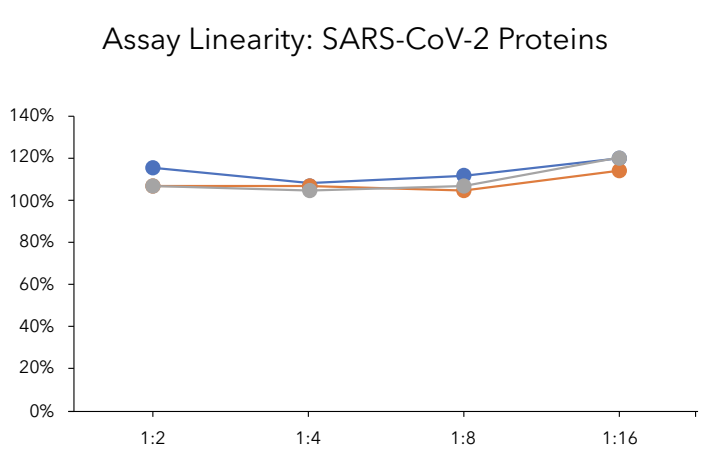

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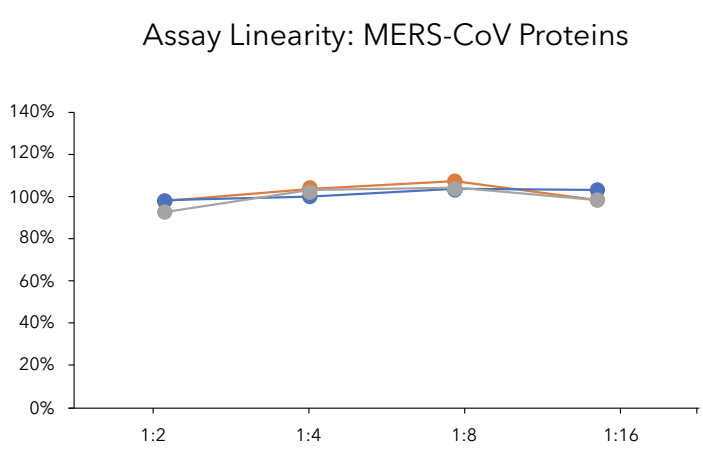

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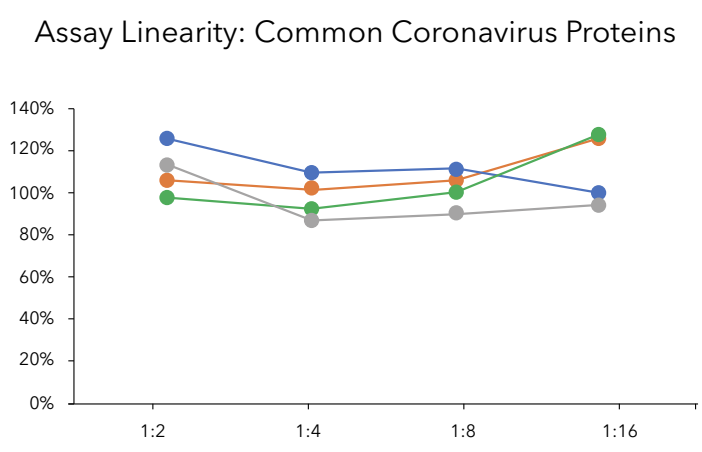

FIGURE 2. Natural Sample Assay Linearity. To show that the proteins used in the multiplex serological assay to capture anti-coronavirus antibodies bound specifically to their intended target, linearity was measured by diluting high positive serum samples with negative serum samples. Linearity plots were generated for: (**A**) SARS-CoV-2 proteins (Nucleocapsid-Blue, Spike S1-Orange, Spike RBD-Gray), (**B**) MERS-CoV proteins (Nucleocapsid-Blue, Spike S1-Orange, Spike RBD-Gray), and (**C**) common human coronavirus proteins (HKU1 RBD-Blue, OC43 RBD-Orange, NL63 RBD-Gray, 229E RBD-Green).

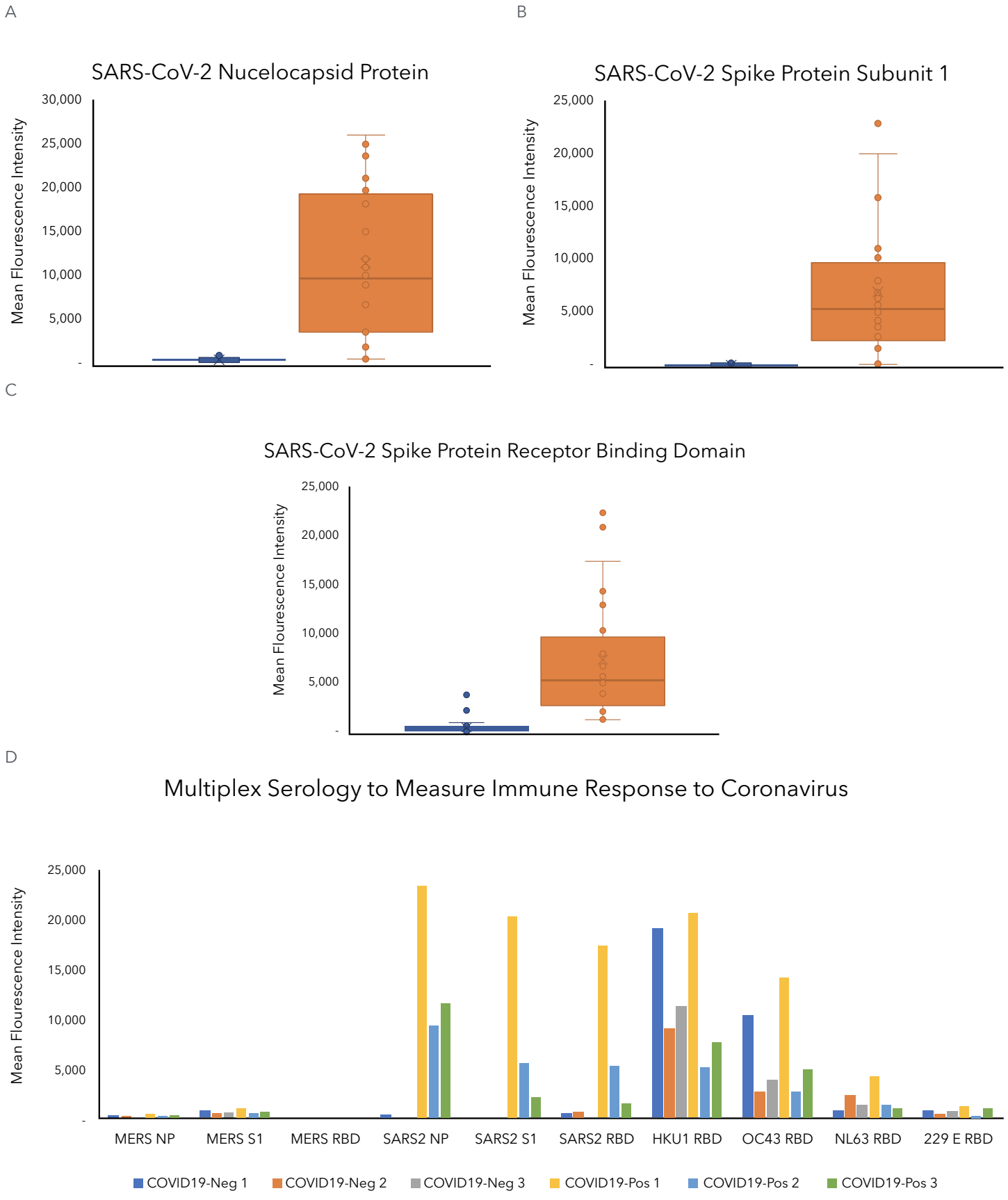

FIGURE 3. Patient Serum Sample Antibody Measurements. Forty pre-COVID-19 and COVID-19 PCR-negative samples (blue), and 24 COVID-19 PCR-positive samples (orange) were tested using the multiplex serological assay to determine the human IgG response to (**A**) the Nucleocapsid, (**B**) Spike Glycoprotein and (**C**) Spike Protein Receptor Binding Protein. (**D**) Six representative COVID-19 patient serum samples show the variable IgG immune response each patient generates to coronavirus.

PCR NEGATIVE (N=8)/PRE-COVID19 SAMPLES (N=32)										
	SARS2 NP	SARS2 S1	SARS2 RBD	MERS NP	MERS S1	MERS RBD	HKU1 RBD	OC43 RBD	NL63 RBD	229E RBD
Negative	40	40	37	40	40	40	03	00	04	19
Positive	00	00	03	00	00	00	37	40	36	21
Total Samples	40	40	40	40	40	40	40	40	40	40
Negative Agreement	100%	100%	93%	100%	100%	100%	8%	0%	10%	48%

PCR POSITIVE COVID19 SAMPLES										
	SARS2 NP	SARS2 S1	SARS2 RBD (N=24)	MERS NP	MERS S1	MERS RBD	HKU1 RBD	OC43 RBD	NL63 RBD	229E RBD
Negative	02	02	03	21	20	21	01	01	01	12
Positive	22	22	21	03	04	03	23	23	23	12
Total Samples	24	24	24	24	24	24	24	24	24	24
Positive Agreement	92%	92%	88%	13%	17%	13%	96%	96%	96%	50%

TABLE 2. Negative and Positive Agreement. To determine the clinical performance of the SARS-CoV-2 nucleocapsid and spike glycoprotein IgG assay, a positive percent agreement between the IgG assay and a PCR-based diagnostic test was performed. The MERS and common coronaviruses sample agreements were calculated to show little correlation between the SARS-CoV-2 and other coronaviruses.

METHODS

R&D Systems developed human coronavirus proteins were conjugated to distinct fluorescent Luminex MagPlex® Microspheres to generate a multiplex serological assay. Recombinant coronavirus spike protein (**TABLE 1**) conjugated microspheres were pooled generating a 10 analyte multiple plex antibody capture serological assay. Assay positive control samples were prepared using coronavirus-specific antibodies SARS-CoV-2 Nucleocapsid Antibody (MAB10474), SARS-CoV-2 Spike S1 Subunit Antibody (MAB105403), Anti-MERS Spike Protein (Absolute Antibody®), MERS-CoV Nucleocapsid Antibody (Sino Biological®), Human Coronavirus Spike HKU1 (Sino Biological®) or, when an appropriate antibody is unavailable, an anti-histidine tag antibody (MAB050) was used.

A total of 64 archived serum samples were selected, including: pre-COVID-19 (n=32), SARS-CoV-2 PCR-confirmed negative (n=8), and PCR-confirmed positive (n=24) samples. Samples were diluted 1:250 using the assay calibration buffer and incubated with microparticles for 2 hours at room temperature. After washing away any unbound substances, Serotype-specific IgG antibodies were detected with a biotinylated-anti-human IgG antibody (Coming Soon!) or anti-mouse IgG (BAF018) antibody (to detect positive control antibodies) and incubated for 1 hour at room temperature. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. Following washes to remove unbound Streptavidin-PE, the microparticles were chemically fixed by resuspending in 4% formaldehyde (FC004) and were incubated for 1-hour at room temperature. The final wash removes fixation buffer and the microparticles were resuspended in wash buffer and read using a Luminex® instrument.

RESULTS

Calibration curves were generated, using commercially available coronavirus spike and nucleocapsid protein antibodies (**FIGURE 1**). The standard curve ranges are indicated in TABLE 1. Linearity is an important measurement of assay accuracy and specificity as non-specific binding can be identified with each sample dilution if signal does not decrease. Serially diluted samples should have the same concentration once back-calculated, giving you consistency in results and confidence that the assay is measuring the correct analyte. The SARS-CoV-2, MERS-CoV, and common cold coronavirus assay natural linearity was 96-131% which is within the acceptable range for Luminex assays (**FIGURE 2**). To test for accuracy, COVID-19 positive samples were compared to COVID-19 negative samples. Multiple serum samples from COVID-19 PCR positive patients showed strong assay signal for all three SARS-CoV-2 proteins, Nucleocapsid, Spike S1 and Spike RBD, (**FIGURE 3A-C**). PCR-negative samples had no signal. Representative COVID-19 negative and COVID-19 positive samples were run to show specificity of the assay to capture SARS-CoV-2 IgG responses. The MERS-CoV proteins, which had nearly undetectable signal and a variable common cold IgG response showed positivity across COVID-19 positive and negative samples indicating no correlation with SARS-CoV-2 IgG responses (**FIGURE 3D**). Finally, agreement between this custom assay and commercially available ELISA tests was evaluated (**TABLE 2**). The positive and negative agreement showed the ability of a multiplexed R&D Systems coronavirus proteins to accurately detect SARS-CoV-2 IgG responses.

CONCLUSIONS

Taken together we have demonstrated that this custom multiplex antibody capture assay allows for the simultaneous evaluation of SARS-CoV-2, MERS-CoV and common cold coronaviruses. This assay can be used by investigators to determine if immune responses to different coronaviruses has a positive (protective), negative (enhancement), or no effect on the clinical outcome of an infection. Partner with R&D Systems, a Bio-Techne brand, to optimize this and other future assays for your specific needs.

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NOTES

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