A New SARS CoV-2 Dual Purpose Serology Test: Highly Accurate Infection Tracing and Neutralizing Antibody Response Detection

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Abstract

Many SARS CoV-2 serology tests have proven to be less accurate than expected and do not assess antibody function as neutralizing, correlating with protection from reinfection. A new assay technology measuring the interaction of purified SARS CoV-2 spike protein receptor binding domain (RBD) with the extracellular domain of the human ACE2 receptor detects these important antibodies. This cPass™ surrogate virus neutralization test (sVNT) when compared directly with eight SARS CoV-2 IgG serology and two live cell neutralization tests gives similar or improved accuracy for qualitative delineation between positive and negative individuals in a fast, scalable and high throughput assay. The combined data support cPass™ sVNT as a tool for highly accurate SARS CoV-2 immunity surveillance of infected/recovered and/or vaccinated individuals, as well as drug and convalescent donor screening. The data also prevue a novel application for cPass™ sVNT in calibrating the stringency of live cell neutralization tests and its use in longitudinal testing of recovered and/or vaccinated patients.
Introduction

Molecular and serological tests for SARS CoV-2 are a critical component of disease control strategies globally (1, 2). Consequently, the demand for test kits is high and the market has responded with a growing number of commercially available tests but without clear global guidelines to ensure their efficacy and accuracy (3). Unfortunately, the data generated from these tests is widely varied in terms of sensitivity, specificity and accuracy leading to concerns in the actual number of disease carriers that can unknowingly spread the virus throughout the population (4-6).

The majority of serology tests on the market primarily detect the natural IgM and IgG antibodies that are generated in response to SARS CoV-2 infection (5). These assays are typically ELISA based with either full length or a truncation of purified spike or nucleocapsid protein coated to the plate surface with detection via anti-IgG or anti-IgM conjugated to horseradish peroxidase (HRP) or other fluorophores (7). In fact, many of these kits use plates coated with the receptor binding domain (RBD) of the spike protein due to its high immunogenicity (8, 9). Since the coating process involves the passive adsorption of proteins via hydrophobic interactions, conformational changes of the coated protein molecules may occur resulting in newly exposed or altered epitopes that may not be present in the native state (10-14). This can lead to non-specific binding of immunoglobulins to the coated surface and reduced specificity (11, 15).

A novel serology assay termed cPass™ surrogate virus neutralization test (sVNT) directly addresses the potential problems associated with the pre-existing technologies while providing additional functional data (16). Utilizing 96-well plates coated with the purified extracellular domain of the human ACE2 receptor (hACE2) and purified, solubilized, recombinant RBD conjugated to horseradish peroxidase (RBD-HRP), the assay capitalizes on the strong interaction between hACE2 receptor and the RBD coupled with the high immunogenicity of the RBD (8, 17). This permits the direct assessment of the inhibitory
capacity of immunoglobulins, antibody-based drugs and compounds that block (or neutralize) this binding event (Figure 1) [8, 9, 17, 18].

There are several advantages to this assay format over traditional serology tests: It measures the interaction between hACE2 receptor and the RBD to elucidate the function of antibodies (and other molecules) as neutralizing (16). The test is amenable to indirect detection of immunoglobulins that abrogate the interaction between the RBD and hACE2 receptor and is therefore not specific to any isotype (i.e., IgG, IgM, IgA, etc.) (i.e: isotype agnostic). Antibodies generated in all species infected with SARS CoV-2 are detectable with this assay (16, 19). For vaccine and drug development organizations, this test offers the potential application as a high throughput, safe and practical methodology for screening antibodies, proteins, peptides or small molecules that block the interaction between the RBD and hACE2 receptor. As opposed to the more traditional virus neutralization tests (20), this assay does not require a BSL3 containment laboratory. Also, the cPass™ sVNT can be performed in about 1.5 hours per 96-well plate compared with two to four days for virus and pseudovirus tests.

The cPass™ sVNT was compared to eight traditional SARS CoV-2 IgG ELISA tests in two, separate studies that utilize protein-coated plates and to two cell-based, live virus neutralization tests using human serum and plasma samples collected from several cohorts of SARS CoV-2 PCR-confirmed positive, negative and pre-pandemic de-identified samples. Finally, an approach to using the cPass™ sVNT for longitudinal studies to assess changes in neutralizing antibody titers in COVID-19 recovered patients or vaccinated subjects is described.
Materials and Methods

Samples

For Study 1, plasma and serum samples from The Children’s Hospital Colorado’s COVID-19 Convalescent Plasma (CCP) donor program registered with the FDA as eligible to collect CCP on March 31, 2020 were collected. Eligible individuals for the CCP donor program were confirmed PCR-positive for SARS CoV-2 and were symptom-free for at least 14 days prior to plasma donation and met all standard blood donation criteria per FDA requirements. For each donor the number of days from the PCR-positive SARS CoV-2 test to the day of plasma donation and the number of donations was tracked. Positive and pre-pandemic presumed negative samples were de-identified, tested and tabulated (Figure 2 and Table 1).

For study 2, a subset of the identical serum samples collected for a previously published article comparing six commercial serology assays were tested and delineated with cPass sVNT (21). The collection and description of the de-identified patient cohorts for both the positive and pre-pandemic samples are well described (21). The data for the positive samples between 48 and 80 years of age were grouped, summarized and tabulated by sampling days post symptom onset along with the pre-pandemic samples (Table 2).

The data in Table 3 were derived from PCR positive and negative de-identified samples collected and tested in Singapore (Health Sciences Authority conducted by Diagnostic Development Hub (DxD Hub)), DukeNUS, commercial vendors and Granger Genetics with data collated and analyzed by Corgenix Clinical Laboratory.

SARS CoV-2 IgG ELISA Tests:

Study 1
The CE-marked Epitope Diagnostics Inc. (EDI) ELISA, (San Diego, CA), (#KT-1032) test utilizes the SARS CoV-2 recombinant nucleocapsid antigen and samples were diluted, tested, and analyzed according to the kit instructions for IgG. The CE-marked and FDA Emergency Use Authorization (EUA) approved Euroimmun ELISA, (Lubeck, Germany) (#2606) assay utilizes the S1 domain, including the receptor binding domain (RBD) of the SARS CoV-2 spike protein and samples were diluted, tested, and analyzed according to the kit instructions for IgG. The FDA Policy D, IVD status Akston Biosciences ELISA (Beverly, MA) (#600016) assay utilizes the recombinant RBD antigen of the SARS CoV-2 spike protein with samples diluted, tested and analyzed according to the kit instructions for IgG.

For the EDI assay, positive, negative and borderline results were calculated based on the average optical density (OD450) value for the negative control assayed in triplicate for the specific assay. The positive cut-off value was calculated using the formula: positive cut-off = 1.1 x (NC+0.18), where NC is the average OD450 of triplicate negative control OD values. For Study 1, given the day-to-day fluctuation in OD450 values from both the positive cut-off and from our own inter-plate positive control calibrator, the median positive cutoff OD450 for several days of testing (0.44) was used to delineate between positive and negative samples.

Study 2

The six commercial IgG ELISA tests (1. Abbott Laboratories; 2. Epitope Diagnostics Inc.; 3. Affinity Diagnostics Corp.; 4. DRG International Inc. supplied by Bio-Rad; 5. Euroimmun and 6. Roche) used for detection of SARS CoV-2 IgG antibodies and the associated protocols employed for screening the positive and negative study samples have been previously described (21). The positive cutoff defined in the kit instructions for each assay was used to delineate between positive and negative samples compared with cPassTM sVNT using a 30% cutoff.

SARS CoV2 cPass™ Surrogate Virus Neutralizing Test (sVNT):
GenScript sVNT (Piscataway, NJ) (#L00847) utilizes the recombinant RBD of the SARS CoV-2 spike protein to detect antibodies that block the RBD from binding to hACE2 receptor. Plasma or serum samples and the kit supplied positive and negative controls were diluted 1:10 in kit-specific sample dilution buffer according to the kit insert. The diluted standards, samples and controls are pre-incubated with RBD-HRP in a “neutralization reaction” for 30 minutes at 37°C permitting the interaction and binding of neutralizing antibodies to RBD-HRP (Figure 1B). Each neutralization reaction mixture is then added to the capture plate pre-coated with the human ACE2 protein whereby the free RBD-HRP as well as RBD-HRP bound to non-neutralizing antibodies strongly interact with ACE2 and are captured on the plate (Figure 1B). RBD-HRP complexed with neutralizing antibodies (i.e., those blocking the interaction between RBD and ACE2) remain in the supernatant and are removed in a subsequent wash step. After the wash steps, TMB followed by stop solution is added to all wells, permitting visualization of bound RBD-HRP to the plate based on OD450 intensity. Color intensity is inversely proportional to amount of neutralizing antibody in standards or samples (Figure 1B).

Data is interpreted by the percent inhibition of RBD-HRP binding calculated as follows: Percent Inhibition = (1 – OD value of Sample/OD value of Background)*100%. A 30% cutoff is used to delineate positive and negative samples where this cutoff has been calibrated against the gold standard Plaque Reduction Neutralization Test (PRNT) using high stringency PRNT90 (90% plaque reduction) data analysis. Percent inhibition greater than or equal to 30% indicates the presence of SARS CoV-2 RBD-interacting antibodies blocking the RBD-ACE2 interaction.

**Plaque Reduction Neutralization Test (PRNT)**

The Plaque Reduction Neutralization Test (PRNT) is considered the gold standard for characterizing neutralizing antibodies to most viruses, including SARS CoV-2. Sera samples were heat inactivated for 30 minutes at 56°C. Serial 2-fold dilutions of the inactivated samples were prepared in a 96-well plate.
Viral stock (strain hCoV-19/USA/WA1/2020, BEI Resources, Manassas, Virginia) containing approximately 200 plaque-forming units (PFU) per 0.1 mL was added to each well containing serum dilutions. Following a 1 hour incubation period at 37°C in a CO₂ incubator, 6-well plates (Greiner Bio-One, Monroe, North Carolina) containing recently confluent Vero cells (ATCC, Manassas, Virginia) were inoculated with the virus-serum mixtures. After a second incubation period of 45 minutes at 37°C in a CO₂ incubator, 2 mL of overlay (2X agarose. Melt 1% agarose in water using a microwave oven, and cool to 45°C prior to mixing with 2X MEM with 4% FBS [Peak Serum, Wellington, Colorado] and 3 mL of 7.5% sodium bicarbonate per 100 mL solution) was added to each well. Finally, the plates were incubated for 24 h at 37°C in a CO₂ incubator upon which a second overlay containing neutral red (Millipore Sigma, St. Louis, Missouri) was dispensed into each well followed by a 24 hour incubation at 37°C in a CO₂ incubator. The number of plaques were counted 48-72 hours after initial inoculation. The highest dilution of serum that inhibits (reduces) plaque formation by 50%, 75% or 90% (PRNT50, PRNT75 or PRNT90) was calculated based on the titer of the viral stock and the number of plaques present at each dilution.

**Focus Reduction Neutralization Assay (FRNT).**

Vero E6 cells (ATCC, Manassas, VA) were seeded in 96-well plates. Serum samples were heat inactivated and serially diluted (2-fold, starting at 1:10) in DMEM (ThermoFisher, Pittsburgh, PA, USA) plus 1% FBS in 96-well plates. Approximately 100 focus-forming units (FFU) of SARS CoV-2 USA-WA1/2020 (deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH) was added to each well and the serum plus virus mixture was incubated for 1 hour at 37°C. Post incubation, medium was removed from cells and the serum sample plus virus mixture was added for 1 h at 37°C. After 1 hour, samples were removed and cells overlaid with 1% methylcellulose (MilliporeSigma, St. Louis, MO) in MEM (ThermoFisher, Pittsburgh, PA, USA)/2% FBS and incubated 30 h at 37°C. Cells were fixed with 4% paraformaldehyde (Acros Organics, Pittsburgh, PA, USA) and probed with 1 μg/mL of an
anti-SARS CoV spike monoclonal antibody (CR3022, Absolute Antibody, Boston, MA, USA) in Perm Wash 148 (1X PBS/0.1% saponin/0.1% bovine serum albumin [BSA]) for 2 hours at RT. After washing, cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Southern Biotech, Birmingham, AL, USA, 1:1,000) for 1.5 hour at RT. After washing, SARS CoV-2-positive foci were visualized with TrueBlue substrate (ThermoFisher, Pittsburgh, PA, USA) and counted using a CTL Biospot analyzer and Biospot software (Cellular Technology Ltd, Shaker Heights, OH, USA). The FRNT<sub>50</sub>, FRNT<sub>75</sub> and FRNT<sub>90</sub> titers were calculated relative to a virus only control (no serum) set at 100%, using GraphPad Prism 8 (La Jolla, CA, USA) default nonlinear curve fit constrained between 0 and 100%.
Results

RBD soluble cPass™ sVNT versus eight IgG-specific serology tests including RBD, Nucleocapsid or Spike (S1)-coated plates for COVID-19 diagnosis and classification

For Study 1, sixty-eight (forty-five SARS CoV-2 PCR presumed positive, and twenty-three pre-pandemic presumed negative) human serum samples were directly compared across four tests (see Materials and Methods) using either the RBD (Fig. 2A), nucleocapsid (Fig. 2B), spike (S1-RBD) (Fig. 2C) coated ELISA plates and the RBD soluble cPass™ sVNT (Fig. 2D). Of the PCR positive samples (Figure 2 – circles, left side of each graph), six were categorized as false negative and shared between three tests (nucleocapsid (Fig. 2B), spike S1 (Fig. 2C) and cPass™ sVNT (Fig. 2D) (red circles)). The RBD-coated ELISA (Fig. 2A) assay only gave two false negative samples (shared between the four tests (red circles)) but also exhibited three false positive samples (Fig. 2A – squares, right side of graph above cutoff line), suggesting a lower specificity for this assay. The remaining negatively classified samples (ie: false negatives) within the forty-five PCR presumed positive (Figure 2 – black circles, left side of each graph below cutoff line) for the nucleocapsid (Fig. 2B) and Spike S1 (Fig. 2C) protein-coated ELISA tests were likely undetectable by these assays. For the twenty-three pre-pandemic presumed negative samples (Figure 2 – squares, right side of each graph), the RBD (Fig. 2A) and nucleocapsid (Fig. 2B) coated ELISA tests misclassified three and one samples respectively as positive (squares above cutoff line) whereas the Spike S1 (Fig. 2C) and cPass™ sVNT (D) classified all samples correctly as negative (squares below cutoff line). In summary, for Study 1, the GenScript cPass™ sVNT delivered comparable or improved accuracy, negative and positive predictive values versus the other serology tests (Table 1).

For Study 2, the data from previously tested human serum samples (21) (see Materials and Methods) were directly compared with cPass™ sVNT for SARS CoV-2 positive (blood samples drawn at different interval periods post symptom onset) and pre-pandemic de-identified individuals (Table 2). Consistent
with Study 1, the cPass™ sVNT gave similar or superior comparative results to the other serological tests and also demonstrated the presence of neutralizing antibodies within five days post symptom onset.

A large cohort of well characterized, PCR verified positive and negative samples was screened with the cPass™ sVNT (Table 3). The comparable or superior specificity and sensitivity versus other commercial serology tests (7, 22) translates to a comparable or higher positive (94.5%) and negative (99.7%) predictive value and overall accuracy (99.2%), which is critical in population monitoring and contact tracing.

cPass™ sVNT ELISA versus live cell viral neutralization tests (PRNT and FRNT)

The CDC’s Interim Guidelines for COVID-19 Antibody Testing specific to SARS CoV-2 neutralizing antibody detection includes two assays for neutralizing antibody screening: 1) the virus neutralizing test (VNT) such as the plaque reducing neutralization test (PRNT) and the focus reduction neutralization test (FRNT) and 2) pseudovirus neutralization test (pVNT) (23). These tests require live cells and virus with a multi-day procedure that necessitates a BSL2 or BSL3 containment laboratory. Since the RBD for both SARS CoV-1 and SARS CoV-2 is immunodominant (8, 17), it has been postulated and shown that the cPass™ sVNT gives comparable results (19) and can potentially be used in lieu of VNT or pVNT.

Comparing PRNT50, 75 and 90 values with sVNT on 66 well-characterized samples gave high correlation in delineating positive and negative samples for PRNT75 and 90 where one sample (red dot) of sixty-six did not corroborate (Figure 3A). However, when using a lower stringency analysis for the PRNT (i.e., the reciprocal dilution that inhibited 50% of infection) two of the samples found to be negative by the cPass™ sVNT assay had detectable PRNT50 titers (Figure 3A - blue dots).

The same forty-five PCR-confirmed, presumed positive samples from Study 1 (Figure 2 and Table 1) were also tested for live virus neutralizing activity using a SARS CoV-2-specific FRNT. FRNT50, 75, and 90 titers were determined giving excellent correlation between cPass™ sVNT and FRNT75 (Figure 3B). However,
when comparing FRNT75 to FRNT50, five of the six samples found to be negative by the cPass\textsuperscript{TM} sVNT assay had detectable FRNT50 titers. Reciprocally, 16 samples found to be positive by the cPass\textsuperscript{TM} sVNT assay did not have detectable FRNT90 titers.

**Temporal persistence in circulating neutralization antibodies in longitudinal studies (experimental design is critical)**

Serum samples from three COVID-19 recovered individuals were collected over three months to assess the persistence of inhibitory antibodies using the cPass\textsuperscript{TM} sVNT assay (Figure 4). In order to determine the true quantitative difference between the time points, a serial dilution series of each sample was performed on the same plate to uncover a dilution whereby the signals were within the linear, quantitative range. For sample 20, the third point (1:90) was within the linear range of each dilution series and a decrease in inhibitory antibodies of approximately 2.5-fold was measured over a three-month period. For Sample 85, the fifth dilution (1:810) was within the linear range and gave a 1.7-fold decrease over three months. However, if the samples had only been diluted by 10-fold, where the signal was within the upper plateau of the dilution curves for all three time points, very little difference in inhibitory antibodies would be quantified for either sample 20 or 85 (compare the first point in the dilution series at each month for samples 20 and 85). Sample 74 exhibited almost overlapping dilution curves over three months indicating no change and thus persistence of immunity over that time period.
The quality of serology test data has been widely variable and resulted in lower levels of sensitivity and specificity for some commercial tests that has led to reduced confidence in serological testing. This can directly contribute to increased spread of disease (5, 7, 20, 22, 25). The root cause of reduced accuracy is likely consequent to the choice of antigen, associated post-translational modifications (26, 27) and/or protein-coated surface of the ELISA plates. Since the coating process of ELISA plates relies primarily on hydrophobic interactions, coating plates with proteins such as the spike S1 and S2 domains, the RBD or nucleocapsid proteins of the SARS CoV-2 can lead to various subpopulations of structurally altered antigen in each well (14). This in turn can lead to the exposure of antigenic sites that would not otherwise be present in the native state giving increased false positives from non-specific immunoglobulin binding (11, 15). This was observed for nucleocapsid and RBD-coated plates with pre-pandemic samples in Study 1 (Figure 2A and Table 1) and for the Abbott and Diasorin tests in Study 2 (Table 2). This issue has also been described for other serology assays (4, 7, 22, 25). Ideally the “bait” protein used to capture circulating immunoglobulins should be in a native or near-native conformation to ensure the antigenic sites of the protein are correctly and consistently exposed to the disease-related antibodies. This is likely the case for cPass™ sVNT because the purified RBD-HRP is supplied and applied in solution (Figure 1) (16) and evidenced by the high specificity of the assay in this work (Tables 1, 2 and 3). Although the cPass™ sVNT utilizes hACE2 protein-coated plates, which can lead to structural perturbations of this protein, there is evidence that immobilized ACE2 receptor maintains a strong interaction with the RBD suggesting minimal loss of structural integrity (16, 27-29). Furthermore, since immunoglobulins from any isotype that recognize RBD antigenic sites can bind and will be measured as a total antibody response, the sensitivity and negative predictive value of this versus immunoglobulin-specific tests (ie: IgG/IgM) should be similar or improved for cPass™ sVNT as was shown here (Figure 2, Table 1 and Table 2) and others (30).
Taken together, these points help explain the similar or improved specificity, sensitivity, positive and negative predictive values and accuracy obtained for the cPass™ sVNT versus other popular commercial SARS CoV-2 IgG tests (Tables 1 and 2) (7, 30). Furthermore, these data support the notion that a binding antibody response as measured through the presence of circulating immunoglobulins coincides with neutralizing antibodies.

Although, for Study 1, the cPass™ sVNT categorized a total of six PCR-positive samples as negative (Figure 2D – red circles), the Spike (S1) (Fig. 2C) and nucleocapsid (Fig. 2B) assays also coincided with their negative classification suggesting that these “false negatives” were in fact true negatives or possibly did not seroconvert. Since these samples were categorized as positive by qPCR testing, this raises the question about the accuracy of qPCR. Some recent SARS CoV-2 and MERS studies suggest that PCR false positives can range from about 2% to 30% with an average of 8% (31, 32). This may be attributed to using a cycle threshold cutoff that is too high and beyond the limit of detection for qPCR (6) accounting in part for the six false negative samples delineated by cPass™ sVNT and the other two serology tests (Figure 2B, C and D – red dots). In fact, at Cq values above 35, many of the technical replicates for a given sample are negative and single copy numbers of contaminating DNA can result in a false positive call (33).

**Application of the cPass™ sVNT as a high throughput-screening tool for COVID-19 drug or vaccine development**

In order to abrogate viral entry, replication and spread of infection, a vaccine should induce the production of antibodies that block (or neutralize) the interaction between the RBD and ACE2 receptor (34-36). Some antibody-based drug candidates are similarly targeting this interaction (37, 38).

To date, the gold and silver standards in assessing the neutralization activity from drugs or antibodies are viral neutralization (VNT) and pseudovirus neutralization tests (pVNT) (39, 40). The VNT requires live
SARS CoV-2 virus and cells that express the ACE2 receptor and therefore a BSL3 containment laboratory, personal protective equipment and highly trained personnel to conduct the experiments whereas the pVNT can be performed in a BSL2 laboratory. Both tests involve sample incubations and manipulations that give results in two and four days and are therefore relatively low throughput, expensive and time-consuming, requiring aseptic technique and personal protective equipment. The early phases of vaccine or drug development typically require screening large numbers of compounds and/or serum samples from candidate vaccine clinical trials to uncover those that neutralize the virus-host cell interaction with the greatest efficiency and efficacy (41). Furthermore, once a good potential vaccine or drug candidate has been selected, clinical trials involving thousands of individuals are required to assess protection against infection and longevity of the neutralizing antibody response post-vaccination (42). Thus, thousands of samples must be collected at regular time points and screened for neutralizing antibody titers, which would be challenging, expensive and time-consuming using VNT or pVNT.

Since the cPass™ sVNT utilizes the purified protein components of the RBD and ACE2 interaction in a high throughput ELISA test requiring about 1.5 hours for each 96-well plate assay in a BSL2 laboratory (Figure 1), it can potentially be used to screen for the best neutralizing drug and/or antibodies generated by vaccination (16). The cPass™ sVNT was compared directly with FRNT and PRNT using serum from COVID-19 recovered patients. An excellent correlation with FRNT75, PRNT75 and PRNT90 in detecting the presence of neutralizing antibodies post-infection (Figure 3 and others (19)) was revealed supporting its application as a reliable tool for vaccine development and longitudinal studies tracking immune response post-vaccination. These data are likely owing to the immunodominance of the RBD versus other antigenic sites of the spike protein (8, 9).

There is no consistency in the literature concerning the analysis stringency that should be applied to cell-based neutralization assays (ie: PRNT50 vs PRNT90 or FRNT50 vs FRNT90) to assure accurate delineation between positive and negative samples. Recent concerns have emerged concerning the ensuing
variability and confidence in the results when different stringencies are applied to the data analysis of these live cell assays (43, 44). The corroboration of the cPass™ sVNT with FRNT and PRNT was examined between 50% and 90% foci and plaque reduction. Significant changes in the analysis were observed between PRNT50 and PRNT75 (Figure 3A - two samples shifting from negative to positive (blue dots)) but no change between PRNT75 and PRNT90. For the FRNT analysis there were large differences between FRNT50, FRNT75 and FRNT90 (Figure 3B) making it difficult to accurately determine the true delineation between positive and negative samples. The corroboration of cPass™ sVNT with the higher stringency PRNT75 and PRNT90 test (Figure 3A) is supported by recently published work (19) and underlines the benefit of this test in accurately delineating between neutralization antibody positive and negative individuals.

**Experimental design for comparative drug or vaccine testing**

cPass™ sVNT was used to assess dynamic changes in neutralizing antibodies from SARS CoV-2 recovered patient samples. Within the linear range, there was a significant decrease in inhibitory antibodies over time for samples 20 and 85 with no decrease observed for sample 74 (Figure 4). However, outside the linear range, near the upper or lower plateau of the dilution curves, the data points for each time point were almost overlapping. This underlines the importance of producing a dilution series from each sample when dissecting the quantitative difference in neutralization titers over time. The cPass™ sVNT offers a much higher throughput, lower cost and safer option to achieve high quality longitudinal data versus more traditional VNT and pVNT especially considering the close correlation with high stringency PRNT90 (19).
The cPass™ sVNT provides a newly structured, high throughput assay (Figure 1) that permits augmented specificity, sensitivity and accuracy for serological assessment of disease versus pre-existing IgG tests (Figure 2 and Tables 1, 2 and 3) (7). The test also permits the functional delineation of virus neutralization for patient recovery that correlates strongly with live cell neutralization (PRNT90) (Figure 3) (19) and for high throughput screening of drug and vaccine immune response antibodies that neutralize the interaction between the RBD and hACE2 receptor (Figure 4).

**Regulatory Status**

The cPass™ SARS CoV-2 Neutralization Antibody Test is CE Marked for diagnostic use in European Union and authorized for emergency use by Health Sciences Authority in Singapore and the US Food and Drug Administration for qualitative delineation between positive and negative patient samples. The quantitation and automation protocols have not yet been authorized by FDA, European Union or Singapore and are Research Use Only.
Figure Legends

Figure 1. cPass™ sVNT Design and Description. A. sVNT Design. The test consists of purified RBD-HRP conjugate (brown) in solution and ELISA plates coated with hACE2 receptor (green) which form a strong complex. When mixed with a sample containing proteins, small molecules or antibodies that block the interaction between the RBD and hACE2 receptor, a low OD450 will be measured after incubation with TMB and stop solution. B. Performing the sVNT. Sample dilutions are initially mixed with the RBD-HRP solution with incubation for 30 minutes at 37°C to permit binding of components to the RBD. If the sample does not contain constituents that bind and block the RBD-hACE2 interaction (bottom four wells) the RBD-HRP will bind to the hACE2-coated wells giving a yellow color after incubation with TMB for 15 minutes at 37°C followed by stop solution. If the sample does contain blocking constituents, they will bind to the RBD during the initial 30 minutes and inhibit the interaction with hACE (top four wells) giving a light yellow color after addition of stop solution.

Figure 2. Study 1: Direct comparison between nucleocapsid, RBD and spike (S1) coated IgG ELISA plates with RBD-soluble sVNT. A. RBD Coated Plate, B. Nucleocapsid Coated Plate, C. Spike (S1) Coated Plate, D. cPass™ sVNT. Forty-five PCR-positive samples with blood drawn more than 14 days post PCR testing were categorized by the four tests (round symbols – left side of each chart). Of the negative delineated samples, six were shared between three tests (red dots). Twenty-three pre-pandemic presumed negative samples were categorized by the four tests (square symbols – right side of each chart). Both the nucleocapsid and RBD-coated IgG ELISAs gave false positives.

Figure 3. Direct comparison between PRNT, FRNT (at different analysis stringencies) and sVNT. A. PRNT. Sixty-six samples were assayed between PRNT50, PRNT75, PRNT90 and sVNT. One sample was discordant between sVNT and PRNT75 and PRNT90 (red dot). Two samples were discordant between PRNT50 and PRNT75 (blue dots). For PRNT, negative samples below 10 were randomly assigned values
of 2, 5 or 8 to more easily visualize the number of negative samples. B. FRNT. Forty-five presumed positive samples were tested between FRNT50, FRNT75 and FRNT90 and sVNT. The same six samples were categorized negative for sVNT, FRNT75 and FRNT90 (red dots). One sample was discordant between FRNT75 and sVNT. For FRNT, all samples with a value of zero were assigned a value of 1 to more easily visualize the negative samples.

Figure 4. Longitudinal assessment of viral titers by cPass™ sVNT for serum samples taken at different time points post-infection. Samples were initially diluted 1:10 according to the kit instructions and then serially 1:3 for an additional five dilutions to generate a competition curve for each sample at each time point. Samples 20 and 85 were compared for titer within the linear range of each curve or by the OD450 ratio.

Table 1. Combined data from Study 1 (Figure 2) comparing assay performance of three commercial serology tests with cPass™ sVNT. Sensitivity, specificity, accuracy, positive and negative predictive values are shown. A prevalence of 10% was used for the calculations.

Table 2. Combined data from Study 2 comparing assay performance of six commercial serology tests with cPass™ sVNT. Sensitivity (Sens), specificity (Spec), accuracy (ACC), positive (PPV) and negative (NPV) predictive values are shown. Pos: positive; Neg: negative; CI: 95% confidence interval; n: sample number; Equ: equivocal results. Prevalence of 10% was used for the calculations.

Table 3. Combined clinical data for cPass™ sVNT. 186 positive and 480 negative samples were verified by PCR and then screened by sVNT. Sensitivity, specificity, accuracy, positive and negative predictive values are shown. A prevalence of 10% was used for the calculations.
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Figure 1A

Receptor Binding Domain of SARS-CoV-2 conjugated to HRP (RBD-HRP)

ACE2 Receptor Protein (ACE2) of Host Cells

Neutralizing Antibodies from Patient Sample blocking the binding of RBD-HRP to ACE2

Complexed RBD-HRP with ACE2

Non-Nitrogenizing Antibodies from Patient Sample that do NOT block the binding of RBD-HRP to ACE2
Figure 1B

Sample containing mixed COVID-19 antibodies: (Neutralizing and Non-Neutralizing)

Pre-Mix

+ RBD-HRP

Patient samples

Neutralizing Antibodies

Non-Neutralizing Antibodies

→ Transfer to sVNT kit plate

Sample with only non-neutralizing COVID-19 antibodies

→ Transfer to sVNT kit plate

→ Wash plate

→ Add TMB followed by stop solutions and read plate

→ Wash plate

→ Add TMB followed by stop solutions and read plate
Figure 2

A. IgG - RBD Coated Plates (Study 1: 68 Samples)
- 45 Presumed Positive
- 23 Presumed Negative
- Cutoff = 1313

B. IgG - Nucleocapsid Coated Plates (Study 1: 68 Samples)
- 45 Presumed Positive
- 23 Presumed Negative
- Cutoff = 0.44

C. IgG - Spike (S1) Coated Plates (Study 1: 68 Samples)
- 45 Presumed Positive
- 23 Presumed Negative
- Cutoff = 1.1

D. cPass™ sVNT (Study 1: 68 Samples)
- 45 Presumed Positive
- 23 Presumed Negative
- Cutoff > 30%

Presumed Positive Samples Negatively Classified:
- Red dot: Shared between three tests: either false positive PCR or no seroconversion
- Black dot: False negatives
Figure 3A

PRNT Viral Neutralization Test
(66 Samples)

Cutoff ≤ 10

CPass™ sVNT
(66 Samples)

Cutoff = 30%
Figure 3B

FRNT Viral Neutralization Test
(45 Presumed Positive, >14 Days Post-PCR)

- FRNT50
- FRNT75
- FRNT90

Cutoff ≤ 10

% Neutralization

cPass™ sVNT
(45 Presumed Positive, >14 Days Post-PCR)

Cutoff = 30%
Figure 4

3-month Longitudinal Study (Sample 20)

3-month Longitudinal Study (Sample 85)

Longitudinal Study (Compared at 3rd Dilution)

Longitudinal Study (Compared at 5th Dilution)

3-month Longitudinal Study (Sample 74)
|                     | Nucleocapsid Coated Plates | Spike (S1) Coated Plates | RBD Coated Plates | cPass™ sVNT       |
|---------------------|----------------------------|--------------------------|------------------|-------------------|
|                     | Positive (n=45) | Negative (n=23) | Positive (n=45) | Negative (n=23) | Positive (n=45) | Negative (n=23) |
| Positive            | 28            | 1               | 34              | 0                | 43              | 3                |
| Negative            | 17            | 22              | 11              | 23               | 2               | 20               |
| Accuracy            | 92.31%        | 97.36%          | 87.82%          | 98.67%           |
| PPV                 | 61.39%        | 100.00%         | 44.87%          | 100.00%          |
| NPV                 | 95.80%        | 97.36%          | 99.44%          | 98.54%           |
| Sensitivity         | 62.22%        | 75.56%          | 95.56%          | 86.67%           |
| Specificity         | 95.65%        | 100.00%         | 86.96%          | 100.00%          |
### Table 2

| Assay         | Analyte          | 5-9 days Neg | 5-9 days Pos | Sens CI | 10-19 days Neg | 10-19 days Pos | Sens CI | >19 days Neg | >19 days Pos | Sens CI | All time points Neg | All time points Pos | Pos Sens CI | n | Neg Equ Pos Spec Cl | PPV | NPV | ACC  |
|---------------|------------------|--------------|--------------|---------|----------------|----------------|---------|--------------|--------------|---------|---------------------|---------------------|------------|----|---------------------|-----|-----|------|
| Abbott IgG    |                  | 0            | 6            | 100     | 54-100         | 3              | 9       | 75           | 43-95        | 0       | 9                   | 100                 | 66-100     | 3  | 24                 | 89  | 71-98 | 27   |
| Affinity IgG  |                  | 0            | 7            | 100     | 59-100         | 2              | 11      | 85           | 55-98        | 0       | 8                   | 100                 | 63-100     | 2  | 26                 | 93  | 71-98 | 28   |
| BioRad IgG    |                  | 0            | 6            | 100     | 54-100         | 3              | 9       | 75           | 43-95        | 0       | 9                   | 100                 | 66-100     | 3  | 24                 | 89  | 71-98 | 27   |
| Diasorin IgG  |                  | 4            | 2            | 33      | 04-78          | 4              | 8       | 67           | 35-90        | 0       | 9                   | 100                 | 66-100     | 3  | 24                 | 89  | 71-98 | 27   |
| Euroimmun IgG |                  | 3            | 3            | 50      | 12-88          | 4              | 8       | 67           | 35-90        | 0       | 8                   | 100                 | 63-100     | 7  | 19                 | 73  | 71-98 | 26   |
| Roche Total Ab|                  | 1            | 6            | 86      | 42-100         | 4              | 9       | 69           | 39-91        | 0       | 9                   | 100                 | 66-100     | 5  | 24                 | 83  | 71-98 | 29   |
| GenScript RBD-ACE2 |               | 1            | 6            | 86      | 42-100         | 1              | 12      | 92           | 64-100       | 0       | 9                   | 100                 | 66-100     | 2  | 27                 | 93  | 71-98 | 29   |

Days Post Symptom Onset:
- Negative Samples (serum collected pre Nov 2019)
- PPV: Positive Predictive Value
- NPV: Negative Predictive Value
- ACC: Accuracy
| Overall clinical data collected by June 1st, 2020 | GenScript cPass<sup>TM</sup> SARS-CoV-2 sVNT (Calculations based on a disease prevalence of 10%) | RT-qPCR |
|-----------------------------------------------|-------------------------------------------------------------------------------------------------|---------|
| Positive                                      | 181                                               | Positive (n=186) |
| Negative                                      | 5                                                  | Negative (n=480) |
| Sensitivity                                   | 97.30%                                             |         |
| Specificity                                   | 99.40%                                             |         |
| Accuracy                                      | 99.20%                                             |         |
| PPV                                           | 94.50%                                             |         |
| NPV                                           | 99.70%                                             |         |

Sensitivity 97.30%
Specificity 99.40%
Overall clinical data collected by June 1st, 2020

RT-qPCR

99.20%
94.50%
99.70%

GenScript cPass<sup>TM</sup> SARS-CoV-2 sVNT (Calculations based on a disease prevalence of 10%)