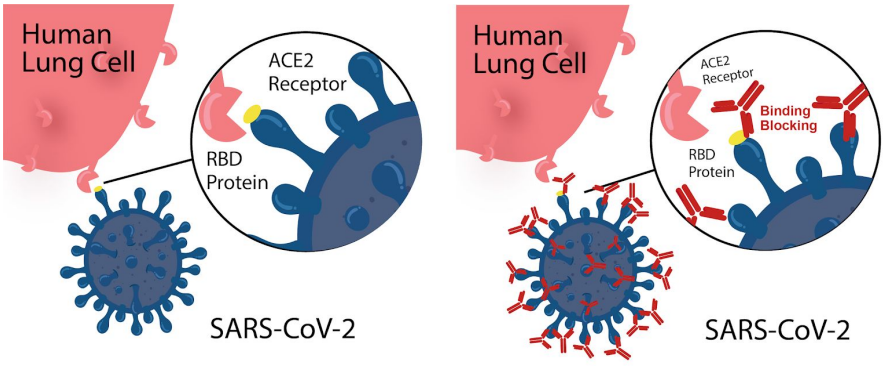
Development and Validation of a Novel SARS-CoV-2 High-throughput Surrogate Neutralization Assay

Introduction

In December 2019, a novel coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, and caused the coronavirus disease 2019 (COVID-19) pandemic. In early February, Leinco started developing recombinant antibodies from COVID-19 survivors. In addition, we began manufacturing SARS-CoV-2 proteins and assays for use in research and diagnostics; all play an important role in research and development for dozens of assays and therapeutics. As of October 31, 2020, over 45 million cases have been reported worldwide, contributing to more than 1.2 million deaths[1]. COVID-19 presentation ranges from mild flu-like symptoms that typically resolve within 1-2 weeks to fatal respiratory failure, shock, and multiorgan dysfunction[2,3].

Antibodies against SARS-CoV-2 begin to appear during the first week following symptom onset, with seroconversion occurring approximately one week later[5]. These antibodies are primarily specific for the nucleocapsid (N) and spike (S) structural proteins[6-8]. The primary function of the N protein is to package the viral RNA genome into a helical ribonucleoprotein complex[9]. The S protein mediates viral attachment, fusion, and entry into host cells[10]. Specifically, the receptor-binding domain (RBD) of the S protein binds to the angiotensin-converting enzyme 2 (ACE2) receptor on host cells[10-12]. The S protein, specifically the RBD, is highly immunogenic and can elicit neutralizing antibodies (NAbs) that disrupt the RBD-ACE2 interaction and prevent SARS-CoV-2 cell entry[8, 13, 14] **(Figure 1)**. Although NAbs are correlated with protective immunity in other viral infections, the exact role of NAbs in SARS-CoV-2 immunity is still not clear.

Figure 1. Neutralizing antibodies targeting the SARS-CoV-2 spike protein, specifically the RBD, prevent cell entry by disrupting the interaction between the RBD and angiotensin-converting enzyme 2 (ACE2).



Several diagnostic tests are available to detect active SARS-CoV-2 infection, including molecular diagnostic and viral antigen tests. In addition, serological assays are available that detect serum antibodies against SARS-CoV-2 in individuals who have an active or have had a prior infection and developed an adaptive humoral immune response. These serological assays detect antibodies targeting the N and S proteins, although evaluating the anti-SARS-CoV-2 RBD antibodies has been shown to be the most reflective and sensitive indicator of past SARS-CoV-2 infection[15]. While the N protein elicits binding antibodies, these antibodies are primarily not neutralizing. It is the NAbs that are the protective antibodies. Live virus neutralization assays, including the plaque reduction neutralization test (PRNT) and focus reduction neutralization test (FRNT), are the standard methods of evaluating NAbs. These assays are laborious, require biosafety level 3 containment facilities, and take several days to complete. More rapid, thigh-throughput assays that measure NAbs are essential for monitoring the levels of protective antibodies present in infected patients or vaccinated subjects.

Convalescent plasma from recovered COVID-19 patients has been used as a treatment option in patients with COVID-19 infection. Data from clinical studies are conflicting with some studies showing a reduction in viral loads and increased survival[4], while others show convalescent plasma offering little clinical benefit. These conflicting results may reflect the fact that the plasma used in these clinical trials was not adequately screened for the presence of high titer NAbs; thus protective levels of NAbs were not achieved in treated patients. Current FDA guidelines recommend measuring the NAb titers in convalescent plasma and indicate that donor plasma should have a NAb titer of greater than or equal to 1:160[16]. Rapid, high-throughput screening assays for selecting convalescent plasma containing protective levels of antibodies are not available. Furthermore, the correlation of traditional serologic assays with NAb titers is still unknown. Therefore, a high-throughput, easy-to-use, surrogate neutralization assay that correlates with live virus neutralization testing is urgently needed to identify convalescent plasma with potent anti-SARS-CoV-2 NAbs for efficiently treating patients with COVID-19. Additionally, with the advent of vaccines, it is imperative to have a high-throughput assay that could evaluate the adequacy of one's response to the vaccine and mounted protective antibodies.

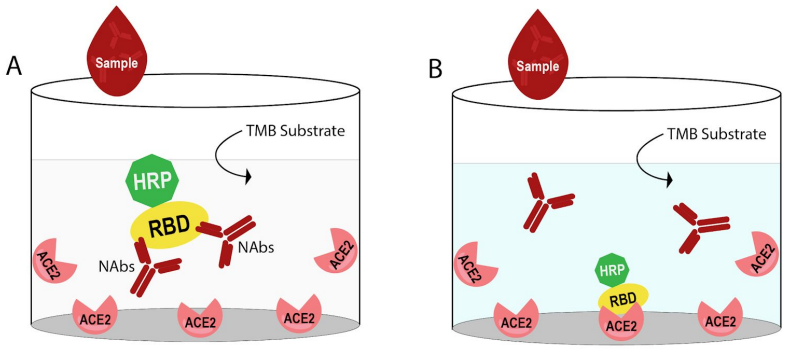
Principles of the ImmunoRank™ Assay

ImmunoRank™ is an ELISA-based assay that allows semi-quantitative detection of anti-SARS-CoV-2 RBD NAbs of all isotypes in plasma or serum. Unlike other neutralization assays, which require live virus, ImmunoRank™ uses purified RBD and ACE2 to mimic the virus-host interactions, allowing rapid detection in standard research or clinical diagnostic labs that do not have a Biosafety Level 3 (BSL3) rating.

The ImmunoRank™ test kit contains all the reagents needed to detect anti-SARS-CoV-2 NAbs. This includes one ELISA plate pre-coated with recombinant human ACE2. In a separate incubation plate, recombinant RBD conjugated to horseradish peroxidase (RBD-HRP) is incubated with plasma or serum samples in question. When added to the pre-coated ELISA

plate, the RBD-HRP will bind to ACE2 in the absence of NAbs, resulting in a change of color upon the addition of a chromogenic substrate **(Figure 2)**. If the sample contains NAbs, they will bind to the RBD-HRP and block RBD-ACE2 binding, resulting in a reduced change of color upon the addition of a chromogenic substrate. Therefore, the intensity of the color is inversely proportional to the concentration of NAbs. The less intense the color, the more NAbs present in the sample.

Figure 2. Detection of anti-SARS-CoV-2 RBD neutralizing antibodies using ImmunoRank™. ELISA well A shows antibodies bound to SARS-CoV-2 RBD, preventing binding to ACE2, indicating no color. ELISA well B shows SARS-CoV-2 RBD bound to immobilized ACE2, indicating a blue color.



ImmunoRank™ Preparation and Workflow

Specimens

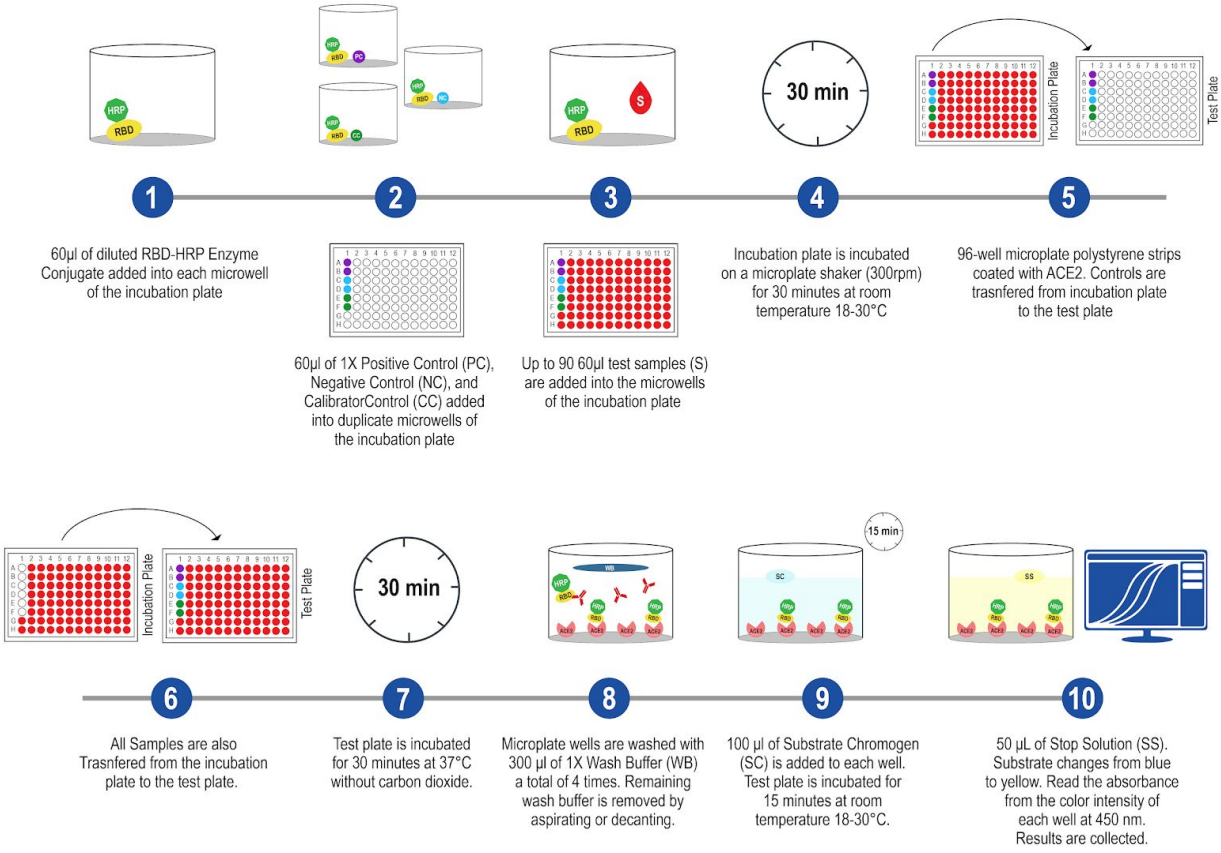
Both natural and contrived positive plasma specimens were used to validate the ImmunoRank™ assay. Contrived positive samples were made by spiking negative human plasma collected prior to the COVID-19 outbreak (December 1, 2019) with a cocktail of human recombinant monoclonal antibodies (IgG_1) sequenced from plasma B cells of COVID-19 survivors that tested positive for anti-SARS-CoV-2 RBD antibodies.

Procedure

NAbs were quantified using ImmunoRank™, according to Leinco's product insert **(Figure 3)**. 60uL of RBD-HRP was added into each well of the incubation plate, followed by 60uL of controls and test samples. After a 30 min incubation at room temperature and shaking at 300rpm, 100uL from each well was transferred from the incubation plate to the test plate containing immobilized recombinant human ACE2. The plate was incubated without shaking for 30 minutes at 37°C, followed by a total of four washes with 300uL of wash buffer. 100uL of

substrate chromogen was added to each well and incubated for 15 minutes at room temperature, protected from direct light. Immediately after the incubation, 50uL of stop solution was added to each well, and absorbance from the color intensity was read at 450nm.

Figure 3. ImmunoRank™ workflow.



Interpretation of Results

The percent neutralization, or Sample Neutralization Index (SNI), for each sample was determined using the assay's negative control and positive control. The positive control contains a highly neutralizing recombinant human monoclonal anti-SARS-CoV-2 antibody (IgG_1) sequenced from the plasma B cells of a COVID-19 survivor.

Algorithm to Determine % Neutralization/SNI

% Neutralization = [1 - (Sample OD_{450nm} / Negative Control OD_{450nm})] / [1 - (Positive Control OD_{450nm} / Negative Control OD_{450nm})] x 100

A cutoff value for NAb positivity was determined as ≥ 20%. This cutoff value was assigned based on the mean neutralization of 531 negative plasma samples collected before the COVID-19 outbreak plus 4 times the standard deviation. Based on the percent neutralization, the samples were categorized as negative or positive for NAbs, with positivity ranging from low, moderate, to high levels **(Table 1)**.

Table 1. Interpretation of percent neutralization values.

% Neutralization/SNI	Test Result
% < 20%	Negative
20% ≤ % < 50	Low levels
50% ≤ % < 75	Moderate levels
75% ≤ % ≤100	High levels

Validation

Accuracy

We first verified that purified recombinant ACE2 and RBD could mimic the virus-receptor interactions by incubating RBD-HRP with immobilized ACE2. As expected, RBD bound to immobilized ACE2 in a dose-dependent manner **(Figure 4)**. Furthermore, this interaction was dose-dependently neutralized by recombinant human anti-SARS-CoV-2 RBD monoclonal antibodies, but not by anti-SARS-CoV-2 N-terminal domain (NTD) monoclonal antibodies **(Figure 5)**.

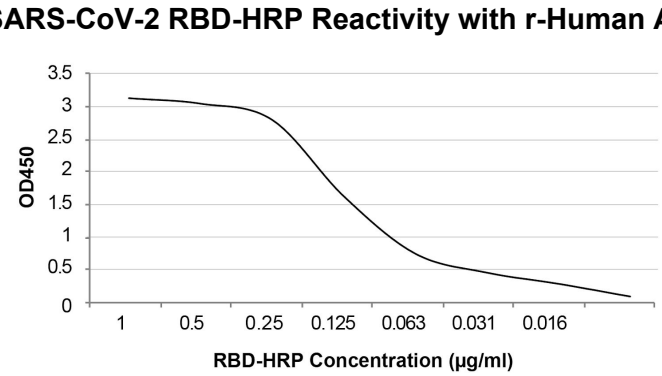


Figure 4. Dose-dependent binding curve of SARS-CoV-2 RBD-HRP binding to immobilized ACE2

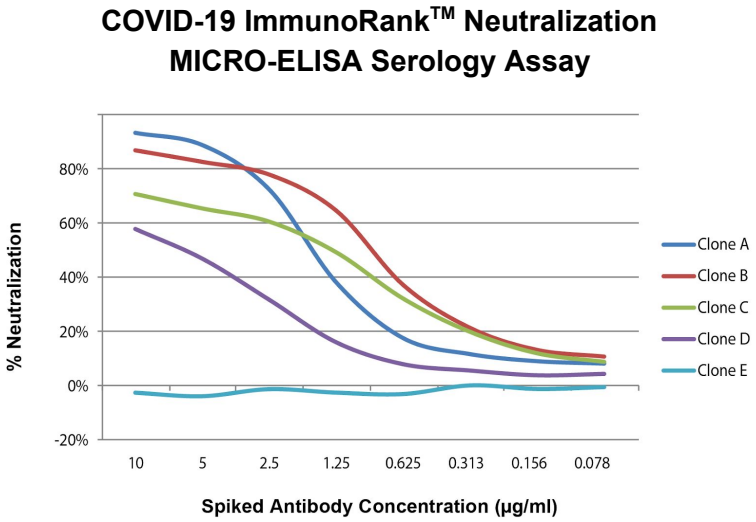


Figure 5. Neutralization of SARS-CoV-2 RBD binding to ACE2 by a panel of monoclonal antibodies sequenced from COVID-19 survivors. Clones A-D recognize the SARS-CoV-2 receptor-binding domain (RBD), and clone E is specific for the SARS-CoV-2 N-terminal domain (NTD) of the spike protein. Antibodies were spiked into negative human plasma collected before December 1, 2019.

To confirm the potential of ImmunoRank™ as a high-throughput surrogate neutralization assay, we compared the percent neutralization of 15 plasma samples from convalescent plasma donors using ImmunoRank™ to the live virus FRNT neutralization titers[17]. 12 of the 15 plasma samples were previously identified as positive for anti-SARS-CoV-2 IgG/IgM antibodies. NAbs were identified in 12 (100%) and 11 (92%) of the convalescent plasma samples by the FRNT and ImmunoRank™, respectively, resulting in a positive percent agreement of 92.0% **(Table 2 and Figure 6)**. Both the FRNT and ImmunoRank™ assays did not detect NAbs in any of the 3 negative plasma samples, resulting in a negative percent agreement of 100.0% (**Table 2 and Figure 6)**. A larger clinical study is in process comparing PRNT titers and ImmunoRank™ SNI values. Based on preliminary results, the comparisons of these two assays suggest a strong correlation in detecting positive and negative samples for the presence of NAbs with high accuracy.

Table 2. Accuracy of the ImmunoRank™ assay compared to the standard live virus focus reduction neutralization test (FRNT).

Samples	FRNT		ImmunoRank™		Percent Agreement	95% CI*
	No. Pos	No. Neg	No. Pos	No. Neg		
Positive	12	0	11	1	92.0%	64.6-98.5%
Negative	0	3	0	3	100%	43.9-100%

*95% CI determined using the Wilson Method[19].

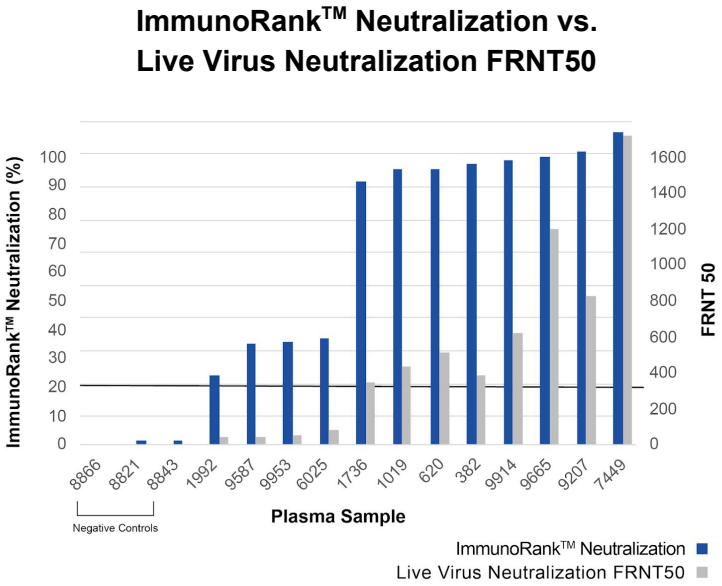


Figure 6. Accuracy of the ImmunoRank™ assay compared to the standard live virus focus neutralization test (FRNT) in determining neutralization titers of anti-SARS-CoV-2 antibody negative (n=3) and positive (n=12) convalescent plasma samples. Line represents the positivity cutoff for both assays.

Specificity

We evaluated potentially cross-reacting antibodies by analyzing a total of 55 specimens with specificity for 11 different categories of microorganisms, including the common human coronaviruses NL63, 2293, OC43, HKU1. All samples tested negative, demonstrating that ImmunoRank™ is highly specific for detecting the presence of neutralizing antibodies to SARS-CoV-2 **(Table 3)**. To confirm the specificity of the ImmunoRank™ assay, we evaluated 531 presumed negative plasma samples collected from healthy US donors before the COVID-19 outbreak (December 1, 2019). Of the negative plasma samples, 527 out of 531 samples were negative for NAbs, resulting in a 99.3% specificity **(Table 3)**.

Table 3. Specificity of the ImmunoRank™ assay.

Antibody Source	n	Number of Negatives (%)
Human Coronavirus NL63	5	5 (100)
Human Coronavirus 229E	5	5 (100)
Human Coronavirus OC43	5	5 (100)
Human Coronavirus HKU1	5	5 (100)
Influenza A	5	5 (100)
Influenza B	5	5 (100)

Antibody Source	n	Number of Negatives (%)
Hepatitis C	5	5 (100)
Hepatitis B	5	5 (100)
Haemophilus Influenzae	5	5 (100)
Respiratory Syncytial Virus	5	5 (100)
HIV	5	5 (100)
Negative Human Plasma	531	527 (99.3)

Precision

To determine intra-assay repeatability and within-laboratory precision, we calculated the percent neutralization values of the positive and negative controls, as well as a contrived positive plasma sample using one ImmunoRank™ kit lot. The assay was performed in triplicate at two separate times per day and on 5 different days[18]. To test the intra-assay repeatability, we calculated the standard deviation (SD) and coefficient of variation (CV) of the triplicate samples within one assay **(Table 4)**. The %CV ranged from 0.6-3.1%, demonstrating a high degree of intra-assay repeatability. We determined the within-laboratory precision by comparing the percent neutralization values within-assay, between assays, and between days **(Table 4)**. Based on the %CV values, which ranged from 0.9%-13%, ImmunoRank™ also shows a high degree of within-laboratory precision.

Table 4. Intra-assay repeatability and within-laboratory precision of percent neutralization (SNI%) values obtained using ImmunoRank™.

Sample	n	Mean SNI%	Intra-assay Repeatability		Within-laboratory Precision	
			SD	%CV	SD	%CV
Positive Control	30	94%	0.005	0.6%	0.09	0.9%
Negative Control	30	0%	0.025	N/A	0.03	N/A
Contrived Positive Plasma Panel	30	44.9%	0.014	3.1%	0.058	13.0%
Contrived Positive Plasma Panel Near Cutoff	21	28.1%	0.025	8.9%	0.033	11.8%

Case Study

Presence of anti-SARS-CoV-2 RBD antibodies in convalescent donor samples

To evaluate whether ImmunoRank™ could screen convalescent donor samples for NAbs, we tested 100 plasma samples of PCR-confirmed COVID-19 convalescent donors using ImmunoRank™. Only 61% of the convalescent plasma samples contained anti-SARS-CoV-2 RBD NAbs **(Table 5)**. The majority of the positive samples exhibited low-to-moderate neutralization activity (80%). Of these positive samples, 20% showed high neutralization activity, demonstrating highly variable levels of NAbs in convalescent donor samples.

Table 5. Percent neutralization of 100 plasma samples from convalescent donors as determined by ImmunoRank™.

% Neutralization/SNI	Number of samples
< 20% (Negative)	39
21 - 50% (Low positive)	33
51 - 75% (Moderate positive)	16
> 75% (High positive)	12

Conclusion

Validation Studies

The studies presented here demonstrate that the ImmunoRank™ surrogate neutralization assay provides precise, repeatable, and specific quantization of anti-SARS-CoV-2 RBD NAbs. In addition, ImmunoRank™ correlates well with the live virus FRNT, and is an assay that could replace labor-intensive, expensive, live virus assays that require biosafety containment and take multiple days to complete. In contrast, ImmunoRank™ can be completed in 1-2 hours, does not use live biological materials, and is amenable to high-throughput testing.

ImmunoRank™ Applications

Although ImmunoRank™ is not suitable for detecting acute SARS-CoV-2 infection, the assay has several research applications. In our case study, we highlight the application of ImmunoRank™ in screening COVID-19 recovered patients for the presence of potent NAbs, which is crucial to ensure optimal efficacy of convalescent plasma therapy. These potent NAbs could also be used in downstream analyses, such as structural studies to define the epitopes recognized by these antibodies. A deeper understanding of how SARS-CoV-2 antibodies bind to the RBD and neutralize the virus is crucial to determine novel vaccines and antibody-based therapies. Furthermore, ImmunoRank™ can be used in epidemiological studies, to determine protective immunity, contact tracing, and measuring vaccine efficacy.

ImmunoRank™ is currently sold as Research Use Only but is under Emergency Use Authorization (EUA) review as an *in vitro* diagnostic.

Acknowledgments

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