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Homogeneous Surrogate Virus Neutralization Assay to Rapidly Assess Neutralization Activity of Anti-SARS-CoV-2 Antibodies

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The COVID-19 pandemic triggered the development of numerous diagnostic tools to monitor infection and to determine immune response. Although assays to measure binding antibodies against SARS-CoV-2 are widely available, more specific tests measuring neutralization activities of antibodies are immediately needed to quantify the extent and duration of protection that results from infection or vaccination. We previously developed a ‘Serological Assay based on a Tri-part split-NanoLuc® (SATiN)’ to detect antibodies that bind to the spike (S) protein of SARS-CoV-2. Herein, we expand on our previous work and describe a reconfigured version of the SATiN assay that can measure neutralization activity of antibodies directly from convalescent or vaccinated sera. The sensitivity is comparable to cell-based pseudovirus neutralization assays but with significantly shorter preparation and assay run time. As the assay is modular, we further demonstrate that Neutralization SATiN (Neu-SATiN) enables rapid assessment of the effectiveness of vaccines and level of protection against existing SARS-CoV-2 variants of concern and can therefore be readily adapted for emerging variants.

SARS-CoV-2 continues to threaten the world’s health as emerging variants of concern have the potential to circumvent deployed vaccines. Simple and rapid serological tests are needed to accurately measure the level and duration of neutralization activity of antibodies that arise from natural infection or vaccination. Currently, there are several FDA-approved serological tests under Emergency Use Authorizations (EUA),

many of which can detect IgM or IgG against SARS-CoV-2, but do not measure their neutralization efficacy specifically¹⁻³. Functional neutralizing antibody titers are often measured with pseudotyped viruses, however, long assay time and discrepancy in published assay protocols have limited their use⁴⁻⁶. Alternatively, surrogate virus neutralization assays have been developed to circumvent the use of pseudovirions⁷⁻¹⁰. Although some of these assays have shown successful measurement for serosurveillance of clinical samples, they often resemble ELISA, requiring multiple time-consuming binding and washing steps, while others have not yet reported successful measurement of clinical samples, likely due to the instability of recombinant proteins in serum and plasma. Here, we report the development of a homogeneous surrogate virus neutralization assay (hsVNA) called “Neu-SATiN” by reconfiguring our previously designed serological assay (Serological Assay based on split Tri-part Nanoluciferase; SATiN)¹¹ to quantify the degree of neutralization from antibodies directly from plasma or serum.

In our previous report, we developed SATiN as a homogeneous serological assay platform that utilizes a tri-part NanoLuc[®], which is split into two small peptide tags, b9 and b10 (each about 1 kDa), and one large fragment, $\Delta 11S$ (18 kDa). Using the same tri-part NanoLuc[®], we now show the development of a homogeneous neutralization assay version of SATiN (Neu-SATiN). In Neu-SATiN, enzyme fragment peptides, b9 or b10, are fused to the ACE2 receptor, the target of infection, and to the SARS-CoV-2 spike (S) protein. We hypothesized that when the ACE2 and the S proteins interact, the fused split-NanoLuc[®] fragments are driven to within ~ 100 Å of each other, allowing $\Delta 11S$ to reconstitute into fully functional NanoLuc[®]. Importantly, when the interaction between the ACE2 and S proteins are blocked by neutralizing antibodies, the tags are prevented from interacting and subsequent complementation of NanoLuc[®] is blocked (Fig 1a). In Neu-SATiN, the level of neutralization correlates with the decrease of luminescence. As the assay is intentionally modular, full-length ectoderm of the S protein of SARS-CoV-2 variants of concern can be quickly produced and swapped with wild type S protein to assess immunological protection. Moreover, as Neu-SATiN is designed to be a “mix-and-read” assay, the actual hands-on time is less than 30 minutes, significantly improving turnaround time.

Fig 1. General schematic of the Neu-SATiN COVID-19 neutralization assay and molecular modeling of spike (S) protein and ACE2 interaction. **a)** Tri-part NanoLuc[®] peptide fragments are individually fused to recombinant S protein (purple) and ACE2 (tan). Interaction of S protein and ACE2 induces complementation of the split-luciferase and ‘turns on’ luminescence (left). In the presence of neutralizing antibodies, the interaction between S protein and ACE2 is blocked, preventing luminescence (right). Figure generated using BioRender. **b)** Molecular model of the predicted refolding of NanoLuc[®] (PDB ID: 5IBO) is shown (green) after complementation of fragments b10 and b9 is driven by the interaction between full spike protein (trimer) and ACE2 (PDB ID: 7A97).

86 **RESULTS**

87 We considered three options for fusing the split-luciferase fragments to the SARS-CoV-2 spike (S) protein
88 and its target, the human ACE2 receptor: 1) N-terminus, 2) C-terminus, or 3) both termini. Although
89 surface loops are possible fusion points, modifying these domains may interfere with neutralizing
90 antibody binding. Based on our previous experience, we know that reconstitution of split-NanoLuc® is
91 most efficient when the fusion locations are within ~50-100 Å¹²⁻¹⁴. We used molecular modeling to
92 determine possible fusion points on S protein and ACE2 that place fragments within this proximity (Fig 2a
93 and Fig 3a).

94 To test feasibility, we initially built the system using only the receptor binding domain (RBD) of the SARS-
95 CoV-2 spike protein and ACE2. Based on molecular modeling of RBD and ACE2 (Fig 2a)¹⁵, the distance from
96 the N-terminus of ACE2 to the N-terminus of RBD is ~60 Å and to the C-terminus is ~53 Å (PBD ID: 6M0J).
97 In contrast, the C-terminus of ACE2 is more than 100 Å away from either terminus of RBD and thus was
98 excluded as a potential tag fusion site. The complete list of binders used in this study is listed in Extended
99 Data Table 1. Using purified recombinant proteins, we confirmed that the engineered RBD and ACE2
100 binders with complementary tags produced detectable luminescent signal when combined with the large
101 fragment of the split luciferase, Δ11S, in human serum. Each binder combination shows substantial
102 luminescence; however, there are differences in signal-to-background ratios (Extended Data Fig 1a). We
103 also confirmed substantial decrease in the signal when the binders were incubated with neutralizing
104 antibody (NAb) (Sino Biological, 40592-MM57), indicating inhibition of RBD binding to ACE2 to prevent
105 complementation of the split-luciferase fragments. For any given pair, the fractional decrease in
106 luminescent signal as a function of increasing concentration of NAb displays a typical dose-response curve
107 for inhibition that is specific to the NAb (Extended Data Fig 1b). This suggests that, although the maximum
108 signal intensity produced by each binder pair is different, the proportional decrease in the signal in the
109 presence of NAb is similar. Therefore, the ability to measure NAB antiviral activity is feasible with several
110 different pairs. We characterized binder pairs further by calculating IC₅₀ of the NAb using each pair and
111 stability of the complex in high concentrations of non-binding IgG (Extended Data Fig 1c and 1d). We
112 selected the pair (S)RBD-b9 and b10-ACE2 based on the lowest discrepancy in IC₅₀ value compared to the
113 manufacturer's report and in resolving concentration to response.

114 After validating Neu-SATiN's ability to detect NAb activity, we validated the assay with clinical plasma
115 samples from actively infected (from ICU) and convalescent patients (n=18). The plasma samples were
116 tested and binned into 5 different groups in respect to their relative luminescence compared to the control
117 NAb (Fig 2d). Of the convalescent patient samples (PS 16, 17, 18), patient sample 17 (PS 17) showed
118 inhibition similar to 100 µg/mL of the control NAb. PS 16 and PS 18 were not as effective as PS 17, but still
119 showed significant decrease in luminescence intensity compared to 0 µg/mL of NAb. As the neutralization
120 activity depends on the epitope, affinity and concentration of the antibodies, the comparison between
121 the control antibody and the antibodies in patient plasma is only relative. Nevertheless, our data confirm
122 that Neu-SATiN is able to distinguish the presence or absence of antiviral antibodies and quantify the
123 relative level of neutralization directly in clinical samples (Figure 2).

124

Fig 2. Binder pair screening in human serum and patient samples (PS 1-18). **a)** Molecular modeling of the distances between N-terminus of ACE2 to the N-terminus of RBD is ~60 Å and to the C-terminus is ~53 Å (PBD ID: 6MOJ). As such, luciferase fragments can be fused at either terminus of RBD and N-terminus of ACE2. **b)** (S)RBD-b9 and b10-ACE2 binder pair was screened with increasing concentrations of neutralizing Ab (NAb, Sino Biological 40592-MM57) in human serum. **c)** Using (S)RBD-b9 and b10-ACE2 binder pair, the signal between 0 μg/mL of NAb (darker color) vs. 100 μg/mL of NAb (lighter color adjacent bar) shows a 10-fold decrease upon neutralization. **d)** Testing 18 patient plasma samples: (S)RBD-b9 and b10-ACE2 were mixed directly with plasma samples, followed by the addition of the detection solution (Δ11S and substrate). Samples 16, 17, and 18 (indicated by ‡ above bar) are known to be convalescent whereas other samples are from ICU patients with unknown antibody presence/levels. Representative samples (colored) were carried forward to test titer. **e)** IgG was purified from the patient plasma samples and serially diluted to mimic plaque-neutralization and other assays, then tested with Neu-SATiN (left) N=4 and also with PSV assay (right) N=4. Average activity shown (symbols), CV is ≤0.15 for each sample (not shown) **f)** Average signals from each assay were plotted and show high correlation between the two assays with an r value of 0.8121. NAb was used as an internal positive control.

Next, we compared Neu-SATiN to a pseudovirus neutralization (PSV) assay based on a human immunodeficiency virus (HIV) system with luciferase gene reporter. As PSV assays are sensitive to protein content and often require dilution of clinical samples (typically one-half dilutions or more) with specific matrix (e.g., cell culture compatible media and buffer), performing the assay using direct plasma samples was not feasible. Instead, we selected ten samples (non-black bars on Fig 2d) and extracted plasma IgG using protein G magnetic beads. The purified samples were serially diluted and tested with PSV assay (Fig 2e right). The results obtained from Neu-SATiN and PSV assay show good correlation (Pearson's r value of 0.81), with better correlation at higher IgG concentrations. Both PSV assay and Neu-SATiN showed that PS 17 had the highest inhibition activity similar to the positive control (IgG purified from commercial human serum spiked with 100 μg/mL NAb). One potential source of discrepancy seen between the results is lack of IgMs in PSV assay. Protein G is effective only in purifying IgGs, therefore purified clinical plasma mainly contained IgGs to be tested on PSV assay. As Neu-SATiN is performed directly using whole plasma, the antiviral activities of both IgGs and IgMs may contribute to the level of inhibition.

Even though many of the antibodies produced by current vaccines target the RBD of S protein^{16, 17}, the more pressing concern is protection against variants of concern which often have mutations outside of the RBD.^{18, 19} In response, vaccines are being developed to target full-length S protein²⁰. As such, we expanded Neu-SATiN using ACE2 and the full ectodomain of S protein, including known amino acid substitutions stabilize conformation²¹. The rationale for using full-length S protein should provide a more comprehensive measurement of the neutralization effect. Molecular modeling was used again to examine the distances between the termini of two proteins (Fig 3a) using PDB ID: 7A97²². Based on this analysis, we decided to fuse b10 to the N-terminus of S protein and b9 to the N-terminus of ACE2. The length of linker connecting the proteins to the tags was also doubled from nine to eighteen amino acids to increase the flexibility of the tags and to encompass a broader range of potential binding confirmations. Consistent with previous studies²³, we found the interaction between S ectodomain and ACE2 monomer is relatively low, likely due to ACE2 instability, and decided to use a dimerized ACE2 binder created by fusing the ACE2 N-terminal domain (a.a. 16-614) with the human IgG Fc fragment (Extended Data Table 1). These two proteins, denoted as “b10-(S)-WT” and “b9-ACE2-Fc”, were validated for binding-induced luminescence (Fig 3b). The full wild-type spike protein (WT) version of the binder pair shows average luminescence signal of 1.6×10^6 RLU indicating successful complementation of the split-NanoLuc[®] fragments and shows a robust 3000-fold signal-to-background ratio. We produced mutated S proteins for each of the major SARS-CoV-2 variants of concern: Alpha, Beta, Gamma, and Delta^{18, 19} (mutation sequences in Extended Data Table 2). The mutations were made throughout the ectodomain of S protein and not just limited to the RBD. Additional mutations were made to the furin cleavage site to prevent unwanted degradation of the binders by cell culture proteases and to enable purification of recombinant proteins^{24, 25}. The orientation of the tags on each protein were kept the same: b10-(S)-Variant and b9-ACE2-Fc. All four variants (Alpha, Beta, Gamma, and Delta) produced distinct luminescence signals with high signal-to-background (Fig 3b). The neutralization of the WT pair (WT S protein with ACE2) corresponds well with concentrations of a NAb (Sino Biological, 40592-R001) (Fig 3c); however, there was limited neutralization of most of the variants using this NAb (Extended Data Fig 2).

We obtained another set of serum samples ($n = 43$) that have been tested with two different Emergency Use Authorized COVID-19 S protein binding antibody assays: 1) COV2G Siemens 1st Gen (target antigen: RBD, cutoff: ≥ 1.0), and 2) EUROIMMUN (target antigen: S1, cutoff: ≥ 1.1). The samples can be further categorized into three different groups: 1) SARS-CoV-2 exposed with no vaccination history (post-infection, Samples 1 – 18), 2) SARS-CoV-2 exposed with vaccination history (post-infection and post-vaccination, Samples 19 – 28), and 3) neither exposed nor vaccinated (no infection and no vaccination, Samples 29 – 43). All of the samples were screened with our full-length-WT S protein Neu-SATiN for the presence of neutralizing antibodies. Commercial human serum spiked with NAb (Sino Biological, 40592-R001) was used as a positive control and to determine relative neutralization activity of the patient samples.

Samples with previous infection history (Samples 1 – 28, $n = 28$) showed normalized activity score < 0.1 (Fig 3d - indicated by red dotted line) in Neu-SATiN, with the exception of Sample 2 (frequency = 27/28, 96%). These were also the samples that scored > 20.00 in Siemens 1st Gen and > 8 in EUROIMMUN EIA, both indicating the presence of anti-SARS-CoV-2 antibodies against the WT S protein. Intriguingly, when the patients were previously infected and then vaccinated (Samples 19-28, $n = 10$), their sera showed normalized activity scores of 0.05 or below (frequency = 10/10, 100%). The various levels of neutralization shown in Samples 1 – 18 are possibly due to difference in sample collection day post-infection, as it is known that antibodies levels are highest 4-5 weeks after symptom onset^{26, 27}. Conversely, all SARS-CoV-2 negative patients (Samples 29-43, $n = 15$), based on standard antibody test, showed signals above 0.1 (frequency = 15/15, 100%), indicating no neutralization activity.

Fig 3. Validation of full spike proteins (wild type and variants) and ACE2 binders in the serosurveillance of clinical samples.
a) Molecular modeling of the distances between N-terminus of ACE2 to the N-termini of nearest S protein is ~ 48 Å and ~ 88 Å, respectively (PDB ID: 7A97). **b)** Comparison of signal and background for the full spike (wild type and variants) and ACE2 pairs. **c)** Serial dilution of a commercially available NAb (Sino Biological, 40592-R001) in the presence of b10-(S)WT and b9-ACE2-Fc pair. **d)** Serum samples that have been tested previously on two different COVID-19 detection kits were also tested on Neu-SATiN. Wild type full spike protein with b10 tag (b10-(S)WT) and ACE2 with b9 tag (b9-ACE2-Fc) were used as the binders. Signals from patient samples were normalized to the signal from normal human serum just with the binders (no neutralizing antibodies). The scaled data for individual sample were plotted where the red dotted line at 10% (i.e. 90% neutralization) indicates the cutoff line to distinguish neutralizing samples from the non-neutralizing samples. Bottom table shows the results from two prior tests detecting anti-SARS-CoV-2 antibodies (COV2G Siemens 1st Gen and EUROIMMUN EIA) for corresponding individual samples; interpretation, indicated either as positive (+) or negative (-), denotes the presence of anti-SARS-CoV-2 antibodies. **e)** Neutralization efficacy of patient samples against WT and variant S proteins. Samples used in Fig 3d (n=43) were tested with an additional n=35 patient samples that were positive for anti-SARS-CoV-2 antibodies. Luminescence signals from each patient serum were normalized to the signal from corresponding binder pair in commercial human serum (no NAb). Then, the highest value in each set was used to compute normalized activity. Known post-vaccination, post-infection, and negative (no known infection or vaccination) samples were plotted separately. Red dotted line at 10% indicates the cutoff between the negative samples and the positive samples for distinction of neutralization. **f)** Comparison of serum data based on patients that were vaccinated to patients that were vaccinated following a reported infection (n=40).

Altogether, the data supports that our newly developed Neu-SATiN neutralization assay is a reliable surrogate test that shows congruent results compared to already established tests. As we recognize that immunities generated by vaccination versus infection are known to produce antibodies targeting different parts of S protein²⁸ and that the protection against variants based on vaccination and/or previous infection is variable, we tested the same patient samples used to validate b10-(S)WT/ b9-ACE2-Fc, along with an additional set of infected patients (n=35), for the samples' neutralization efficacies towards different variants of concern. Since a universal NAb that can neutralize WT and all variants is not available, we were not able to determine relative degree of neutralization compared to a known concentration of NAb for each of the variants, but only for the WT. Instead, we compared fractional decrease in luminescence signal from the positive samples to negative samples for each variant pair; the highest signal observed from the negative group was considered 100% activity (i.e., no decrease in signal) and subsequent decrease in signal was determined for the positive groups. As shown in Fig 3e, the NAb negative samples (darkest color violin plots) tend to display a wide range of signal compared to the NAb positive groups. Overall, the mean fractional signal observed from the positive samples (post-infection or post-vaccination) tested either with WT or variant pairs were 10% or lower. In other words, the signal measured from the positive samples were less than 10% of the signal from the negative group. This suggests that most of the samples within the positive groups have some level of neutralization ability towards each of the variant SARS-CoV-2s. In particular, both WT and Gamma variant were neutralized almost fully by immunity generated by vaccination; however, there appears to be a subset of samples within the Delta, Alpha, and Beta variant groups with minimal neutralization even after infection and/or vaccination (Fig 3f). It was also interesting to note that natural immunity generated by infection with SARS-CoV-2 was not sufficient to neutralize the Gamma variant and that vaccination was required for stronger neutralization (Fig 3e). This observation is potentially due to antibodies produced by different immunities targeting different parts of S proteins and can cause differences in recognizing mutation sites^{28, 29}. Without further information on which vaccines were received by these patients or the sample collection dates post onset of symptoms, we are not able to determine the exact correlation between vaccination and difference in protection against variants.

We next sought to expand on our observations and tested serial dilutions of FDA EUA approved neutralizing antibodies Regn10933 (casirivimab), Regn10987 (imdevimab), and JS016 (etesevimab), as well as sera with known vaccination history (n=24). Having such data is informative in detecting the lowest effective concentrations (titers) and therefore determining the potency of NABs. Of the FDA EUA antibodies, imdevimab was the most effective against all variants of the S protein. The other two antibodies, casirivimab and etesevimab, were most potent against WT but showed variable potency against variant S proteins. There was no effective neutralization observed for the Beta strain with either antibody (Fig 4a). For the serosurveillance of patient samples (Fig 4b), no neutralizing activity was observed in patients that were not vaccinated, as expected. The patients with one dose showed negligible potency compared to the patients with two doses with samples collected < 50 days after the last dose. For samples collected > 50 days after receiving two doses, the potency of antibodies significantly reduced as indicated by shift in titration curve towards the right, consistent with recent reports of waning immunity for some vaccines³⁰. In quantifying these patient samples in terms of the 50% neutralization titer (Fig 4c), the majority show strong neutralization against WT and the least potency against Beta variant, with the other variants of concern intermediate.

Fig 4. Assessing the potency of neutralizing antibodies. a) Neutralizing capabilities of FDA EUA approved therapeutic antibodies Regn10933 (left, casirivimab), Regn10987 (center, imdevimab), and JS016 (right, etesevimab) were evaluated using Neu-SATiN against different S variants. b) Evaluation of neutralizing antibodies in individuals with unvaccinated sera (n = 2), with one vaccination dose (n = 4), with two vaccination doses and collected within 50 days after the second shot (n = 13, intervals between shots were 21-37 days), and with two vaccination doses collected more than 50 days after the second shot (n = 5, intervals between shots were 21-36 days). c) Aggregated data showing titers at 50% neutralization (NT50) for each group.

DISCUSSION

Since the outbreak of the COVID-19 pandemic, the importance of a virus neutralization assay has risen more than ever. Virus neutralization assays are the main tools for developing vaccine and therapeutic strategies³¹⁻³³. Although the PSV assay is effective in measuring the degree of infection, maintaining cell cultures and making pseudovirus particles are labor intensive with potential safety concerns³⁴. Moreover,

batch-to-batch variability in virus production and cell transfection efficiency limit standardization and robust assay results³⁵. Numerous immunoassays for rapid detection of anti-SARS-CoV-2 antibodies have been developed, however, these assays mainly focus on capturing and detecting antibodies binding to virus proteins (e.g., spike or nucleocapsid)². Since it is well known that mere binding does not necessarily imply neutralization³⁶, a true neutralization assay for the better understanding of protective immunity against SARS-CoV-2 is needed. To circumvent the use of virus particles and cells, surrogate versions of neutralization assays have been developed⁷⁻⁹. These assays often use ELISA or similar platforms, with multiple time-consuming binding and washing steps, preventing high-throughput screening³⁷. In need of an accurate yet more rapid virus neutralization assay, we developed a homogeneous surrogate virus neutralization assay called Neu-SATiN, using a split-luciferase system.

In the initial design of the assay, similar to the first report using a complementation assay, we investigated ACE2-RBD interactions¹⁰. By using a tri-part split-luciferase platform, we show for the first time that monitoring the decreased interaction of ACE2 receptor with RBD of spike protein was sufficient to quantify the neutralization activity of patient plasma (Fig. 2). The result is in agreement with widely reported NAb and RBD interaction: the neutralization activities of NAb mostly come from anti-RBD immunoglobulins^{36, 38}. Importantly, the results from Neu-SATiN were comparable to the results from PSV assay with a Pearson's *r* value of 0.81 (Fig 2f). Given that these assays were tested in different matrices (full plasma vs. PSV assay cell culture media), this correlation value is very high.

The importance of conformational changes in spike protein altering ACE2 binding has been emphasized in many studies^{22, 39}. Also, with new variants of concern emerging, many of the deployed vaccines and second-wave vaccines in development are targeting full spike protein⁴⁰⁻⁴². Immunologic response to these vaccines may produce antibodies against epitopes outside of the RBD and may require the trimeric structure of the full spike protein for binding. This compelled us to develop stabilized, full-length-spike (both WT and major variants of concern) versions of the binders and stable, dimerized ACE2 receptor. The data presented in Fig. 3 demonstrates that b10-(S)WT/ b9-ACE2-Fc can differentiate the degree of neutralization directly from patient sera and that variant versions of the spike binders can distinguish anti-SARS-CoV-2 NAb positive serum samples from negative samples (Fig 3e,f). In addition, Neu-SATiN can provide quantitative analysis of NT50 and thus enables the measurement of potency of anti-SARS-CoV-2 antibodies against different strains (Fig 4). Combined, this provides a comprehensive screen of a patient's level of protection against the current variants of concern. As the assay is modular, emerging variants of interest can be quickly produced and incorporated.

In summary, the results obtained with Neu-SATiN correlate with HIV-based PSV assay and other antigen-based assays. One advantage of Neu-SATiN is that it can be performed homogenously and directly using plasma or serum, which significantly reduces hands-on assay time to < 30 min and can be run under standard lab conditions. Importantly, we have demonstrated that the split-NanoLuc[®] based Neu-SATiN can be applied to full-length spike proteins of the original strain and variants to test neutralization levels of convalescent patient sera. Having a modular technology as a surrogate assay that can be easily adopted as a point-of-care tool is important in tracing and adapting to the evolution of the current pandemic.

METHODS

Cell culture media, antibodies, and, cloning reagents,

HEK293 cell culture reagents were purchased from Thermo Fisher Scientifics. Two neutralizing antibodies: 40592-MM57 (used for RBD pair screening) and 40592-R001 (used for WT pair screening) were both purchased from Sino Biological. Regn10933 (CPC511A), Regn10987 (CPC512A) and JS016 (CPC516A) were purchased from Cell Sciences.

Clinical samples

Samples were obtained from either the University of Utah School of Medicine, ARUP Laboratories, or from Unity Health. University of Utah School of Medicine (total n=16) were obtained from infected patients within 48 h of admission to ICU (n=13) or within 3-5 weeks of positive PCR test for convalescent patients (n=3). Samples for determining neutralization (total n=63) in uninfected, infected and/or vaccinated were generously provided by ARUP Laboratories (IRB approved protocol 0007740). Samples for antibody titer and serosurveillance studies (total n=24) were collected from Unity Health employees, enrolled through (REB approved protocol REB 20-107, Toronto). All samples were deidentified.

Vector Construction and Transient Transfection in HEK293 Cells

Vector cloning for the binder expression was performed as previously described¹¹. Briefly, all cDNA were cloned into an in-house mammalian expression vector derived from pCMV5 with a signal peptide sequence appended at the N-termini and an octa-histidine stretch at the C-termini. For the constructs that had the tag at the N-termini, either b9 or b10 sequence was placed after the signal peptide, followed by the binder sequence. Likewise, the constructs with C-terminus tag had b9 or b10 sequence right before octa-histidine (Extended Data Table 1). The final products were transfected into HEK293 cells using polyethylenimine Max (Polysciences).

Production and Purification of Binders

HEK293 cells transfected with binder constructs were cultured in DMEM supplied with 10% FBS and 1X antimycotic-antibiotic mixture. Typically, cells were seeded at 50% confluency, and the media was collected every day until the cells became fully confluent. The collected media was filtered through 0.22 µm PES filter before purification. Purification was done on AKTA FPLC using HisPurTM Cobalt Resin. Tween-20 (0.01%), trehalose (0.1%), and glycerol (10%) was added to the final product and kept at -80 °C before use.

Reconstitution of Split-NanoLuc[®] Driven by (S)RBD and ACE2 interaction

For the screening of (S)RBD binders with ACE2 binders, the neutralizing antibody (Sino Biological, 40592-MM57) spiked in commercial human serum (10 µL in various concentrations) was combined with 5 µL of (S)RBD binders (10 pmol) and 5 µL of ACE2 binders (10 pmol), and incubated for 30 min. The incubation was done using white, round bottom 96-well plates at room temperature with vigorous shaking. Then, the “detection solution” which consists of coelentrastazine (substrate) and Δ11S was premixed, and 80 µL of the detection solution was added to each well. The final concentrations of the substrate, coelentrastazine, was 10 µM and the large enzyme fragment, Δ11S, was 500 nM per well in a total volume of 100 µL. Luminescent signal was measured using TECAN Infinite M1000Pro in a kinetic cycle. The results reported here are from the 30-min timepoint.

Split NanoLuc[®]-based Virus Neutralization Assay: Testing Spiked Samples and Convalescent Samples

Measuring neutralization activity of clinical samples was done in a similar fashion as described above: 10 µL of clinical sample with 10 pmol of Spike protein (5 µL; full S or (S)variant) and 10 pmol of ACE2 fusion (5 µL). All three components were incubated together for 30 min with vigorous shaking. Then, 80 µL of the detection solution (defined above) was added (final total volume per well was 100 µL) and the kinetic cycle of luminescent was measured. For serial dilutions to quantify activity of low titer samples, the assays were performed in a buffer containing 20 mM Tris (pH 7.5), 0.1% Tween 20, 2 mM TCEP, 2mM EDTA, 25 mM NaCl and 0.05% BSA. Serum samples (12.5 µL) were first diluted in a volume of 50 µL of buffer,

366 followed by additional one-half serial dilutions up to 6 times. An aliquot (5 μ L) of the diluted sample was
367 mixed with 5 μ L of b10 modified S binder (20 nM; full S or (S)Variant) and incubate for 30 minutes. An
368 aliquot (5 μ L) of the reaction mixture was further mixed with 45 μ L of substrate mixture containing 555.6
369 nM b9-ACE2-Fc, 100 nM Δ 11S, 22.2 μ M furimazine (substrate). After 1 hr incubation, luminescence signals
370 were measured using a microplate reader. The inhibition curve of a sample against S protein variant was
371 obtained by fitting the readings at different dilutions into the normalized response model with variable
372 slope in GraphPad. The titer of 50% neutralization was calculated according to each model.

373 374 ***Testing Spiked Samples and Convalescent Samples with Pseudovirus Neutralization (PSV) Assay***

375 The active sera (from patients in ICU) and convalescent sera were purified using protein G magnetic beads
376 (Promega Corporation, G7471) as per manufacturer's instruction. The concentrations of purified IgG were
377 measured using NanoDrop 2000. Pseudovirions were produced by co-transfecting 293T human embryonic
378 kidney cells using PEI transfection reagent (Polysciences, Inc., Warrington, PA) with NL4-3 HIV-1 genome
379 (pNL4-3.Luc.R-E-, including the firefly luciferase gene inserted into the nef coding sequence and frameshift
380 mutations in Env and Vpr) and a plasmid encoding the desired virus fusion protein (pCAGGS-SARS2-S-cFlag
381 D614G, kind gift of M. Farzan⁴³ for SARS-CoV-2 S or pMDG VSV-G for VSV as a specificity control). Forty
382 hours post-transfection, pseudovirus-containing supernatant was filtered (0.45 μ M) and concentrated by
383 ultracentrifugation (26,000 RPM, 2 hours) through a 20% sucrose/TNE (10 mM Tris pH 7.6, 100 mM NaCl,
384 1 mM EDTA) cushion, and the pellet resuspended in TNE, aliquoted and stored at -80 C. To measure
385 inhibition of infectivity, 50 μ L of 2 $\times$ IgG (purified from patient sera) diluted in media was added to CaLu-3
386 cells (ATCC HTB-55) in a 96-well format, each concentration in triplicate. 50 μ L of pseudovirus diluted in
387 media+ 16 μ g/mL DEAE-dextran was added and plates were spinoculated at 2100 x g, 30 min, 10 $^{\circ}$ C. At 20
388 hours, virus and inhibitor were removed via aspiration, and fresh media was replenished. At 40 hours, the
389 cells were lysed, and the luciferase activity was measured (Bright-Glo luciferase assay system, Promega,
390 Madison, WI). To determine the normalized luciferase value, average luciferase activity for no virus wells
391 were first subtracted and then the luciferase signals were normalized to the average luciferase activity for
392 no inhibitor wells.

EXTENDED DATA

Extended Data Fig 1. Specificity of RBD binder pair in simulated human serum and IC50 values of each binder pair. **a)** Comparison of signals between 0 µg/mL of NAb (darker color) vs. 100 µg/mL of NAb (lighter color adjacent bar) from six different pairs. Although all pairs show good response, some pairs show more distinct fold-difference between without vs. with NAb as indicated by numbers above bars. **b)** Pairs of ACE2 binders and (S)RBD binders were screened with increasing concentrations of neutralizing Ab (NAb, Sino Biological 40592-MM57) spiked in human serum. All pairs show substantial decrease in signal with increasing concentrations of NAb, demonstrating a quantifiable dose-response. **c)** When each binder pair was incubated with control isotype IgG-spiked human serum (additional concentrations of IgG spiked into human serum), less than 20% inhibition from high IgG (100 µg/mL) was observed in all of the pairs. **d)** IC50 (µg/mL) of the same NAb was calculated using six different binder pairs with AAT Bioquest IC50 calculator.

Blinder	Tag Orientation	Sequence	Note
(S)RBD	b9-(S)RBD	MRLPGAMPALALKGELLSSLLLLLEPQISQGGGSMFLRVITNSGGGSGGGSRVQPTEISVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEV RQIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGGVNYNYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGSGHHH HHHH*	
	(S)RBD-b9	MFVFLVLLPVSSQRVPQTESIVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEVRIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGG NINYLYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGGSGGSGGSMFLRVITNSGGSHHHHHHHH*	
	b9-(S)RBD-b9	MRLPGAMPALALKGELLSSLLLLLEPQISQGGGSMFLRVITNSGGGSGGGSRVQPTEISVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEV RQIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGGVNYNYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGGSGSGG SGSMFLRVITNSGGSHHHHHHHH*	
	b10-(S)RBD	MRLPGAMPALALKGELLSSLLLLLEPQISQGGVSGWRFLFKISGGGSGGGSRVQPTEISVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEV RQIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGGVNYNYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGSGHHH HHHH*	
(S)RBD-b10	b10-(S)RBD	MRLPGAMPALALKGELLSSLLLLLEPQISQGGVSGWRFLFKISGGGSGGGSRVQPTEISVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEV RQIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGGVNYNYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGSGHHH HHHH*	
	(S)RBD-b10	MFVFLVLLPVSSQRVPQTESIVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEVRIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGG NINYLYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGGSGGSGGSMFLRVITNSGGSHHHHHHHH*	
	b10-(S)RBD-b10	MRLPGAMPALALKGELLSSLLLLLEPQISQGGVSGWRFLFKISGGGSGGGSRVQPTEISVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEV RQIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGGVNYNYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGGSGSGG GVSVSGWRFLFKISGGSHHHHHHHH*	
Spike (Full)	b10-Spike (18 G/S linker)	MRLPGAMPALALKGELLSSLLLLLEPQISQGGVSGWRFLFKISGGGSGGGSRGVQTEISVRFPNITLPCFGEVFNATRFASVYAWNRKRISNCVADSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSVIRGDEV RQIAPGQTGKIADYNYKLPPDFTGCVIAWNSNLDKSVGGVNYNYLRFKSNLKPFERDISEIYQAGSTPCNGVEGFNCYFPLQSYGFPQTNGVGQYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCNVFGSGHHH HHHH*	
ACE2	b9-ACE2 (9 G/S linker)	MRLPGAMPALALKGELLSSLLLLLEPQISQGGGSMFLRVITNSGGGSGGGSAAQSTIEQAKFLDKFNHEADLFYQSSLASWNYNTNTEENVQNMNNAAGDKWSAFLKEQSTLAQMYPQLQIQLNLTVKLQLQAL QQNGSSVLESDKSKRLNTLNTMTSTYSTGKVCNPNPQCEQLLEPGLNEIMANSIDYNERLVAWESRWEVGKQLRPLYEEVYVYLNKMEARANHEDYDGYRWGDEVNGVDGYDSRGQDLDMCTVHEHTEEIKPLYE HHEMGIQYDMAYAAQFPLLRNAGNEFHEAWEIMSLAATPKHLISGILLSGSPDFQEDNETEINFLKQALITVGLTPFTYMLEKWRMVVFKEIPDKDQWKKWVWEMKREIVGPEVPVHDYDGPASLHFVNSDY SFIRYTRTLTYQFQEQALCAAKEHGLPHKDCISNSTEAGQKLFNMLRLKGSEPWTALENVGAKNMYNVRLLNFEPLFTWLKDQNKNSFVGWSTDSPYAGGSHHHHHHHH*	used in pair with (S)RBD
	b10-ACE2 (9 G/S linker)	MRLPGAMPALALKGELLSSLLLLLEPQISQGGGSMFLRVITNSGGGSGGGSAAQSTIEQAKFLDKFNHEADLFYQSSLASWNYNTNTEENVQNMNNAAGDKWSAFLKEQSTLAQMYPQLQIQLNLTVKLQLQAL QQNGSSVLESDKSKRLNTLNTMTSTYSTGKVCNPNPQCEQLLEPGLNEIMANSIDYNERLVAWESRWEVGKQLRPLYEEVYVYLNKMEARANHEDYDGYRWGDEVNGVDGYDSRGQDLDMCTVHEHTEEIKPLYE HHEMGIQYDMAYAAQFPLLRNAGNEFHEAWEIMSLAATPKHLISGILLSGSPDFQEDNETEINFLKQALITVGLTPFTYMLEKWRMVVFKEIPDKDQWKKWVWEMKREIVGPEVPVHDYDGPASLHFVNSDY SFIRYTRTLTYQFQEQALCAAKEHGLPHKDCISNSTEAGQKLFNMLRLKGSEPWTALENVGAKNMYNVRLLNFEPLFTWLKDQNKNSFVGWSTDSPYAGGSHHHHHHHH*	used in pair with (S)RBD
	b9-ACE2-Fc (18 G/S linker)	MRLPGAMPALALKGELLSSLLLLLEPQISQGGGSMFLRVITNSGGGSGGGSAAQSTIEQAKFLDKFNHEADLFYQSSLASWNYNTNTEENVQNMNNAAGDKWSAFLKEQSTLAQMYPQLQIQLNLTVKLQLQAL QQNGSSVLESDKSKRLNTLNTMTSTYSTGKVCNPNPQCEQLLEPGLNEIMANSIDYNERLVAWESRWEVGKQLRPLYEEVYVYLNKMEARANHEDYDGYRWGDEVNGVDGYDSRGQDLDMCTVHEHTEEIKPLYE HHEMGIQYDMAYAAQFPLLRNAGNEFHEAWEIMSLAATPKHLISGILLSGSPDFQEDNETEINFLKQALITVGLTPFTYMLEKWRMVVFKEIPDKDQWKKWVWEMKREIVGPEVPVHDYDGPASLHFVNSDY SFIRYTRTLTYQFQEQALCAAKEHGLPHKDCISNSTEAGQKLFNMLRLKGSEPWTALENVGAKNMYNVRLLNFEPLFTWLKDQNKNSFVGWSTDSPYAGGSHHHHHHHH*	used in pair with (S)WT and (S)Variants

Extended Data Table 1. Amino acid sequences of the binders. Spike protein and ACE2 sequences were obtained from literature sources. Then, each binder was PCR-modified with appropriate tags and linkers to produce binders. Octa-histidine was used as the purification tag. M denotes the location of the START codon and * denotes STOP codon. PDGFRB signal peptide (RLPGAMPALALKGELLSSLLLLLEPQISQG) was used to promote protein secretion. The sequences for b9 and b10 are GSMLFRVTINS and VSGWRFKKIS, respectively.

Alpha (B.1.1.7)	Beta (B.1.351)	Gamma (P.1)	Delta (B.1.617.2)
	L18F	L18F	
		T20N	
		P26S	
HV69-70del			
	D80A		
		D138Y	
			G142D
Y144del			
			E154K
		R190S	
	D215G		
	LAL242-244del		
	R2461I		
	K417N	K417T	
			L452R
	E484K	E484K	E484Q
N501Y	N501Y	N501Y	
A570D			
	D614G		D614G
		H655Y	
C681H			P681R
R682G	R682G	R682G	R682G
R683S	R683S	R683S	R683S
R685S	R685S	R685S	R685S
	A701V		
T716I			
S982A			
K986P	K986P	K986P	K986P
V987P	V987P	V987P	V987P
		T1027I	
			Q1071H
			H1101D
D1118H			

Extended Data Table 2. Spike protein variant mutations. Mutation information was obtained from literature sources. Additional mutations were made (R682G, R683S, R685S, K986P, and V987P) to the furin cleavage site to prevent degradation of proteins by host cell protease. Del denotes for deletion.

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Extended Data Fig 2. Neutralization of full S protein variant pairs. The efficacy of 100 µg/mL NAb (Sino Biological, 40592-R001) was tested with variant pairs. The Alpha variant showed the most decrease in signal followed by the Delta strain. Almost no neutralization was observed with the Beta or Gamma strain.

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