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Validation and Suitability Assessment of Multiplex Mesoscale Discovery Immunogenicity Assay for Establishing Serological Signatures Using Vaccinated, Non-Vaccinated and breakthrough SARS-COV-2 Infected Cases

Sushant Shengule, Shweta Alai, Sachin Bhandare, Sumant Patil, Manish Gautam, Bhushan Mangaonkar, Sumit Gupta, Umesh Shaligram and Sunil Gairola *

Clinical Bioanalytical Department, Serum Institute of India Pvt. Ltd, Pune, Maharashtra and India- 411028
* Correspondence: sunil.gairola@seruminstitute.com; Tel.: +91-020-2660-2632

Abstract: Antibody responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are multi-targeted and variable over time. Multiplex quantitative serological assays are needed to provide accurate and robust seropositivity data for establishment of serological signatures during vaccination and or infection. We describe here, validation and evaluation of an electro-chemiluminescence (ECL) based Mesoscale Discovery assays (MSD), for estimation of total and functional IgG to SARS-CoV-2 spike, nucleocapsid, and receptor binding (RBD) proteins in human serum samples to establish serological signatures of SARS-CoV-2 natural infection and breakthrough cases. The 9-PLEX assay was validated as per ICH, EMA and US FDA guidelines using a panel of sera samples including the NIBSC/WHO international reference panel (20/268). Assay demonstrated high specificity and selectivity in inhibition assays, wherein the homologous inhibition was more than 85% and heterologous inhibition was below 10 %. Assay also met predetermined acceptance criteria for precision (CV < 30 %), accuracy (70-130 %) and dilutional linearity. Method applicability to serological signatures was demonstrated using sera samples (N=45) representing vaccinated, infected and breakthrough cases. The method was able to establish distinct serological signatures and thus provide potential tool for seroprevalence of SARS-COV-2 during vaccination or infection.

Keywords: Coronavirus; variants of concerns; herd immunity; vaccine efficacy; Electrochemiluminescence

1. Introduction

Amid the Corona virus disease 2019 (COVID-19), caused by the severe acute respiratory coronavirus 2 (SARS-CoV-2), vaccines against variants of concern are currently being developed and licenced as boosters[1,2]. Recently, COVID-19 vaccines against XBB 1.5 variants were licensed as booster vaccine to effectively target the circulating variants of COVID-19[3,4]. Additionally, new variants like JN.1 belonging to parent lineage of BA.2.86 (Pirola) and EG.5 (Eris) have been recently reported globally[5,6]. Global research map for COVID-19, stresses the need for continual global sero-surveilliance to measure the level of infection and vaccine effectiveness[7,8]. Thus, monitoring of serological responses to SARS-CoV-2 variants will be a key to develop rational vaccination strategies to combat against disease[9]. The serological response to SARS-CoV-2 infection codes for multiple structural proteins including trimeric spike protein (S1, S2, RBD regions) and nucleocapsid (N) protein[10]. Antibody response (IgG, IgM, IgA) directed against S, S1-RBD proteins confer protective immune signatures of COVID-19, being key proteins in virus entry and assembly mechanism. Antibody response to nucleocapsid antigen during infection is shown to correlate with seropositivity[9]. Since the emergence of pandemic, 85 different serological test achieved authorization from FDA[11]. However, out of these 85 assays, majority of them are monoplex cut off assays and very few are fully quantitative assays. Multiplex Serology fully quantitative assays are best suited for

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establishing serological signatures as against mono-plex cut off antibody assays, because they allow; simultaneous estimation of serological response to a) multiple virus proteins (antigen) specific antibodies b) high throughput and c) allow easy calibration to international reference standards[12].

Mesoscale Discovery (MSD's) MULTI-ARRAY® electrochemiluminescence detection technology provides quantitative multiplex immunoassay platform for such applications. The V-PLEX product line of MSD provides MULTI-SPOT® (N=10), independents, electrically conductive, well-defined regions coated plates with specific capture antigens/antibodies[13]. MSD platform also offers opportunities for development of surrogate multiplex neutralization assay, which could simultaneously measure ACE blocking antibodies to multiple variants. Thus, a combination of MSD serology assay and surrogate neutralization assays will be the best tool to assess serological signatures [14,15].

We report here method validation and applicability of MSD assays (serology and surrogate neutralization assay; 9 PLEX assay measuring three SARS-CoV-2 antigens, spike (S), receptor binding domain (RBD) of S1 and nucleocapsid (N) and four different variants of spike protein and RBD-S1 protein i.e. Wuhan, B.1.351, P.1 and B.1.1.7 to study serological signatures following infection or vaccination and in breakthrough cases. The study involves use of a sera samples collected during 2021-22, following infection and/or vaccination. The evaluation also covered studies with NIBSC reference standard (NIBSC 20/268), WHO reference panel for anti-SARS-CoV-2 immunoglobulin [16]. Assay demonstrated establishment of distinct serological signatures in different groups and thereby establishing the usefulness of multiplex assays in generation of robust data on seropositivity which will be useful during serosurveillance studies.

2. Method

2.1. Human Serum Samples for Method Validation

Serum samples used for method validation were collected from healthy volunteers aged > 18 years reported at SIIPL, India, after obtaining informed consent. The selected sera (n =19) samples used for the study are mentioned in Table 1. The selected panel also includes WHO reference standards (20/268) panel members such as (NIBSC 20/150, 20/148, 20/140), negative standard 20/142, antibody depleted human sera and sera samples representative of negative, low, medium, high antibody concentrations. Haemolytic (Hb levels at 2.02 g/dL) and lipemic sera samples (cholesterol: 172 mg/dL; triglycerides (TG): 255 mg/dL) (Haemo Service Laboratories, Hyderabad), were also used during selectivity study. A total 16 samples [6 SARS-CoV-2 positive samples; 4 (High, mid, low, negative) NIBSC panel members; 6 SARS-CoV-2 negative samples] were assigned for IgG concentrations in AU/ml, by performing 6 consecutive runs. All serum samples were used in accordance with local regulations and guidelines and approved by the Independent Research Ethics Committee, Pune (IEC No. IRECP/004/2021).

Table 1. Sera sample panel used for assay validation.

Sr. No.	Sample ID	Sample Description	Test Details
1	Sample 1	SARS-CoV-2 positive samples	Samples used for specificity, accuracy, precision, robustness, and stability study
2	Sample 2		
3	Sample 3		
4	Sample 4		
5	Sample 5		
6	Sample 6		
7	Sample 7		
8	Sample 8	NIBSC samples	
9	Sample 9		
10	Sample 10	SARS-CoV-2 negative samples	Samples used for selectivity study
11	Sample 11		
12	Sample 12		

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13	Sample 13		
14	Sample 14		
15	Sample 15		
16	Sample 16	Haemolytic and Lipemic samples	
17	Sample 17		
18	Sample 18	NIBSC Negative sample	
19	Sample 19	Sigma ADHS	

Table 1 shows the list of coded sera samples used during validation study. The third column indicates sample descriptions and last column suggest the samples used for respective validation parameter.

2.2. MSD Serology Assay procedure: Total IgG

V-PLEX MSD COVID-19 Panel 7 serology assay measures antibodies against nine SARS-CoV-2 antigens as N, S1 RBD, S1 RBD (B.1.1.7), S1 RBD (B.1.351), S1 RBD (P.1), Spike, Spike (B.1.1.7), Spike (B.1.351), Spike (P.1). V-PLEX COVID-19 serology assay was chosen by Operation Warp Speed (OWS), as the basis of its standard binding assays for immunogenicity assessments in all Phase III clinical trials of vaccines[17]. The V-PLEX COVID-19 serology assay was performed as per manufactures instruction[18]. Briefly, Blocker A solution was added to the pre-printed 10 spot 96-well plate at 150 µl/well. The plates were sealed and incubated at room temperature for 30 min on continuous shaking at 700 rpm for 30 mins. The plates were washed three times with 1X wash buffer (150ul/well). Sera samples were prepared with four different dilutions in MSD Diluent 100. The kits contain a serum-based standard, Reference Standard 1 (Lot No.: A0080286), which was used to establish a calibration curve in the assay. The reference standard was provided by MSD with arbitory assigned concentration (AU/mL) for each antigen. The calibration curve was used to calculate IgG concentration against multiple antigens. The calibrators were prepared for 7-point calibration curve with 10-fold diluted reference standard with a zero-calibrator blank on each assay plate. Each serum sample and blank were tested in duplicates. After addition of the sample, reference standard, serology assay controls (1.1, 1.2, 1.3), plates were sealed and further incubated at room temperature with continuous shaking at 300 rpm for 120 min. After incubation, plates were washed 3 times with 1X wash buffer. Then the detection antibody, SULFO-TAG (anti-human IgG 1X) concentration was added to the wells (50 µl/well). The plates were sealed and incubated at room temperature with continuous shaking at 300 rpm for 60 mins. After washing, 150 µl of a working solution of MSD GOLD Read buffer B, was added to each well and plates were read on MESO QUICKPLEX SQ 120 (MSD) reader, as per manufacturer's instruction.

2.3. MSD Assay Procedure: ACE-2 Neutralization (Surrogate)Assay

For estimation of ACE-2 neutralization antibodies against nine antigens, plates were blocked with blocking buffer (MSD Blocker A) following incubation and washing with MSD wash buffer, reference standard (MSD Calibrator), and human sera samples were added [19]. The human sera samples were analysed at 1:10, 1:25, 1:100 dilutions in dilution buffer (MSD Diluent-100). After incubation and washing with MSD wash buffer, ACE-2 detection antibody was added (MSD SULFO-TAG™ Human ACE-2 Antibody). Further after incubation and washing, MSD GOLD™ read Buffer B, was added and plates were read using an MSD plate imager Meso Quick Plex SQ 120. Percentage inhibition was calculated relative to the assay calibrator (maximum 100% inhibition) using the equation below.

% Inhibition = [1 – (Average Sample ECL Signal/Average ECL signal of Calibrator 8)] * 100.

2.4. Assay Validation

The assay was analytically validated according to ICH, EMA, and US FDA guidelines on bioanalytical methods and as per International Council for Harmonization (ICH) guideline Q2 (R1), and bioanalytical method validation guidelines[20–22]. Assays were tested in multiple runs, across

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multiple days, and by multiple analysts. Parameters such as robustness, sensitivity, accuracy, precision, specificity, recovery, linearity, and selectivity were optimized[23].

2.5. Sera Panel for Method Validation Studies

A total of 19 samples [6 SARS-CoV-2 positive samples; 4 NIBSC samples (High, mid, low, negative); 6 SARS-CoV-2 negative samples; Haemolytic (Hb levels at 2.02 g/dL) sample; lipemic sera sample (cholesterol: 172 mg/dL; TG: 255 mg/dL); and antibody depleted human sera sample (Sigma)] (Supplementary Table S1), were used for the validation of this assay method.

2.5.1. Specificity

Assay specificity was evaluated using panel of 5 sera samples. These samples were evaluated using a single dilution (1:5000) for inhibition specificity against Wuhan antigens by mixing a neat aliquot of virus with sera sample in a 1:1 ratio to demonstrate homologous antigen inhibition. Cross reacting material (CRM $_{197}$) having 10 µg/mL in a 1:1 ratio was used to demonstrate heterologous specificity. Specificity was determined based on % inhibition of IgG against homologous Wuhan variant and heterologous CRM$_{197}$ antigen >75% and < 25% of value obtained in sample without any treatment respectively.

2.5.2. Selectivity

The method's selectivity was evaluated using three human serum matrices: (i) matrix 1— Sera from healthy volunteers (samples 10–15), (ii) matrix 2—haemolytic and lipemic matrix (samples 16–17), and (iii) matrix 3—NIBSC negative sample (sample 18), and (iv) antibody-depleted human sera (sample 19) as mentioned in Table 1. These matrices are representative of 10 negative or low concentration sera.

Matrices 1 - 4 were spiked with different concentrations of reference standard and tested at concentrations of 1:1000 (high), 1:5000 (medium), and 1:20000 (low). Recovery of spiked samples from the different matrices was calculated with the acceptance criteria within the range of 70%–130% of expected concentrations.

The % recovery at each level was calculated as follows:

% Recovery = [(Observed Spike Samples – Concentration of unspiked samples) / Concentration of Spike] * 100

2.5.3. Precision

The assay precision was evaluated over 3 days and six runs for different analysts, and days. Intra-assay precision refers to the variability observed for the same day. Inter-assay precision refers to the variability in experiments performed on different days by different analysts. The assay precision was reported in terms of the % CV.

2.5.4. Accuracy

Accuracy was assessed over 3 days and six runs using a panel of sera samples. These samples were tested at different concentrations in six assays spread over 3 days read by two analysts. The estimates were compared with the assigned values to determine the accuracy. The resulting IgG concentration of each serum sample was calculated and compared with the assigned values, with an acceptance criterion of percent agreement between 70 and 130 %.

2.5.5. Dilutional Linearity

Dilution linearity was evaluated in twelve different runs using panel of 9 samples. Assay dilutability was assessed in twelve independent runs, using four dilutions (1:500,1:5000, 1:25000 and 1:50000). Recovery was calculated as a percentage difference between the observed and assigned concentrations. Linearity was considered acceptable if said dilution complied with an acceptable %

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CV of duplicates (i.e., <20%) and if the dilution-corrected concentrations were within 70– 130% of the assigned values (Figure 1).

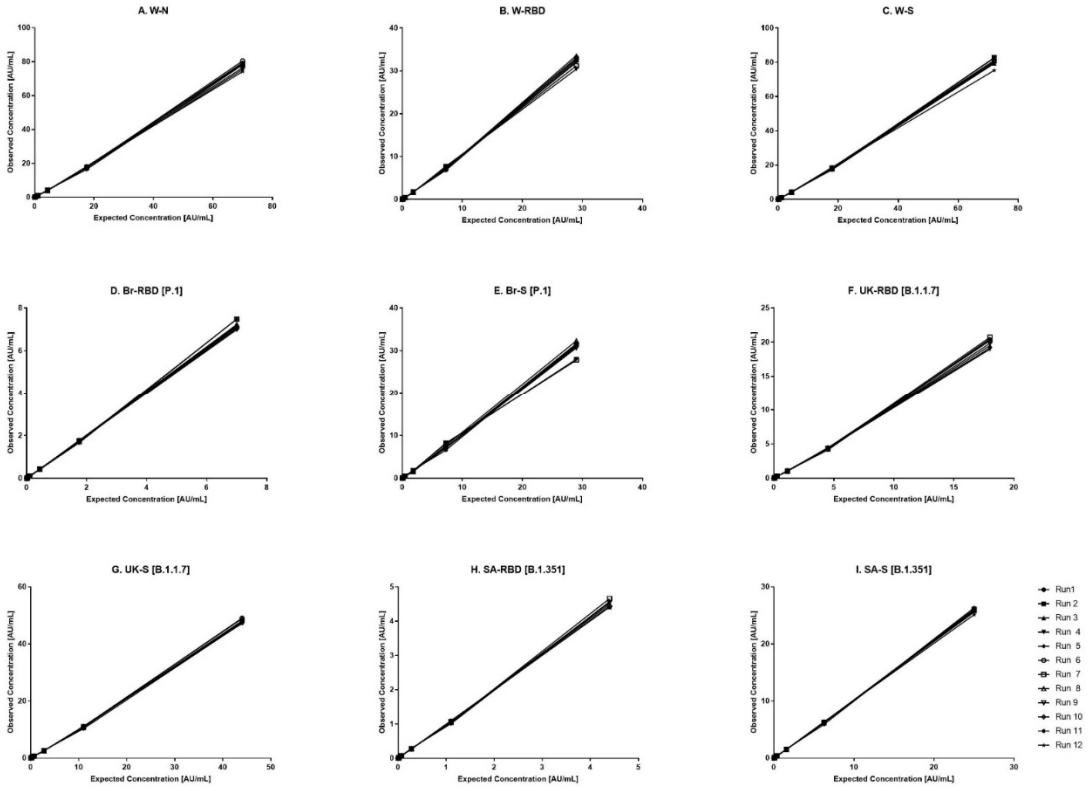

Figure 1. Dynamic range of reference standard for each antigen. (A–I) represents the assay range of reference standard for nine antigens. The X-axis represents expected concentration (AU/mL) whereas Y-axis represents obtained concentration (AU/mL.). Data is representative of 12 runs. W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

2.5.6. Robustness

The robustness of the assay was evaluated using nine sera samples at single dilution i.e. 1:5000. Robustness data on samples concerning incubation time of Ag-Ab and sulfo tag (secondary antibody) was assessed. The % agreement of observed versus expected concentration was calculated for each sample. The following parameters were studied during the robustness assessment: assay sample incubation time and secondary antibody incubation time by (±)30 mins.

2.5.7. Assay Range

The reference standard for the determination of the assay range was evaluated in six runs by four-fold serial dilutions of the reference standard (Supplementary Table S2). The assay range for each antigen was determined using estimates from precision, accuracy, and dilution linearity, after which the most stringent lower and upper concentration limits complying with acceptable accuracy (70–130%) and precision (< 20% CV) and dilutional accuracies of between 70 and 130 % were selected. The assay range was also supported by back-calculated concentrations of calibration standards. The back-calculated concentrations were to be within 70-130 %.

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2.6. Calibration Curve

MSD kit provides reference standard and assay controls. Calibration curve was fitted using 7 point and 4-fold dilution series and a zero-calibrator blank of the reference standards on each assay plate, as specified by manufacturer.

2.7. Panel 7 Assay Calibration with WHO International Panel and MSD Reference Standard (20/268)

A series of six runs were performed to calibrate the MSD reference standard and Panel 7 assay to the first international WHO reference panel (NIBSC code: 20/268). The WHO reference panel consists of individual panel members denoted as; NIBSC 20/150 (high), 20/148 (mid), 20/140 (low), 20/142 (negative human plasma)[24].

2.8. MSD Serology Applicability Study: Development of Serological Signatures

2.8.1. Study Samples

The study uses sera samples from subjects reported at Serum Institute of India Pvt. Ltd., (SIIPL), Pune, selected by Occupational Health Centre (SIIPL), with criteria of above 18 years during the period of year 2021-22. The sera samples were collected with written, informed consent for collection of demographic and clinical data. A total of forty-five sera samples were selected for this study. Study samples represent three groups representing Group 1: Breakthrough infection cases; Group 2: Convalescent and Group 3: Vaccinated with no infection. All vaccinated individuals are immunized with COVISHIELD™ vaccine manufactured by SIIPL, Pune[25]. Samples were collected within 4-6 months of vaccination. The breakthrough infection and convalescent group samples were collected within 4-6 weeks and 1-2 weeks after SARS-CoV-2 infection respectively. A confirmed case of COVID-19 was defined with positive result for real-time reverse transcriptase-polymerase chain reaction assay of nasal and nasopharyngeal swab specimens[26]. Blood samples were collected from the patients at the time of first visit after detection of symptomatic disease and later in accordance with routine biochemical tests[27]. For total IgG estimation against nine different antigens of SARS-CoV-2 similar MSD V-Plex COVID-19 serology assay procedure was used.

2.8.2. Ethics

The study compiled with the declaration of Helsinki, written informed consent was obtained from all participants and the study was approved by the Independent Research Ethics committee, Pune (IRECP/004/2021)[28].

2.8.3. Method Applicability: Development of Serological Signatures

MSD assay provides sera profile with respect to IgG concentrations against nucleocapsid, RBD and spike protein of Wuhan and other variants. Such data can be used to develop serological signatures which can allow assessment of vaccine immunogenicity, breakthrough cases and seropositivity profiles during course of infection[19,29]. The assay offers quantitative read outs in AU/mL. The data in three different groups of infection, vaccinated and breakthrough cases was analysed with respect to ratios of spike protein and nucleocapsid IgG antibody concentration (S/N) and ratio of antibody concentration against variant/antibody concentration against vaccine strain[25].

2.8.4. Statistical Analysis

The MSD assay provides a readout in units of mean luminescence intensity and all readouts were directly log transformed and interpolated using a 4-parameter logistic curve fit directly using MSD Discovery Workbench and GraphPad Prism version 7.0 (GraphPad, San Diego, CA)[18]. For analysis of disease severity various demographic, hematological and laboratory parameters were compared between convalescent group and breakthrough infected group by Student t-test. p values of <0.05 were considered as significant.

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3. Results

3.1. MSD Assay Validation and Characterization

3.1.1. Sera Panel Establishment

Target values for sera panel were established using an average of six consecutive runs, which was further used during validation study. The established values for sera panel is provided in Supplementary Table S3.

3.2. Reference Standard Curve

The kit-based Reference standard 1, was used to establish a calibration curve in the assay. The calibration curve was used to calculate concentration of human IgG against nine antigens. Reference standard 1 was diluted 10-fold to create highest calibrator point (CAL-01). Overall, seven separate four-fold dilutions of the reference standard were performed and were fitted using a 4-PL fit. Figure 1 shows the reference standard dilutional profiles for each of the nine antigens. Linearity of response was demonstrated using back-fitted recoveries, all nine antigens showed 70-130% recoveries for all calibration levels. The lower limit (LL) and upper limit (UL) of the assay range were determined using estimates from accuracy, precision, and robustness analysis (Table 2). The assigned values for the concentration of IgG antibodies to nine antigens in Reference Standard 1 is provided in Supplementary Table 2.

Table 2. Assay range with a lower and upper limit of quantification.

Antigen	Calibration Curve range (AU/mL)		Precision (AU/mL)		Accuracy (AU/mL)		Robustness (AU/mL)		Selectivity (AU/mL)	
	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
W-N	0.01710	70	0.01914	86.1	0.01914	86.1	0.01957	80.8	0.02022	83.2
W-RBD	0.00708	29	0.00777	35.4	0.00777	35.4	0.00812	33.1	0.00813	35.6
W-S	0.01760	72	0.01960	82.7	0.01960	82.7	0.02110	82.1	0.02212	86.2
Br-RBD (P.1)	0.00171	7	0.00119	7.9	0.00119	7.9	0.00152	7.4	0.00210	7.6
Br-Spike (P.1)	0.00708	29	0.00700	32.3	0.00700	32.3	0.00417	31.0	0.00732	32.7
UK-RBD (B.1.1.7)	0.00439	18	0.00337	23.0	0.00337	23.0	0.00044	21.3	0.00453	21.8
UK-S (B.1.1.7)	0.01070	44	0.01071	49.0	0.01071	49.0	0.01300	50.2	0.01342	52.7
SA-RBD (B.1.351)	0.00107	4.4	0.00077	4.8	0.00077	4.8	0.00086	5.4	0.00108	4.6
SA-S (B.1.351)	0.00610	25	0.00682	26.3	0.00682	26.3	0.00629	26.3	0.00733	27.7

Table 2: W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.3. Method Validation

The validation study was designed based on the FDA, EMA, and ICH M10 guidelines for bioanalytical methods. The SARS-CoV-2 serology assay (MSD) was validated for specificity, selectivity, precision, accuracy, dilutional linearity, LOQ and stability using sera samples (Table 2).

3.3.1. Specificity

Specificity is demonstrated for SARS-CoV-2 serology assay in inhibition experiments using homologous and a heterologous competitor antigen. For inhibition experiments, the % inhibition of RFU of a positive serum sample following the addition of an individual antigen was assessed for all nine antigens. The addition of homologous and heterologous antigens, resulted in an >75% and <25%

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inhibition of signal for all 9 antigens respectively (Table 3), indicating the high specificity of the assay in capturing the respective antibodies in the serum sample.

Table 3. Specificity.

Sample.	% inhibition																	
	W-N		W-RBD		W-S		Br-RBD [P.1]		Br-S [P.1]		UK-RBD [B.1.1.7]		UK-S [B.1.1.7]		SA-RBD [B.1.351]		SA-S [B.1.351]	
	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He
Sample 1	89	1	88	2	89	1.3	89	2	89	4	88	1.6	88	2.3	88	1.3	89	2
Sample 3	89	0.6	88	3.3	88	2	89	1.6	89	3.3	88	0.3	87	1	87	4	88	1
Sample 6	89	0.6	88	1.3	89	0.6	89	2.6	89	2	88	1	89	0.6	88	1.3	88	0.3
Sample 8	90	2	89	1	89	0	89	0	90	2.3	89	1.3	89	1.6	88	0.3	89	3.6
Sample 9	90	1	90	1.3	90	1.3	89	0	90	3.6	89	2.3	89	2.3	88	0	89	0.6

Table 3: W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.3.2. Precision

Precision analysis suggested that the assay was precise for different analysts on different days. The % CV for the combined precision of the two analysts was below 30 % for all nine antigens (Table 4). Based on the data, the precision-based LLs and ULs ranged from 0.01914 to 86.1 AU/mL for W-N, 0.00777 to 35.4 AU/mL for W-RBD, 0.01960 to 82.7 AU/mL for W-S, 0.00119 to 7.9 AU/mL for Br-RBD (P.1), 0.00700 to 32.3 AU/mL for Br-S (P.1), 0.00337 to 23.0 AU/mL for UK-RBD (B.1.1.7), 0.01071 to 49.0 AU/mL for UK-S (B.1.1.7), 0.00077 to 4.8 AU/mL for SA-RBD (B.1.351), and 0.00682 to 26.3 AU/mL for SA-S (B.1.351) (Table 2).

Table 4. Precision and accuracy estimates.

Precision	*Analyst (% CV)									**Days (% CV)								
	W-N	W-RBD	W-S	Br-RBD [P.1]	Br-S [P.1]	UK-RBD [B.1.1.7]	UK-S [B.1.1.7]	SA-RBD [B.1.351]	SA-S [B.1.351]	W-N	W-RBD	W-S	Br-RBD [P.1]	Br-S [P.1]	UK-RBD [B.1.1.7]	UK-S [B.1.1.7]	SA-RBD [B.1.351]	SA-S [B.1.351]
Sample 1	15	6	6	5	10	6	5	6	6	15	12	10	8	9	15	8	9	10
Sample 2	16	10	11	10	9	14	12	13	12	16	14	13	12	9	17	13	14	12
Sample 3	11	6	8	7	19	7	6	9	8	11	11	13	11	18	14	10	14	11
Sample 4	18	12	12	11	11	12	12	12	13	18	19	16	15	12	19	15	16	16
Sample 5	13	19	15	12	15	14	15	15	16	13	17	16	19	15	18	20	15	16
Sample 6	8	8	10	10	4	9	10	9	12	8	11	10	11	18	14	10	11	13
Sample 7	20	15	16	13	18	16	17	15	15	20	19	20	17	17	19	18	19	20
Sample 8	12	13	17	13	18	11	16	12	16	12	12	14	15	14	13	13	12	14
Sample 9	15	14	15	16	17	15	15	15	16	15	14	17	14	16	15	16	13	16
Accuracy	***Analyst (% Recovery)**									****Days (% Recovery)**								
Sample 1	94	101	104	101	110	101	104	104	104	94	100	100	101	106	100	102	103	100
Sample 2	105	100	95	105	93	112	97	116	93	105	93	88	102	89	105	90	110	88
Sample 3	87	93	95	77	107	94	95	96	89	87	93	91	79	99	93	94	95	85
Sample 4	75	97	90	104	87	110	92	115	89	75	91	84	100	84	103	86	109	84
Sample 5	93	89	91	82	85	88	90	102	82	93	92	102	90	91	90	94	107	81
Sample 6	88	97	92	95	89	100	94	94	88	88	96	88	99	98	99	91	95	86
Sample 7	73	95	94	103	93	104	97	106	101	73	90	82	90	82	88	91	94	92
Sample 8	84	87	85	90	91	114	87	90	88	84	86	81	89	87	112	83	89	84
Sample 9	91	87	86	89	80	91	91	90	86	91	92	88	93	86	94	95	96	90

Table 4: Precision and Accuracy results are determined concerning different analysts and days. W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S. Precision is determined in terms of % CV. Accuracy is reported in terms of % recovery. *Combined precision (% CV) and accuracy (% recovery) of analysts' 1 and 2, **Combined precision and accuracy of 4 runs over 3 days.

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3.3.3. Accuracy

Acceptable recoveries were observed within the range of 70-130 % for all 9 antigens (Table 4). The accuracy-based LLs and ULs ranged from 0.01914 to 86.1 AU/mL for W-N, 0.00777 to 35.4 AU/mL for W-RBD, 0.01960 to 82.7 AU/mL for W-S, 0.00119 to 7.9 AU/mL for Br-RBD (P.1), 0.00700 to 32.3 AU/mL for Br-S (P.1), 0.00337 to 23.0 AU/mL for UK-RBD (B.1.1.7), 0.01071 to 49.0 AU/mL for UK-S (B.1.1.7), 0.00077 to 4.8 AU/mL for SA-RBD (B.1.351), and 0.00682 to 26.3 AU/mL for SA-S (B.1.351) (Table 2).

3.3.4. Selectivity

The selectivity of the method was evaluated with respect to the use of different serum matrices for SARS-CoV-2 negative samples, NIBSC negative, haemolytic sera, antibody depleted human sera, and lipemic sera. The assay showed high selectivity, as excellent spike recoveries (70-130%) were observed in all the matrices (Table 5). No interference was observed in the assay for haemolytic and lipemic matrices covering up to 2.02 g/dL of haemoglobin and 275 mg/mL of total cholesterol respectively.

Table 5. Selectivity.

Samples	Sample Description	Reference Standard Spike Level	% Recovery								
			W-N	W-RBD	W-S	Br-RBD [P.1]	Br-S [P.1]	UK-RBD [B.1.1.7]	UK-S [B.1.1.7]	SA-RBD [B.1.351]	SA-S [B.1.351]
Sample 10	SARS-CoV-2 negative samples	High	90	88	87	87	92	98	85	98	95
		Mid	103	107	105	92	109	130	106	120	118
		Low	99	108	104	125	102	123	123	112	128
Sample 11		High	91	86	89	87	94	97	89	94	99
		Mid	108	103	109	115	111	88	105	123	115
		Low	120	110	82	105	75	95	80	107	74
Sample 12		High	92	89	94	89	91	113	105	96	104
		Mid	114	113	116	127	97	125	86	81	83
		Low	105	95	86	102	86	122	118	86	89
Sample 13		High	90	93	89	96	95	96	89	96	96
		Mid	86	91	98	85	95	75	101	90	102
		Low	95	97	81	82	84	80	73	83	104
Sample 14		High	91	91	88	90	93	99	88	103	96
		Mid	102	98	97	111	106	104	103	130	114
		Low	124	95	84	106	95	123	124	112	115
Sample 15		High	87	90	87	104	92	95	88	102	96
		Mid	105	128	105	122	124	120	115	125	114
		Low	97	109	121	108	118	73	85	123	115
Sample 16	Haemolytic and Lipemic samples	High	116	83	103	127	97	86	80	70	90
		Mid	105	86	108	115	90	82	76	75	96
		Low	113	95	116	128	96	81	71	80	88
Sample 17		High	86	85	88	101	97	94	89	118	95
		Mid	86	130	110	111	116	118	119	111	123
		Low	86	70	111	125	75	78	118	120	73
Sample 18	NIBSC Negative Sample	High	92	82	94	97	95	95	92	87	95
		Mid	96	104	99	108	103	90	106	104	106
		Low	82	71	104	112	101	82	89	125	90
Sample 19	Sigma antibody	High	88	87	92	102	92	95	89	96	93
		Mid	90	105	95	106	99	94	95	97	97

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depleted human serum	Low	89	100	94	112	105	102	91	96	94

Table 5: W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.3.5. Robustness

The robustness of the assay was studied using sera samples covering the entire range. The critical assay parameters studied included Ag-Ab incubation time and sulfo tag (secondary antibody) incubation time. The % agreement of observed versus expected concentration was calculated for each sample. The results demonstrated that concentration of samples generated from the assays with deliberate variations were within the acceptable range of < 30% agreement for all the antigens (Table 6).

Table 6. Assay Robustness.

Antigen	% Agreement Range			
	Ag-Ab incubation		Sulfo Tag Incubation	
	150 Minutes	90 Minutes	90 Minutes	30 Minutes
W-N	72 - 121	72 - 115	70 - 121	93 - 119
W-RBD	89 - 130	88 - 130	83 - 126	100 - 129
W-Spike	93 - 125	93 - 125	86 - 124	101 - 122
Br-RBD [P.1]	90 - 114	84 - 115	81 - 110	83 - 114
Br-Spike [P.1]	70 - 126	71 - 115	85 - 128	92 - 124
UK-RBD [B.1.1.7]	88 - 113	83 - 130	80 - 129	87 - 126
UK-S [B.1.1.7]	97 - 112	90 - 121	88 - 122	98 - 123
SA-RBD [B.1.351]	86 - 112	78 - 123	80 - 126	90 - 125
SA-S [B.1.351]	91 - 120	83 - 127	87 - 113	90 - 125

Table 6: The % agreement of observed versus expected concentration was calculated for each sample. The range of %agreement was represented for nine samples. The results demonstrated that concentration of samples generated from the assays with deliberate variations were within the acceptable range of < 30% agreement for all the antigens. W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.4. Dilution Linearity

The panel samples were tested in twelve independent runs across a series of sera samples ranging from a dilution of 1:500 to 1:50000. No loss in dilution integrity was observed with 1:5000, 1:25000 and 1: 50000 dilution range recorded for all antigens (Figure 2).

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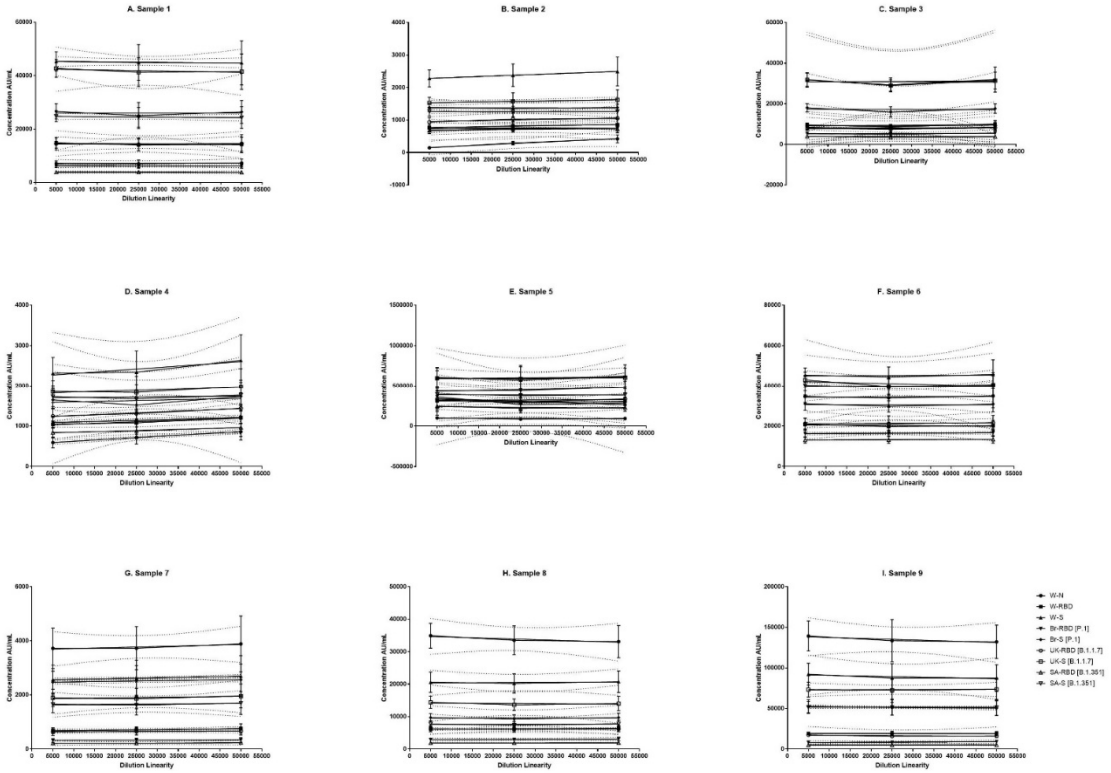

Figure 2.: Dilution linearity of assay in SARS-CoV-2 infected sera samples and NIBSC samples for W-N, W-RBD, W-S, Br-RBD [P.1], Br-S [P.1], UK-RBD [B.1.1.7], UK-S [B.1.1.7], SA-RBD [B.1.351], SA-S [B.1.351] antigens. The X-axis represents the sample's dilutions, and the Y-axis represents the concentration observed in (AU/ml). (A–F) represent dilution linearity graphs for SARS-CoV-2 infected sera samples. (G-I) represents dilution linearity data for NIBSC samples. SARS-CoV-2 infected sera samples and NIBSC samples shows no loss of dilution integrity 5000-50000 dilution range. The dotted line in the figure represents the 95% confidence interval. W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.5. Assay Range

The assay range was selected based on the estimates from precision, accuracy, and study sets. The LL and UL of the assay range were established as ranging from 0.01914 to 86.1 AU/mL for W-N, 0.00777 to 35.4 AU/mL for W-RBD, 0.01960 to 82.7 AU/mL for W-S, 0.00119 to 7.9 AU/mL for Br-RBD (P.1), 0.00700 to 32.3 AU/mL for Br-S (P.1), 0.00337 to 23.0 AU/mL for UK-RBD (B.1.1.7), 0.01071 to 49.0 AU/mL for UK-S (B.1.1.7), 0.00077 to 4.8 AU/mL for SA-RBD (B.1.351), and 0.00682 to 26.3 AU/mL for SA-S (B.1.351) (Table 2).

3.6. Studies with WHO International Reference Panel (NIBSC/WHO)

We report here calibration factors (MSD reference standard to WHO international reference standard; NIBSC 20/268) which will be useful for harmonization of assays across different laboratories. NIBSC 20/268 reference panel is recommended by WHO for assessment and development of assays used in the detection and quantitation of antiSARS-CoV-2 antibodies. The panel provides the unitages for spike, RBD and Nucleocapsid antibodies. The reference panel was further characterized for values against different variants. We provide here factors against reference standard provided by MSD which can be used for harmonization of assay across different laboratories (Table 7).

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Table 7. NIBSC Standards (20/268) values using Panel 7 in BAU/ml.

	NIBSC High BAU/mL			NIBSC Mid BAU/mL			NIBSC Low BAU/mL			Conversion factor
	AU/mL	Assigned values	BAU/mL	AU/mL	Assigned values	BAU/mL	AU/mL	Assigned values	BAU/mL	
W-N	130339	713	308	45344	295	107	4896	45	12	0.00236
W-S	94318	832	2047	27350	241	593	3297	53	72	0.0217
W-RBD	21859	817	509	7971	205	186	790	12	18	0.0233
UK-S [B.1.1.7]	79280	-	1720	17918	-	389	2334	-	51	0.0217
UK-RBD [B.1.1.7]	20147	-	469	5658	-	132	694	-	16	0.0233
SA Spike [B.1.351]	61017	-	1324	9616	-	209	1882	-	41	0.0217
SA RBD [B.1.351]	5195	-	121	2155	-	50	233	-	5	0.0233
Br Spike [P.1]	58047	-	1260	11526	-	250	2961	-	64	0.0217
Br RBD [P.1]	9520	-	222	3381	-	79	352	-	8	0.0233

Table 7: Evaluation of NIBSC standards with MSD SARS-CoV-2 Panel 7 in BAU/ mL using assigned conversion factors for three antigens of SARS-CoV-2. W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.6.1. Method Applicability for Development of Serological Signatures with Respect to Nucleocapsid, RBD and Spike Protein of Ancestral Strain

A total of forty-five sera samples were analyzed for total IgG antibodies against N, S and S1 RBD antigens, using mesoscale discovery (MSD) COVID-19 Serology Assay panel 7. Total IgG antibodies concentration was evaluated using MSD COVID-19 Serology Assay kit provided reference standard (Calibrators) (Supplementary Table S3). Assay reported significantly higher antibodies against N protein in convalescent patient cohort [68887 AU/mL (1088-455149 AU/mL)], as compared to the breakthrough infected group [568 AU/mL (47-2649 AU/mL)] and vaccinated non-infected group [361 AU/mL (160-1040 AU/mL)] [30]. This is consistent to reports wherein antibody levels to nucleocapsid are shown to correlate with viral loads[29,31]. Trend in vaccinated and non-vaccinated samples was found further consistent with RT-PCR predictions, wherein convalescent subjects showed positivity and virologic symptoms in other parameters (Table 8).

Antibodies against spike protein and its subunits S1 RBD are reported to correlate with virus neutralization activities[32,33]. In vaccinated group, S1 RBD antibodies were found to be in the range of [1936 AU/mL (512-5426 AU/mL)]. These levels were on expected lines as these samples were collected 4-6 months post vaccination[34]. These levels are further consistent with reports wherein antibodies are known to wane with time[35]. Higher level of antibodies was observed against S1 RBD in breakthrough infection group [35780AU/mL (918-459708) AU/mL)] as compared to vaccinated group.

For Spike [S1 and S2] antigen too, a similar trend was observed wherein a significant increase in IgG antibodies were observed in vaccinated breakthrough infected patient cohort [94780 AU/mL (4515-1170950 AU/mL)] as compared to the convalescent group; [49670 AU/mL (585-1420159AU/mL)][36]. This increase is further consistent to reports on robust recall responses in vaccinated subjects[37]. This is further continual with other virologic characterization, wherein the breakthrough subjects did not experience severe infection outcomes and hospitalization was limited.

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Table 8. Serological signatures (Total IgG) in convalescent, breakthrough infected and vaccinated non-infected groups.

Sr. No.		W-S	Br-S [P.1]	UK-S [B.1.1.7]	SA-S [B.1.351]	W-RBD	Br-RBD [P.1]	UK-RBD [B.1.1.7]	SA-RBD [B.1.351]	W-N
Convalescent	Mean	153142	96724	149779	107637	61700	44362	57755	41650	121550
	GM	38340	24558	40571	25313	9846	6166	9344	4221	60727
	Min	585	368	521	385	244	146	243	143	1088
	Max	1420159	841413	1296289	892359	678787	484588	640622	537828	455149
	Median	49670	31791	51075	25313	9846	6166	9344	3618	68887
Breakthrough Infected	Mean	231286	204313	190332	137319	77452	79452	83267	118569	704
	GM	71419	50328	57198	34032	23650	22385	25059	20413	380
	Min	4515	1344	3651	1700	918	830	1047	618	47
	Max	1170950	1031308	976125	680803	459708	483341	518697	874411	2649
	Median	94780	68982	82654	54032	35780	35951	36734	29289	568
Vaccinated and Non-Infected	Mean	8555	4303	6347	4131	2366	2097	2610	1504	365
	GM	6707	3306	5056	3468	1813	1607	2020	1095	321
	Min	2433	976	2045	1577	512	634	596	287	160
	Max	15193	10680	14085	7598	5426	5440	6248	3923	1040
	Median	7211	3438	5040	3517	1936	1495	2083	1117	361

Table 8: IgG Estimation using Panel 7 SARS-CoV-2 Serology Assay: IgG titre estimated against nine antigens using Panel 7 SARS-CoV-2 Serology Assay (AU/ml) in Convalescent, Breakthrough Infected and vaccinated non-infected groups. W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.6.2. Serological Signatures: Immune Responses to Different Variants- Total IgG

Multiplex assays such as MSD allows simultaneous measurement of IgG responses against parent and variant strains. Sera panel comprising of samples from convalescent, breakthrough and vaccinated subjects were evaluated for IgG concentrations against Brazil. SA and UK variant. It was noted that all the subjects received vaccine containing Wuhan variant[38]. The assay predicted immune response against the different variants in vaccinated subjected wherein the level of antibodies against Wuhan, Brazil, SA and UK variant of spike antigen was 7211 AU/mL (2433-15193 AU/mL), 3438 AU/mL (976-10680 AU/mL), 3517 AU/mL (1577-7598 AU/mL) and 5040 AU/mL (2045-14085 AU/mL) respectively. For RBD antigen, the level of antibodies against Wuhan, Brazil, SA and UK variant were 1936 AU/mL (512-5426 AU/mL), 1495 AU/mL (634-5440 AU/mL), 1117 AU/mL (287-3923 AU/mL) and 2083 AU/mL (596-6248 AU/mL) respectively [39].

The sera of breakthrough and convalescent subjects are representative of infections during second wave of pandemic. A significantly higher number of antibodies (median) against Brazil, SA and UK variants was observed in breakthrough group. The order of levels was UK>Brazil >SA. This trend is consistent with published reports wherein vaccination with Wuhan did provide protection against different variants[40]. This was further supported by reduced levels of nucleocapsid antibody levels in these groups.

The sera of convalescent groups showed the level of antibodies against different variants in the order of UK>Brazil >SA respectively (Table 9).

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Table 9. Spike: Nucleocapsid IgG antibody ratios for developing serological signatures.

	IgG [Median]							
	W-S: W-N	**Br-S [P.1]: W-N**	**UK-S [B.1.1.7]: W-N**	**SA-S [B.1.351]: W-N**	**W-RBD: W-N**	**Br-RBD [P.1]: W-N**	**UK-RBD [B.1.1.7]: W-N**	**SA-RBD [B.1.351]: W-N**
Convalescent	0.72	0.46	0.74	0.37	0.14	0.09	0.14	0.05
Breakthrough Infected	166.97	121.52	145.61	95.19	63.03	63.33	64.71	51.6
Vaccinated and Non-Infected	20	9.54	13.98	9.75	5.37	4.15	5.78	3.1

Table 9: W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.6.3. Serological Signatures with Respect to Surrogate Neutralization Antibodies Using MSD COVID-19 ACE-2 Neutralization Assay

The V-plex panel 7 measures % inhibition of ACE-2 neutralizing antibodies against Wu-N, Wu-S, Br-S, UK-S, SA-S and Wu-S1 RBD, Br-S1 RBD, UK-S1 RBD, SA-S1 RBD antigens. A significantly higher % of ACE-2 inhibition against Brazil, SA and UK variants was observed in breakthrough group. The order of inhibition was UK>SA>Brazil for spike antigen and SA>UK>Br for RBD antigen. This trend is consistent with published reports wherein vaccination with Wuhan did provide protection against different variants[31,41].

The sera of convalescent groups showed the % inhibition against RBD and spike antigen for different variants in the order of UK>Brazil >SA and UK>SA>Brazil respectively (Table 10).

Table 10. Serological signatures (functional antibodies) in convalescent, breakthrough infected and vaccinated non-infected groups.

ACE-2 Neutralisation	**W-S**	**Br-S [P.1]**	**UK- S [B.1.1.7]**	**SA-S [B.1.351]**	**W-RBD**	**Br-RBD [P.1]**	**UK-RBD [B.1.1.7]**	**SA-RBD [B.1.351]**	**W-N**
Convalescent	**93.9**	**68.1**	**83.5**	**70.7**	**93.6**	**62.7**	**86.4**	**52.0**	56.5
Breakthrough Infected	99.0	92.8	95.9	93.8	98.5	93.0	95.8	97.1	58.0
Vaccinated and Non-Infected	30.5	12.0	19.6	10.8	21.3	22.4	17.4	54.0	52.8

Table 10: SARS-CoV-2 ACE-2 neutralization: The percent inhibition by anti-SARS-CoV-2 antibody measured by MSD ACE-2 neutralization assay. Percentage inhibition was calculated relative to the assay calibrator (maximum 100% inhibition) using the equation below: % Inhibition = [1 – (Average Sample ECL Signal/Average ECL signal of Calibrator 8)] * 100; W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.7. Method Applicability to Predict Distinct Signatures

3.7.1. Spike Protein: Nucleocapsid IgG Antibody Concentration Ratios Were Distinctive among Groups

Ratio of IgG concentration of the spike and nucleocapsid protein [S/N] and RBD and nucleocapsid protein [R/N] of the vaccinated and non-vaccinated infected groups were compared[42].

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These ratios were found distinctive among groups wherein highest ratios were observed in the ascending order of breakthrough > vaccinated > convalescent groups. These ratios were also monitored for RBD and Spike IgG concentrations of different variants and similar pattern was observed (Table 9).

3.8. Method Applicability to Develop Profiles against Variants

All the subjects in vaccinated and breakthrough cases received the vaccine manufactured using Wuhan strain. Sera samples were profiled for total and functional IgG antibodies against different variants[12]. Ratio of antibodies (antibody concentration against variant/antibody concentration against vaccine strain) were determined for each of the group. These ratios were found distinctive among groups wherein highest ratios were observed in the ascending order of breakthrough > convalescent >vaccinated groups. These ratios were also monitored for Spike IgG concentrations of different variants and similar pattern was observed for all variants except UK variant were highest ratios observed in the ascending order of convalescent > breakthrough > vaccinated groups. (Table 11).

Table 11. Impact of vaccine on different variants.

	ACE-2 Neutralisation			IgG		
	Br-S [P.1]: W-S	**UK-S [B.1.1.7]: W-S**	**SA-S [B.1.351]: W-S**	**Br-S [P.1]: W-S**	**UK-S [B.1.1.7]: W-S**	**SA-S [B.1.351]: W-S**
Convalescent	**0.72**	**0.89**	**0.75**	**0.64**	**1.03**	**0.51**
Breakthrough Infected	**0.94**	**0.97**	**0.95**	**0.73**	**0.87**	**0.57**
Vaccinated and Non-Infected	**0.39**	**0.64**	**0.35**	**0.48**	**0.7**	**0.49**

Table 11: SARS-CoV-2 ACE-2 neutralization: The percent inhibition by anti-SARS-CoV-2 antibody measured by MSD ACE-2 neutralization assay. W-N, Wuhan Nucleocapsid; W-RBD, Wuhan receptor binding domain (RBD); W-S, Wuhan Spike (S); Br-RBD [P.1], Brazil RBD; Br-S [P.1], Brazil S; UK-RBD [B.1.1.7], United Kingdom RBD; UK-S [B.1.1.7], United Kingdom S; SA-RBD [B.1.351], South Africa RBD; SA-S [B.1.351] South Africa S.

3.9. Hematological Parameters in Convalescent/breakthrough Subjects

The study also collected the haematological data of convalescents (n=15), breakthrough (n=15) and vaccinated subjects (n=15) to study association with serological signatures if any. Haematological parameters included markers such as D-Dimer, CRP, Ferritin and LDH[43–45]. The demographic and haematology for all samples are summarized in Table 12. The results suggest significant differences in CRP, D-Dimer, Ferritin and LDH levels among groups of convalescent and breakthrough cases which is on expected lines (Table 12). It was noted that breakthrough group values were significantly lower for all these parameters suggesting reduced severity of disease in vaccinated subjects [46]. This is further consistent with trends observed in serology wherein breakthrough groups reported significantly reduced levels of nucleocapsid antibodies as compared to convalescent group [47].

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Table 12. Demographic parameters of study groups.

Parameters	Convalescent	Breakthrough Infected	Vaccinated Non-Infected
	n= 15	n= 15	n= 15
Age (years)	33 (23-49)	40 (23-57)	42 (34-61)
Sex (Male/ Female)	5/15	15/0	15/0
Vaccination Status	No	Yes	Yes

Parameter	Convalescent	Breakthrough Infected	Vaccinated Non-Infected	p value [Student t-test]
Ct Value	23	18	-	**p<0.01**
Haemoglobin (g/dL)	12.02	14.9	-	**p<0.001**
MCV (μm³)	90.21	88	-	**p<0.01**
WBC (/mm³)	10600	7200	-	**p<0.001**
Neutrophils (%)	70	60	-	**p<0.001**
Lymphocytes (%)	28	34	-	**p<0.001**
Platelet (/μL)	242000	286000	-	**NS**
D-dimer (ng/mL)	739.34	119	-	**p<0.001**
Ferritin(ng/ml)	370.33	149	-	**p<0.001**
LDH (U/L)	717.99	379	-	**p<0.001**
CRP (mg/L)	25	6.57	-	**p<0.01**

Table 12 shows demographic and clinical breakthrough and vaccinated non-infected groups. For analysis of disease severity various demographic, hematological and laboratory parameters were compared between convalescent group and breakthrough infected group by Student t-test. p values of <0.05 were considered as significant.

4. Discussion

COVID-19 vaccine based on spike protein continues to provide protection against disease outcomes, even in presence of emerging variants[48]. Serological responses to SARS-CoV-2 infection is multi-targeted and therefore bring challenges in effective monitoring. Multiplex assays bring opportunities as it allows simultaneous estimation of antibody responses against multiple proteins of ancestral as well as variant strains. Such multi-dimensional data further offers opportunities in establishment of serological signatures. We report here the validation of multiplex MSD platform based serological assay and its applicability to establish serological signatures of SARS-CoV-2. The signatures were identified for total and functional IgG levels against parent and different variants. The functional IgG was determined using ACE receptor blocking antibodies on MSD platform. Neutralization assay is reported as surrogate neutralization assay for quantification of virus neutralizing antibodies[49].

The performance of any multiplex assay depends on many factors[12]. MSD assay was therefore validated using a panel of sera samples. The validation parameters were designed based on guidelines of US FDA, EMA and ICH guidance on bioanalytical methods. This study for the very first time reports the performance evaluation of MSD assay with respect to different variants. The assay performance against different variants was slightly variable. It was noted that assay showed relatively high variability against Brazil and SA variants[50]. This could be due to multiple reasons as technology involves spotting and single detection antibody for all the variants. Additionally, the assay reports the values for all the variants against one reference serum. The binding of different variants can vary. It is recommended that each laboratory should perform validation and establish the performance metrics in their laboratories using international reference standards as this will allow pooling of data across the laboratories. We report here calibration factor of MSD assay reference standard international reference standard. The study here reports suitable calibration factors which

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could be used to report the estimated IgG concentration in international units for other emerging variants used in panel 7 assay.

Serological signatures involving multiple proteins of different variants offers comprehensive understanding of serologic responses against infection, vaccination and recall responses. For applicability to sero-signatures, results were reported from a pilot scale study using N=45 seropositive individuals representing vaccination, infection and vaccinated individuals following breakthrough infection. The vaccinated samples represent immunization with COVISHIELD™ vaccine. The vaccine is based on adeno-vector platform (ChAdOx1 ncov-19 Corona Virus particles) which is a recombinant, replication-deficient chimpanzee adenovirus vector encoding the SARS-CoV-2 spike glycoprotein of Wuhan variant[38]. The study uses sera samples collected during the duration, 2021- 22 and thus represent infections with Delta or Omicron variants of concern (VOCs). Method was able to clearly establish distinct serological signature between infection, vaccinated and breakthrough responses. Antibodies against nucleocapsid proteins were found to be highly indicative of infection wherein breakthrough and infection groups showed significantly higher antibodies against nucleocapsid protein. The diagnostic significance of antibodies against nucleocapsid protein is already reported and established[10]. An attempt was also made to study the ratios of antibodies against nucleocapsid and spike protein in these groups. It was noted ratios were also predictive of distinct signatures in the groups used in the study. Antibodies against S protein and the RBD of SARS-CoV-2, serve as a target for the development of vaccines and therapy[51,52]. The assay was able to predict different levels of reactivities to spike protein and S1-RBD in different groups. Vaccinated breakthrough cases showed significantly higher number of antibodies against spike protein and RBD supporting a strong recall response with vaccine. This is further consistent with reports on COVID vaccines wherein a robust recall response was reported in breakthrough cases[53].

Several variants of concern (VOC) of SARS-CoV-2 have emerged[54]. There is an urgent need to quantify the breadth of immune responses generated by any vaccine against these variants[35,55]. Between the 3 groups, vaccinated breakthrough infection showed highest median antibody titre values of IgG followed by convalescent and vaccinate non-infected for all variants. This further supports the reports of robust recall responses with Covid vaccines. The assay reported antibodies against variants in the order of Wuhan > Br >UK > SA for both spike and RBD. Thereby supporting the reports on variable degrees of protection against variants of SARS-CoV-2.

Neutralizing or functional antibodies against SARS-CoV-2 virus were reported using different assay platforms including plaque reduction neutralization test (PRNT), microneutralization test and pseudo neutralization test[56]. However, all these tests are laborious and requires considerable amount of serum. Determination of functional IgG levels using surrogate neutralization tests such as MSD ACE receptor inhibition assay offers opportunities of high throughput, simultaneous estimation against different variants and most importantly uses minimal amount of serum sample. The study analyzed ACE2 binding inhibition within serum samples from vaccinated infected, non-infected and non-infected alone individuals. The percentage inhibition of ACE-2 receptor binding to all four variants for the vaccinated breakthrough infected cohort was significantly higher than the convalescent. Among recently emerged strains (alpha, beta, gamma), ACE2 binding inhibition compared to wild type was reduced for all[6]. Although, all RBD mutants except wild type (Wuhan) showed slightly reduced ACE2 binding inhibition (Table 10) in serum samples. This specific mutation has been reported in multiple studies as an escape mutation that enhances the RBD-ACE2 affinity which may also be confirmed from the study results[57]. Additionally, results generated also showed positive correlation between ACE2 binding inhibition and S1/trimeric spike antibody production. Therefore, the signatures developed by assays were able to distinctively classify the groups of convalescent, breakthrough, and vaccinated groups.

The present study has limitations are with respect to number of samples in this study. The study reports result from N=45 subjects which is very limited. Nevertheless, the study provides sufficient evidence on capability of the multiplex methods to establish serological signatures. Another limitation of the study is that the study is done on a panel of sera samples available at occupational

health center. Though, suitable care was taken to identify the best panel of samples for this study, however a time course study would have further helped to establish the kinetics of the immune responses. The study uses panel 7 MSD kits which was the most updated kits available during the study. It is noted that recently MSD also introduced kits with the most current XBB variant in panel. It will be further interesting to profile the sera samples against the XBB variant[8,40]. It is also noted that at the time of data analysis NIBSC also introduced a new reference standard for VoCs. It will be further helpful to develop calibration factors against the international standard for SARS-CoV-2 to allow reporting the results in international units.

Multiplex assays such as MSD will be essential in large scale serosurveillance and deciphering vaccination strategy[12]. By comparing the results of serologic assays that detect antibodies to either spike (S1), the spike glycoprotein receptor-binding domain (RBD), or the nucleocapsid (N), it is possible to study vaccine effectiveness between previously SARS-CoV-2-infected (either infected alone or infected and then vaccinated), vaccinated and uninfected individuals. By comparing the titer of antibodies to either spike (S1), the spike glycoprotein receptor-binding domain (RBD), or the nucleocapsid (N), it will be possible to distinguish between previously SARS-CoV-2-infected (either infected alone or infected and then vaccinated), vaccinated (with no evidence of prior infection), and uninfected individuals using MSD platforms. The study also demonstrated the usefulness of platform to profile the sera reactivity to different variants which will help in better understanding of impact of vaccines in real-world settings.

5. Conclusions

In conclusion, findings presented here presents opportunities for multiplex SARS-CoV-2 serological diagnostics in sero-surveillance studies as it allows efficient classifications among different groups of vaccinated, infected and breakthrough cases. The precision and accuracy estimate of assay especially the range and sensitivity further support the use of multiplex assays in regions and studies where low seroprevalence is expected. The study suggests method suitability to establish serological signatures in different groups based on vaccination and infection status. The study further supports the concept and merits of serological signatures which will be helpful in better understanding of diversity of immune responses against SARS-CoV-2.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Supplementary Table 1. Antigen details used for specificity study, Supplementary Table 2. Values of Reference Standard 1 in MSD Arbitrary units (AU/ml), Supplementary Table 3 Assigned values (AU/ml) for sera panel.

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