

ORIGINAL ARTICLE

Validation and performance of a multiplex serology assay to quantify antibody responses following SARS-CoV-2 infection or vaccination

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Abstract

Objectives. Robust, quantitative serology assays are required to accurately measure antibody levels following vaccination and natural infection. We present validation of a quantitative, multiplex, SARS-CoV-2, electrochemiluminescent (ECL) serology assay; show correlation with two established SARS-CoV-2 immunoassays; and present calibration results for two SARS-CoV-2 reference standards. **Methods.** Precision, dilutional linearity, ruggedness, analytical sensitivity and specificity were evaluated. Clinical sensitivity and specificity were assessed using serum from pre-pandemic and SARS-CoV-2 polymerase chain reaction (PCR)-positive patient samples. Assay concordance to the established Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay and a live-virus microneutralisation (MN) assay was evaluated. **Results.** Standard curves demonstrated the assay can quantify SARS-CoV-2 antibody levels over a broad range. Assay precision (10.2–15.1% variability), dilutional linearity (≤ 1.16 -fold bias per 10-fold increase in dilution), ruggedness (0.89–1.18 overall fold difference), relative accuracy (107–118%) and robust selectivity (102–104%) were demonstrated. Analytical sensitivity was 7, 13 and 7 arbitrary units mL⁻¹ for SARS-CoV-2 spike (S), receptor-binding domain (RBD) and nucleocapsid (N) antigens, respectively. For all antigens, analytical specificity was > 90% and clinical specificity was 99.0%. Clinical sensitivities for S, RBD and N antigens were 100%, 98.8% and 84.9%, respectively. Comparison with the Elecsys[®] immunoassay showed $\geq 87.7\%$ agreement and linear correlation (Pearson r of 0.85, $P < 0.0001$) relative to the MN assay. Conversion factors for the WHO International Standard and Meso Scale Discovery[®] Reference Standard are presented. **Conclusions.** The multiplex SARS-CoV-2 ECL serology assay is suitable for efficient, reproducible measurement of antibodies to SARS-CoV-2 antigens in human sera, supporting its use in clinical trials and sero-epidemiology studies.

Keywords: antibodies, COVID-19, electrochemiluminescence, immunoassay, SARS-CoV-2, serology

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an enveloped, positive-sense RNA virus, was identified as the causative agent of coronavirus disease 2019 (COVID-19) in January 2020.^{1,2} SARS-CoV-2 codes for various structural proteins, including the highly immunogenic spike protein (S) that forms characteristic club-like spike projections from its surface, and the nucleocapsid protein (N) that plays a key role in transcription and viral assembly.^{3–5} At the time of manuscript preparation, there have been more than 445 million confirmed cases of COVID-19 worldwide, and approximately six million people are known to have died from COVID-19.⁶

In the months following declaration of the pandemic, there was a rapid increase in the development and availability of SARS-CoV-2 diagnostics, which was followed by the development and rollout of vaccines and monoclonal antibodies (mAbs) against SARS-CoV-2.^{4,7–10} To facilitate the development of vaccines and therapeutic antibodies and to enable large sero-epidemiology studies, robust serology assays are needed. A robust anti-SARS-CoV-2 serology assay should ideally: (1) differentiate SARS-CoV-2 antibodies from cross-reactive antibodies (e.g. antibodies to other coronaviruses), (2) differentiate antibody responses following vaccination from those following natural infection, (3) be high-throughput and easy to perform, (4) reproducibly quantify antibody levels over time (months and years of follow-up) and (5) be accurate so that results can be compared to other clinical studies and be used to determine correlates of protection.¹¹

Quantitative, multiplex electrochemiluminescence (ECL)-based serology assays allow for sensitive, high-throughput and simultaneous quantification of immunoglobulin G (IgG) levels to multiple antigens and have been shown to correlate with SARS-CoV-2 neutralisation assays.^{12–14} Such assays are highly scalable, optimal for complex matrices (typically yielding low interference from serum or plasma),¹² have a broad dynamic range and have the capacity to multiplex within a single well with small sample

volume requirements. The multiplex SARS-CoV-2 ECL serology assay presented here is based on Meso Scale Discovery® (MSD) technology (MSD, Rockville, MD, USA); it is a quantitative ECL assay¹⁴ that uses disposable multi-spot microtiter plates coated with S, receptor-binding domain (RBD) and N antigens to detect SARS-CoV-2-specific antibodies present in serum samples.¹⁵

In this report, we provide an overview of the strategy and validation results for the multiplex SARS-CoV-2 ECL serology assay and demonstrate the assay's applicability to complement clinical diagnostics. We also correlate the results of this assay with those from the Food and Drug Administration (FDA)-authorised Elecsys® Anti-SARS-CoV-2 immunoassay (Roche Diagnostics, Basel, Switzerland) and with a live-virus microneutralisation (MN) assay developed and validated by the Battelle Biomedical Research Center (BBRC, Columbus, OH, USA). Lastly, we present the assay calibration and potency results of the multiplex SARS-CoV-2 ECL serology assay to the World Health Organization (WHO) International Standard and another commonly used calibration standard.

RESULTS

Standard curve characterisation and assay validation

Standard curve characterisation

A high-titre, pooled, human serum, collected from polymerase chain reaction (PCR)-confirmed SARS-CoV-2-positive convalescent samples (see Methods), was used to create an 11-point reference standard curve for each antigen with concentrations ranging from 0.01 to 100 arbitrary units (AU) mL⁻¹. The ECL signals spanned four logs (Figure 1), and 11 standard curve points were used for the SARS-CoV-2 S and N antigens and nine points for the RBD antigen. Per the prespecified standard curve criteria, the total number of valid standard curve points for SARS-CoV-2 S and N antigens was ≥ 9 and RBD antigen was ≥ 7 , and all 45 plates used in the validation met these criteria (Figure 1).

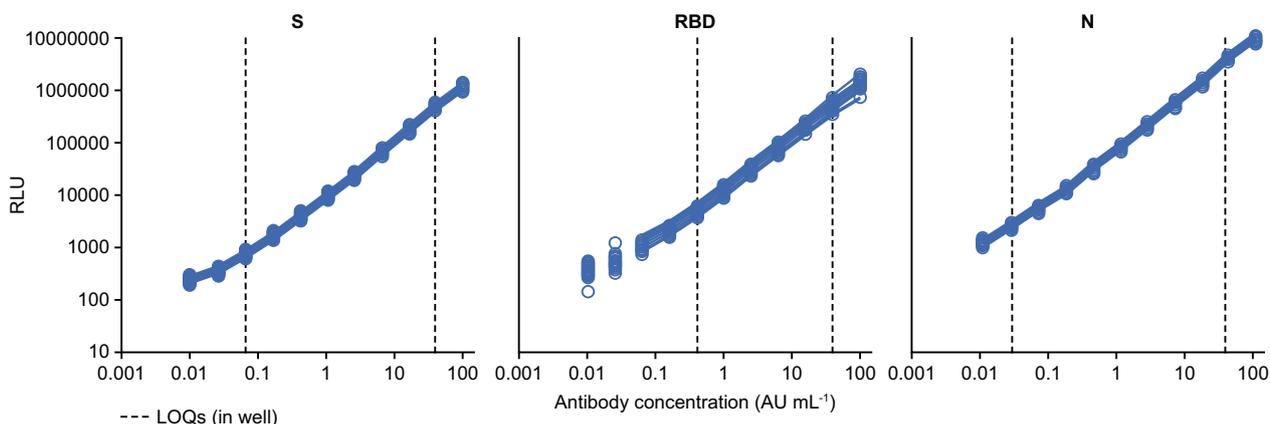


Figure 1. Standard curve precision profiles for SARS-CoV-2-specific S, RBD and N antibodies 11-point dilution series tested 45 times. AU, arbitrary units; LOQ, limit of quantitation; N, nucleocapsid protein; RBD, receptor-binding domain; RLU, relative light unit; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Assay precision, dilutional linearity and ruggedness

Intermediate assay precision was determined to be 15.1%, 10.2% and 14.9% geometric coefficient of variance (GCV) for SARS-CoV-2 S, RBD and N antigens, respectively (Table 1). Dilutional linearity bias ranged from 1.07- to 1.16-fold per 10-fold increase in dilution across the three antigens, and ruggedness between the five analysts and two plate lots ranged from 0.89- to 1.18-fold (Table 1). Based on the intermediate precision of the assay, it was determined that a 1.82-, 1.51- and 1.81-fold increase in antibody levels for S, RBD and N, respectively, were statistically significant (Table 1).

Analytical sensitivity and quantifiable range

The limit of detection (LOD) for each antigen was defined as the lowest antibody concentration where the associated assay signal was statistically higher, with > 95% probability, than a blank sample containing no antibody. This assessment was performed on samples tested at the 500-fold dilution. The LODs for SARS-CoV-2 S, RBD and N antigens were determined to be 7, 13 and 7 AU mL⁻¹, respectively (Table 1). The limits of quantitation (LOQs) were based on acceptable assay performance with regard to precision and accuracy for which values were within concentrations corresponding to the second lowest and second highest standard curve points, but not less than the LOD for each antigen. The lower LOQ

(LLOQ) for SARS-CoV-2 S, RBD and N antigens at 1:500-fold dilution were established to be 33, 204 and 14 AU mL⁻¹, respectively. The upper LOQ (ULOQ) for all three SARS-CoV-2 antigens at the 1:50 000 dilution was 2 000 000 AU mL⁻¹ (Table 1). Therefore, the combination of 500-fold and 50 000-fold dilutions selected for these studies provided an assay range of 4–5 logs for all antigens evaluated (33–2 000 000 AU mL⁻¹ for S, 204–2 000 000 for RBD and 14–2 000 000 AU mL⁻¹ for N).

Analytical specificity

Competition with homologous and heterologous antigens was performed to determine the analytical specificity of the assay to measure SARS-CoV-2-specific antibodies. Serum samples ($n = 8$) spiked with homologous antigens (SARS-CoV-2 S, RBD and N) exhibited > 90% reduction in antibody levels, whereas < 15% reduction in antibody levels was observed when competed with heterologous antigens from other viruses, including the seasonal coronavirus OC43 S and influenza H3 Hong Kong hemagglutinin (Table 1). In addition, when S was used for competition, > 90% reduction in antibody levels was observed for both the full-length S and RBD antigens. Similarly, when samples were spiked with RBD, > 90% reduction in antibody levels was observed for the RBD antigen, but only a partial reduction in antibody levels (> 37%) were observed for the S antigen. Since the RBD comprises part of the S, it was expected that some of the antibodies that bind to the S antigen would also bind to the RBD moiety (Figure 2a and b).

Table 1. Multiplex SARS-CoV-2 ECL serology assay validation summary characteristics

Assay characteristic	SARS-CoV-2 antigen		
	S	RBD	N
LLOQ (AU mL ⁻¹)			
1:500	33	204	14
1:5000	330	2040	140
1:50 000	3300	20 400	1400
ULOQ (AU mL ⁻¹)			
1:500	20 000	20 000	20 000
1:5000	200 000	200 000	200 000
1:50 000	2 000 000	2 000 000	2 000 000
LOD (AU mL ⁻¹)	7	13	7
Proportion of samples with % GCV ≤ 25% ^a	≥ 95.7%	100%	≥ 90.9%
Analyst ruggedness ^b , fold	0.93–1.14	0.89–1.18	0.93–1.10
Plate lot ruggedness ^b , fold	0.97–1.01	0.97–1.02	0.93–0.98
Intermediate assay precision ^c , % GCV	15.1%	10.2%	14.9%
Statistically significant fold increase ^d	1.82	1.51	1.81
Relative accuracy range	112–117%	107–113%	113–118%
Dilutional linearity ^e , fold	1.11	1.16	1.07
Selectivity ^f	102%	104%	103%
Homologous specificity ^g	> 91%	> 94%	> 95%
Heterologous seasonal coronavirus antigen (OC43) and H3 influenza antigen specificity ^g	< 11%	< 5%	≤ 6%

AU, arbitrary units; ECL, electrochemiluminescence; GCV, geometric coefficient of variation; H3, Hong Kong hemagglutinin subtype 3; LLOQ, lower limit of quantitation; LOD, limit of detection; N, nucleocapsid protein; OC43, seasonal coronavirus OC43 spike protein; RBD, receptor-binding domain; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ULOQ, upper limit of quantitation.

^aMinimum % across all dilutions.

^bRange of overall fold difference.

^cMaximum percent GCV across dilutions.

^dCalculated as $e^{3 \times \sqrt{2 \times \Sigma \sigma^2}}$.

^eBias per 10-fold increase in dilution.

^fOverall % recovery.

^gOverall % inhibition.

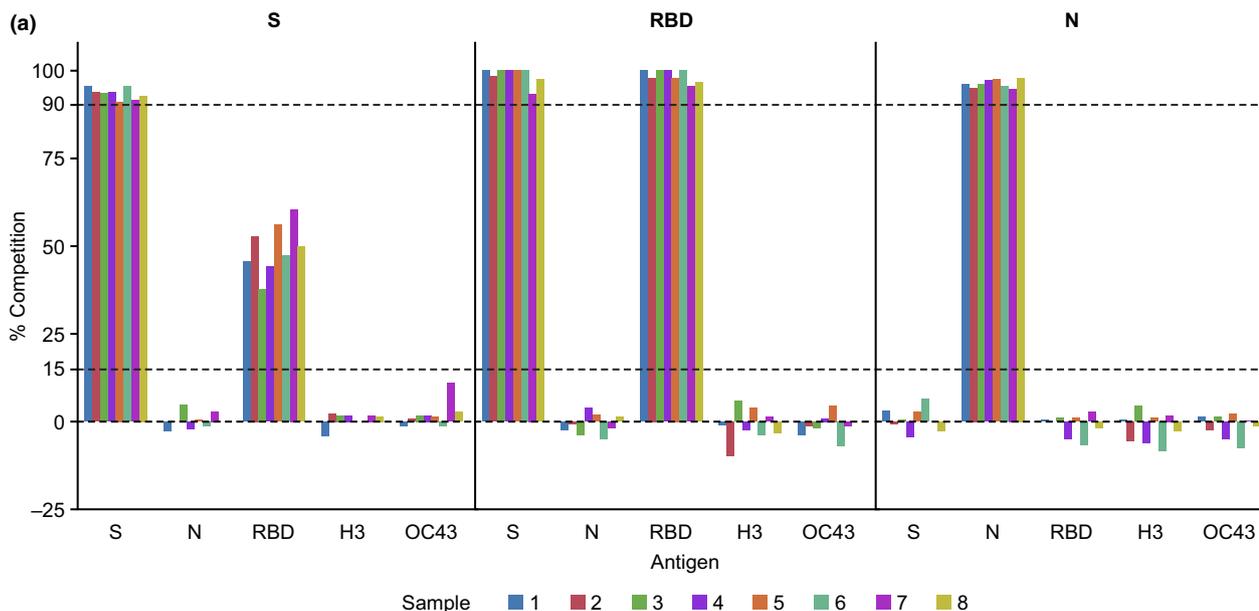
Serostatus cut points

To determine whether an individual had been previously exposed to the SARS-CoV-2 virus, we determined the serostatus cut points for the three antigens to differentiate a seropositive versus a seronegative individual. To determine the baseline for a seronegative status, a set of prepandemic samples ($n = 195$) were tested and used to establish the 99th percentile serostatus cut points for the multiplex SARS-CoV-2 ECL serology assay. The assay cut points for S, RBD and N SARS-CoV-2 antigens were set at 675, 2396 and 9787 AU mL⁻¹, respectively (Figure 3a, Table 2). S, RBD and N antibody concentrations were subsequently compared for all serum samples (258 measurements from 86 samples from SARS-CoV-2 PCR-positive individuals and 584 measurements from 195 SARS-CoV-2-negative, prepandemic samples). The assay cut points were applied to the data to determine the agreement between serostatus and SARS-CoV-2

prior infection status by PCR (Figure 3b, Table 2) and to calculate clinical sensitivity and specificity of the multiplex SARS-CoV-2 ECL serology assay, as described below.

Clinical sensitivity and specificity

A total of 258 measurements from 86 serum samples from SARS-CoV-2 PCR-positive individuals (collected ≥ 14 days after obtaining a positive PCR result) were used to assess clinical sensitivity or the ability to correctly identify the samples from individuals previously diagnosed with SARS-CoV-2 infection (true positive rate). This assessment was performed for each of the three SARS-CoV-2 antigens, and the clinical sensitivities were determined as the proportion of samples at or above the cut point. For SARS-CoV-2 S, RBD and N antigens, the clinical sensitivity of each serology assay was 100% (258/258), 98.8% (255/258) and 84.9% (219/258), respectively (Table 2).



(b)

Sample	Antigen	Spiked with:												
		Antibody concentration (AU mL ⁻¹)							Percent specificity					
		Water	Diluent 100	S	N	RBD	H3	OC43	Diluent 100	S	N	RBD	H3	OC43
1	S	23086	24216	1329	23719	12748	24005	23336	-5.0%	95.6%	-2.8%	45.4%	-4.0%	-1.1%
1	N	23415	23272	22727	1034	23320	23371	23152	0.6%	3.0%	96.2%	0.4%	0.2%	1.1%
1	RBD	23027	22435	< 2040	23456	< 2040	23147	23765	2.8%	100.0%	-2.0%	100.0%	-0.6%	-3.5%
2	S	81535	78591	5176	81465	38930	79705	80983	3.6%	94.0%	0.1%	52.5%	2.3%	0.7%
2	N	66614	68108	67088	3425	66537	70427	68152	-2.2%	-0.7%	95.1%	0.1%	-5.7%	-2.3%
2	RBD	76005	80907	3155	76410	3511	83065	76521	-6.6%	98.5%	-0.5%	98.0%	-9.5%	-0.7%
3	S	48047	49968	3353	45854	30055	47106	47186	-4.0%	93.7%	4.6%	37.7%	2.0%	1.8%
3	N	45388	45293	45181	1966	45019	43383	44829	0.2%	0.5%	96.0%	0.8%	4.4%	1.2%
3	RBD	40484	38195	< 2040	41761	< 2040	38240	41175	6.0%	100.0%	-3.3%	100.0%	5.8%	-1.8%
4	S	24224	24122	1689	24683	13673	23823	23770	0.4%	94.3%	-1.9%	44.2%	1.7%	1.9%
4	N	55482	56829	57814	1675	58232	58817	58160	-2.4%	-4.2%	97.2%	-5.0%	-6.0%	-4.8%
4	RBD	19780	20519	< 2040	19144	< 2040	20210	19623	-4.2%	100.0%	3.6%	100.0%	-2.4%	0.9%
5	S	46734	47715	4369	46468	20722	46647	46061	-2.1%	91.3%	0.6%	56.1%	0.2%	1.5%
5	N	35997	36046	34981	970	35629	35616	35287	-0.1%	2.8%	97.7%	1.0%	1.1%	2.0%
5	RBD	53436	51840	< 2040	52569	3161	51509	51071	3.1%	100.0%	1.7%	97.8%	3.7%	4.6%
6	S	53505	53989	2779	54188	28487	53554	54406	-0.9%	95.4%	-1.3%	47.0%	-0.1%	-1.7%
6	N	70344	76880	65127	3331	75064	76159	75573	-9.3%	7.4%	95.5%	-6.7%	-8.3%	-7.4%
6	RBD	46949	48400	< 2040	49049	< 2040	48610	50013	-3.2%	100.0%	-4.7%	100.0%	-3.7%	-6.8%
7	S	75785	78518	6601	73582	30322	74535	67624	-3.6%	91.7%	2.9%	60.3%	1.7%	10.8%
7	N	41119	40111	41087	2240	40072	40397	41243	2.5%	0.1%	94.9%	2.6%	1.8%	-0.3%
7	RBD	82674	80294	7168	84187	5570	81398	83730	3.0%	93.6%	-1.9%	95.6%	1.6%	-1.3%
8	S	76790	75164	5746	76798	38659	75652	74673	2.1%	92.9%	0.0%	49.9%	1.5%	2.8%
8	N	18142	17985	18661	501	18453	18631	18399	0.9%	-2.9%	98.0%	-1.7%	-2.7%	-1.4%
8	RBD	70047	70969	3546	69132	4144	72138	70031	-1.4%	97.8%	1.3%	96.9%	-3.1%	0.0%

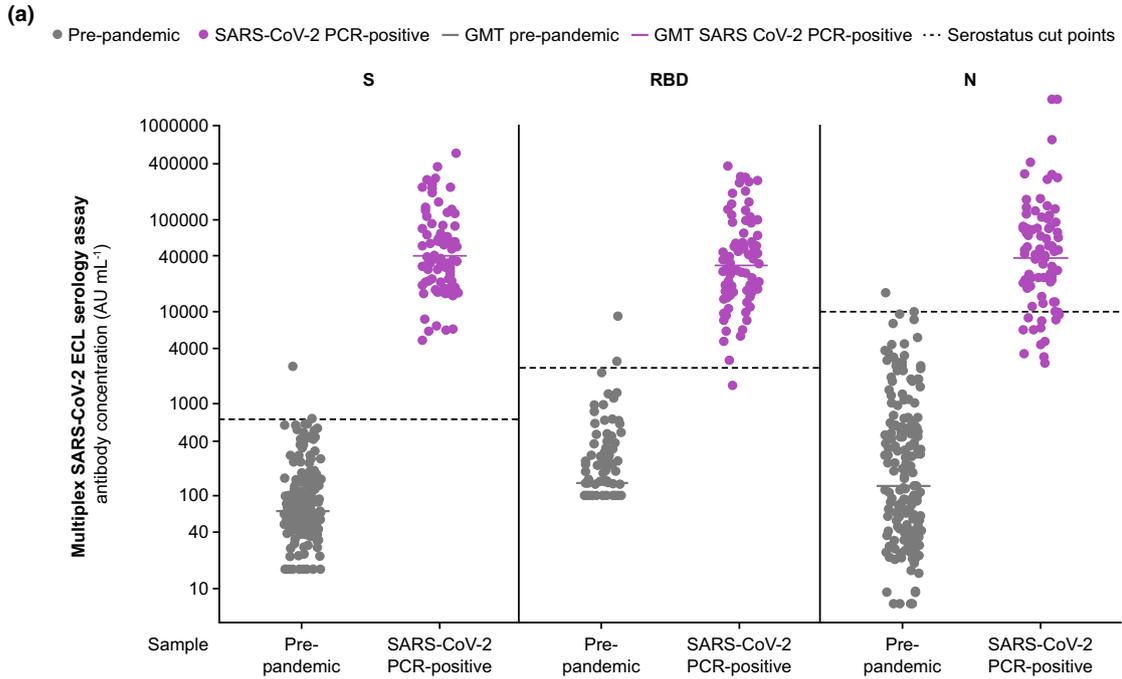
Green shading represents homologous inhibition; yellow shading represents expected cross-reactivity between RBD and S.

Figure 2. Analytical specificity (a) and sensitivity (b) of the multiplex SARS-CoV-2 ECL serology assay to measure antibodies to heterologous and homologous antigens. ECL, electrochemiluminescence; H3, H3 Hong Kong influenza hemagglutinin; N, nucleocapsid protein; OC43, seasonal coronavirus OC43 spike protein; RBD, receptor-binding domain; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Conversely, a total of 584 measurements from 195 SARS-CoV-2-negative, prepandemic samples were used to assess clinical specificity, or the ability to correctly identify those individuals without prior SARS-CoV-2 infection (true negative rate), as determined by the number of measurements below the cut point for SARS-CoV-2 S, RBD and N antigens. For all three antigens, clinical specificity was 99.0% (Table 2).

Concordance with FDA-authorized Elecsys[®] assay and a SARS-CoV-2 neutralisation assay

To evaluate the performance of the multiplex SARS-CoV-2 ECL serology assay with an FDA-authorized sero-diagnostic assay, we compared the serostatus results to the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay, a diagnostic assay for



Multiplex SARS-CoV-2 ECL serology assay result	S		RBD		N	
	<Cut point n (%)	>Cut point n (%)	<Cut point n (%)	>Cut point n (%)	<Cut point n (%)	>Cut point n (%)
Pre-pandemic (n = 584) [†]	578 (99.0)	6 (1.0)	577 (99.0)	6 (1.0)	577 (99.0)	6 (1.0)
SARS-CoV-2 PCR-positive (n = 258)	0 (0.0)	258 (100)	3 (1)	255 (99)	39 (15)	219 (85)

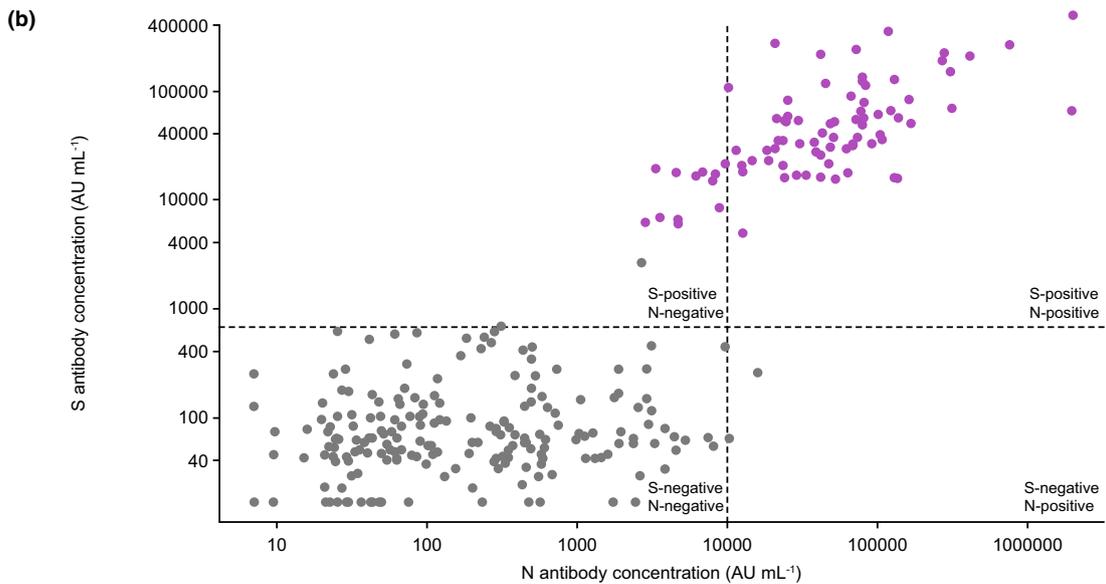


Figure 3. SARS-CoV-2 S, RBD and N antibody distribution by serostatus cut points (dashed lines) in samples from donors according to known SARS-CoV-2 status (a) and number of PCR-positive samples that were seronegative versus seropositive for both S and N (b) [†]One measurement failed run validity criteria. In a, GMT values for each group are shown as solid-coloured lines. In b, RBD versus N distribution is the same as S versus N and is not shown. AU, arbitrary units; ECL, electrochemiluminescence; GMT, geometric mean titre; N, nucleocapsid protein; PCR, polymerase chain reaction; RBD, receptor-binding domain; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Table 2. Multiplex SARS-CoV-2 ECL serology assay clinical sensitivity and specificity

Sensitivity analyses from SARS-CoV-2-PCR-positive individuals ^a				
SARS-CoV-2 antigen	Serostatus cut point, AU mL ⁻¹	Measurements, <i>n</i>	Samples ≥ cut point, <i>n</i>	Sensitivity, % (95% CI)
S	675	258	258	100 (98.6, 100)
RBD	2396	258	255	98.8 (96.6, 99.8)
N	9787	258	219	84.9 (79.9, 89.0)
Specificity analyses from SARS-CoV-2-negative individuals ^b				
SARS-CoV-2 antigen	Serostatus cut point, AU mL ⁻¹	Measurements, <i>n</i>	Samples < cut point, <i>n</i>	Specificity, % (95% CI)
S	675	584	578	99.0 (97.8, 99.6)
RBD	2396	583	577	99.0 (97.8, 99.6)
N	9787	583	577	99.0 (97.8, 99.6)

AU, arbitrary units; CI, confidence interval; ECL, electrochemiluminescence; N, nucleocapsid protein; PCR, polymerase chain reaction; RBD, receptor-binding domain; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

^aSamples for the sensitivity analyses were collected ≥ 14 days after obtaining a positive PCR test result.

^bSamples for the specificity analyses were obtained prepandemic.

detecting antibodies to the SARS-CoV-2 N antigen. The 99th percentile serostatus cut points for each of the SARS-CoV-2 antigens were additionally applied to the antibody concentration of 150 samples with known Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay results (Figure 4a). Distribution of S versus N antibody concentrations as measured by the multiplex SARS-CoV-2 ECL serology assay is shown in Figure 4b.

A separate supplementary sample set (*n* = 150), different from the sample set used to determine the serostatus cut points, and with unknown SARS-CoV-2 PCR status, was also tested in both assays. For the 57 samples that were detected as seropositive with the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay, ≥ 87.7% of them were above all antigen cut points (i.e. positive) in the multiplex SARS-CoV-2 ECL serology assay, with 100% agreement for the S antigen (Figure 4c). For the remaining 93 samples that were classified as seronegative by the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay, ≥ 92% were below all antigen cut points (i.e. negative) in the multiplex SARS-CoV-2 ECL serology assay, with 100% agreement for the N antigen (Figure 4c). For S, RBD and N assays, kappa scores were determined to be 0.90, 0.85 and 0.90, respectively, demonstrating a strong level of agreement between the multiplex SARS-CoV-2 ECL serology assay and the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay (Figure 4c).

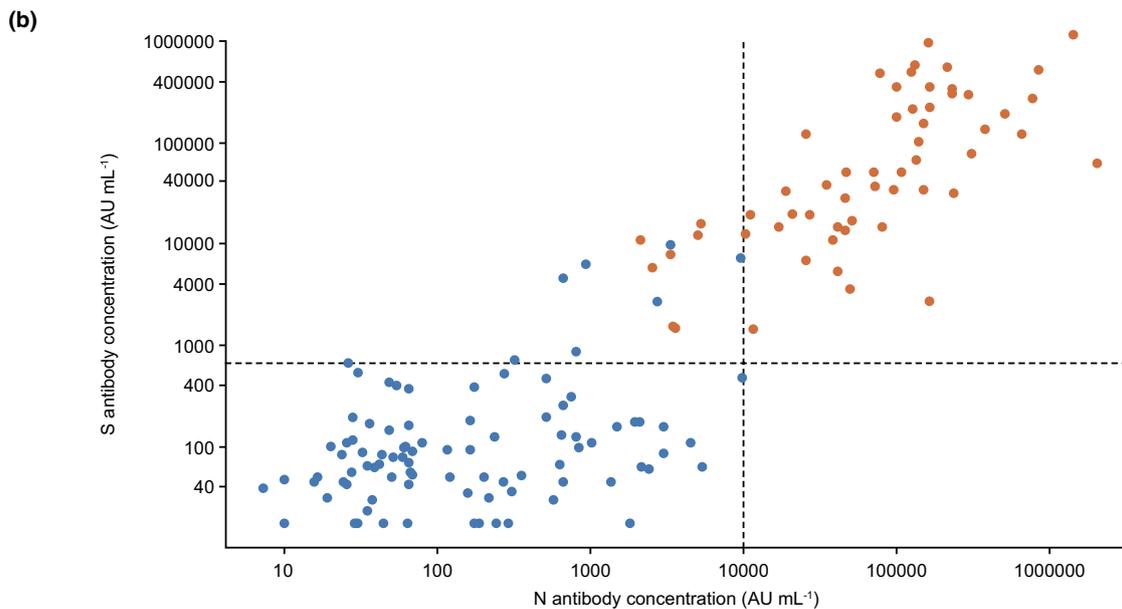
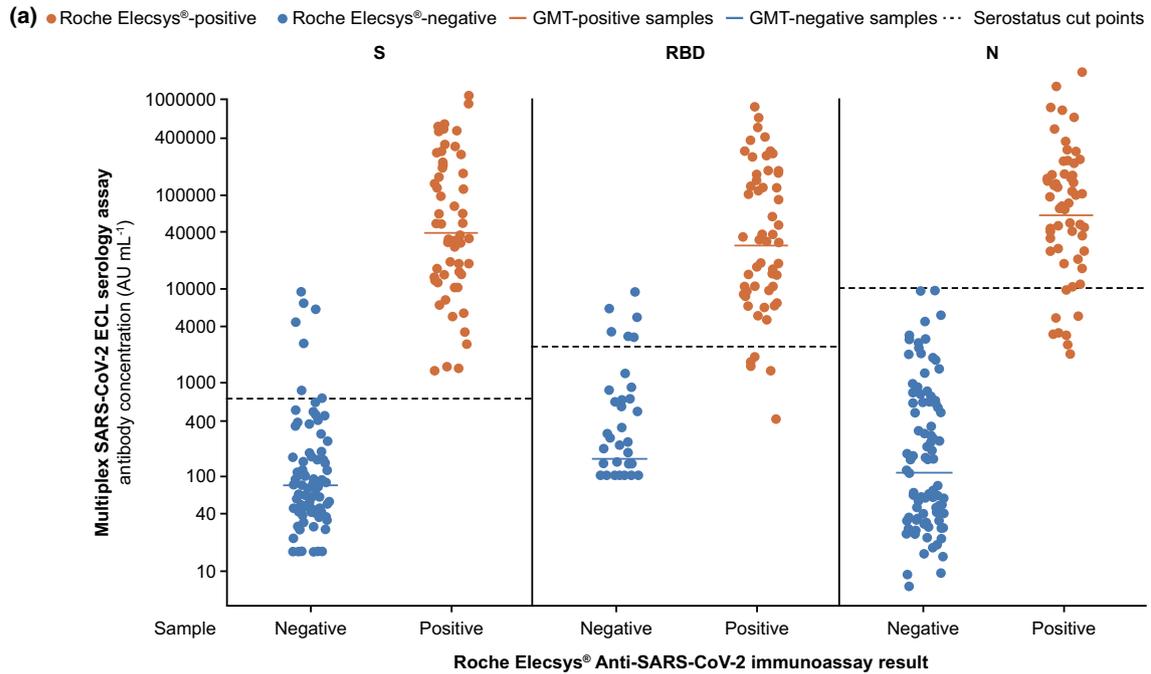
The quantitative values generated in the multiplex SARS-CoV-2 ECL serology assay were

compared with neutralisation antibody titres determined in the BBRC SARS-CoV-2 live-virus MN assay, a cell-based assay that measures the ability of antibodies to neutralise replication-competent SARS-CoV-2. Comparison of the antibody titres and concentrations of 57 samples demonstrated concordance (Pearson $r = 0.85$, $R^2 = 0.72$, $*P < 0.0001$) between the multiplex SARS-CoV-2 ECL serology assay and the BBRC SARS-CoV-2 live-virus MN assay. These 57 samples were a subset of 150 samples tested in the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay as previously discussed. All 24 samples that were detected as seropositive in the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay were above the S antigen cut point for the multiplex SARS-CoV-2 ECL serology assay, and 23 of 24 samples were positive in the BBRC SARS-CoV-2 live-virus MN assay (Figure 5). Of the 33 samples that were detected as seronegative in the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay, six were above the S antigen cut point in the multiplex SARS-CoV-2 ECL serology assay, and five of these showed neutralisation activity in the BBRC SARS-CoV-2 live-virus MN assay (Figure 5).

Assay calibration to the WHO international and MSD reference standards

WHO international standard NIBSC 20/136

A series of experiments were performed to calibrate the AstraZeneca (AZ) reference standard used in the validation experiments performed at



(c)

Roche Elecsys® assay result	S		RBD		N	
	<Cut point n (%)	>Cut point n (%)	<Cut point n (%)	>Cut point n (%)	<Cut point n (%)	>Cut point n (%)
Negative (n = 93)	86 (93)	7 (8)	87 (94)	6 (7)	93 (100)	0
Positive (n = 57)	0	57 (100)	5 (9)	52 (91)	7 (12)	50 (88)
Kappa	0.90		0.85		0.90	

Figure 4. SARS-CoV-2 S, RBD and N antibody distribution by serostatus cut points (dashed lines) in samples from donors with known Roche Elecsys® Anti-SARS-CoV-2 immunoassay results **(a)** and distribution of S versus N antibody concentrations as measured by the multiplex SARS-CoV-2 ECL serology assay **(b)**. In **a**, GMT values for each group are shown as solid-coloured lines. In **b**, RBD versus N distribution is the same as S versus N and is not shown. AU, arbitrary units; ECL, electrochemiluminescence; GMT, geometric mean titre; N, nucleocapsid protein; RBD, receptor-binding domain; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

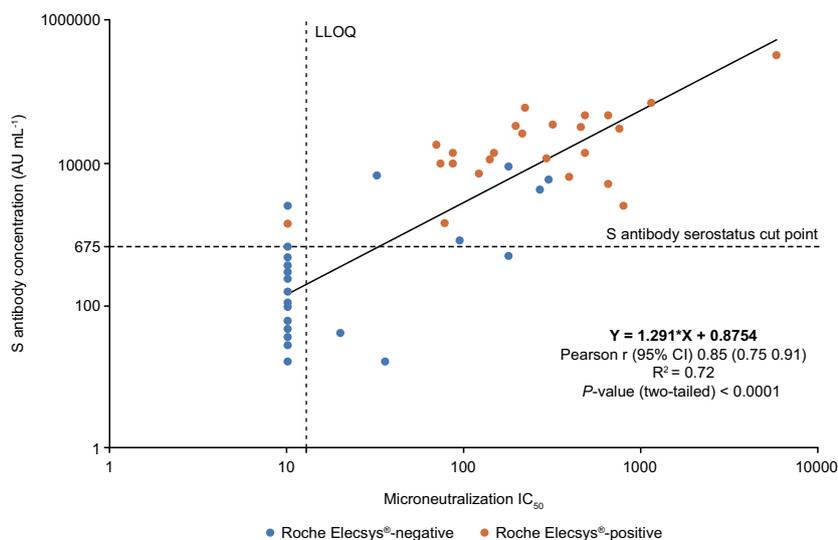


Figure 5. Correlation between the multiplex SARS-CoV-2 ECL serology assay (S assay) and the BBRC SARS-CoV-2 live-virus MN assay. A total of 57 samples were tested in three assays. Horizontal dotted line indicates the multiplex SARS-CoV-2 ECL serology S assay cut point (675 AU mL⁻¹). Vertical dotted line indicates BBRC MN assay LLOQ (IC₅₀ = 20). Colour shows Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay results. AU, arbitrary units; BBRC, Battelle Biomedical Research Center; CI, confidence interval; ECL, electrochemiluminescence; IC₅₀, half maximal inhibitory concentration; LLOQ, lower limit of quantitation; MN, microneutralisation; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

PPD[®] Laboratories (Richmond, VA, USA) to the WHO International Standard 20/136 (National Institute for Biological Standards and Controls [NIBSC], Potters Bar, Hertfordshire, UK).¹⁶ It was determined that a serum sample with an IgG concentration in AU mL⁻¹ calculated from the AZ reference standard in the multiplex SARS-CoV-2 ECL serology assay can be converted to WHO binding antibody units (BAU) mL⁻¹ by multiplying the concentration by the appropriate conversion factor. The WHO International Standard conversion factors established were 0.00645, 0.00798 and 0.00324 for the SARS-CoV-2 S, RBD and N antigens, respectively (Table 3).

MSD reference standard 1

A series of experiments were performed to calibrate the AZ reference standard to the now commercially available and widely distributed MSD Reference Standard 1 (see Methods for further details), which was not available when the validation experiments described in this manuscript were performed. The conversion factors for the AZ reference standard (AU mL⁻¹) to the MSD Reference Standard 1 (MSD AU mL⁻¹) were calculated to be 0.82236, 0.30834 and 2.03375 for the SARS-CoV-2 S, RBD and N antigens, respectively.

Table 3. Conversion factors calibrating the multiplex SARS-CoV-2 ECL serology assay arbitrary units (AU mL⁻¹) to the WHO International Reference Standard units (BAU mL⁻¹)

SARS-CoV-2 antigen	WHO International Standard	
	Conversion factor (AU mL ⁻¹ to BAU mL ⁻¹)	95% CI
S	0.00645	0.00594–0.00701
RBD	0.00798	0.00735–0.00866
N	0.00324	0.00295–0.00356

AU, arbitrary units; BAU, binding arbitrary units; CI, confidence interval; ECL, electrochemiluminescence; N, nucleocapsid protein; RBD, receptor-binding domain; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WHO, World Health Organization.

DISCUSSION

In this study, we describe the validation of a quantitative, multiplexed, high-throughput, sensitive, specific, SARS-CoV-2 IgG serology assay for fast and accurate detection of SARS-CoV-2 antibodies in human sera. This assay has been used to measure antibodies to SARS-CoV-2 S, RBD and N antigens simultaneously, in support of multiple phase three clinical trials of a SARS-CoV-2 vaccine^{17,18} (with > 50 000 clinical trial biospecimens tested to date). This assay may additionally enable large-scale sero-epidemiology

studies to determine the prevalence or incidence of SARS-CoV-2 infections and support immunisation programs around the world.

Although serology assays differ from typical immunoassays, the standard validation parameters (sensitivity, specificity, precision, linearity and accuracy) of the multiplex SARS-CoV-2 ECL serology assay were characterised, and all met the acceptance criteria in the validation plan. Notably, the assay demonstrated a broad quantifiable range, which allows most samples to be tested at a single dilution, significantly increasing the throughput of testing clinical samples. The assay also demonstrated acceptable ruggedness, precision, relative accuracy, dilutional linearity (< 2-fold per 10-fold increase in dilution), and clinical sensitivity and specificity, as well as analytical specificity. With respect to the latter, all evaluable samples showed > 90% reduction in antibody levels when spiked with homologous antigens, and < 15% reduction in antibody levels when spiked with heterologous antigens, including heterologous seasonal coronavirus OC43 spike and H3 Hong Kong influenza hemagglutinin antigens. Expected cross-reactivity of serum antibodies between SARS-CoV-2 RBD and S antigens was demonstrated, given that the S contains the RBD as one of its two protein subunits.⁵

Classification of samples as seronegative or seropositive based on the serostatus cut points for the multiplex SARS-CoV-2 ECL serology assay demonstrated good agreement ($\geq 85\%$) with the previously established SARS-CoV-2 status of the samples based on a documented SARS-CoV-2 infection by PCR. We note that our assay serostatus cut points were developed utilising pre-pandemic samples obtained from male donors. As gender has been associated with modest impacts on immunogenicity from vaccination and viral pathogenesis, we acknowledge this as a potential limitation of our analysis.^{19,20} However, our cut points were tested using confirmed SARS-CoV-2 PCR-positive samples obtained from male and female donors. Furthermore, the assay demonstrated concordance with the FDA-authorized Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay, which is used as a diagnostic assay for detecting antibodies to the SARS-CoV-2 N antigen.¹¹ The multiplex SARS-CoV-2 ECL serology assay also demonstrated concordance with the BBRC SARS-CoV-2 live-virus MN assay. Live-virus neutralisation assays are considered the 'gold standard' for measuring neutralising antibody

levels, but are laborious, in the case of SARS-CoV-2 require biosafety level three facilities,^{21,22} and are subject to assay variation because of different virus lots, cell lots and other key reagents.²³

The multiplex SARS-CoV-2 ECL serology assay was also calibrated to the WHO International Standard (NIBSC 20/136) and the commercially available MSD Reference Standard 1. Calibration to both standards will assist evaluation of vaccine immunogenicity studies and aid comparison of data collected from multiple vaccine manufacturers as part of epidemiological and immunological surveillance studies.^{8,22}

There is broad applicability of a reproducible SARS-CoV-2 serological assay to support COVID-19 vaccine and mAb clinical studies. In particular, measuring antibody levels over time will help determine whether there is a minimum protective level of antibodies associated with protection and help determine the duration of protection afforded by vaccination or administration of mAbs.^{22,24} Furthermore, a multiplex assay that can identify epitope-specific responses following vaccination or infection could provide insight into correlates of protection,²⁵ and high-quality antibody tests can help broaden the understanding of the humoral antibody response.²⁶ SARS-CoV-2 infections are diagnosed predominantly with molecular testing of respiratory samples, which allows early detection of infection but cannot be used to determine the overall exposure in a community to support epidemiology studies or gauge the level of herd immunity.²⁷ Rapid molecular tests are costly with low throughput, while high-throughput tests have longer turnaround times owing to the time requirements of sample extraction.^{28,29} To mitigate these limitations, antibody tests could be incorporated into the diagnostic arsenal for COVID-19.^{22,27} Easy to perform, high-throughput serology assays could also improve understanding of the impact of the SARS-CoV-2 virus through large sero-epidemiologic studies, which is particularly helpful given that as many as 45% of SARS-CoV-2 infections may be asymptomatic³⁰ and those with mild COVID-19 symptoms are mostly undetected³¹ and may prove even more valuable when correlates of protection of vaccines are established and recognised.³²

Having the capability to quantify antibody levels to SARS-CoV-2 would inform the evolving scientific understanding of viral prevalence, transmission dynamics, disease burden and rates of symptomatic and asymptomatic infection, all of

which impact the public health response to this virus.²² Because the multiplex SARS-CoV-2 ECL serology assay measures antibodies to both S and N, it has the ability to distinguish the previously infected/convalescent individuals with antibodies to S, RBD and N antigens from vaccinated individuals who would only have S and RBD antibodies, but not N antibodies. Our investigation complements similar findings by Hicks *et al.*,³³ who developed an enzyme-linked immunosorbent assay (ELISA)-based assay to determine the seroprevalence of SARS-CoV-2 in the Australian population. In order to compensate for poor S1 antibody sensitivity and specificity, Hicks *et al.* employed an alternative methodology by which the mean concentrations of N and RBD antibodies were combined to increase overall assay sensitivity. However, we believe our approach, of keeping the assay cut points separate, is more suitable for characterising the vaccine-induced immune response, for which antigen specificity between N, S and RBD is the most important characteristic. We show N and S antibody levels for each donor within our analysis.

A major limitation of traditional serological assay techniques, such as ELISA, is their narrow dynamic range and low throughput, which do not allow for unequivocal and unambiguous interpretation of results.³⁴ This would be a particular issue for SARS-CoV-2 detection because of potential for significant cross-reactivity to other seasonal coronavirus infections.^{22,35} Specificity can be augmented by evaluating humoral immunity to more than one viral-associated antigen at a time by using multiplexing.³⁶ In contrast to an ELISA, the multiplex SARS-CoV-2 ECL serology assay described herein can measure several SARS-CoV-2 antigen-specific antibodies simultaneously, without requirements for additional sample volume, with added advantages of a large dynamic range, sensitivity and ruggedness of performance.

Validated antibody assays that are sensitive, precise, accurate and reproducible under a variety of test conditions enable robust testing of serum samples from vaccine and therapeutic antibody clinical trials. Validated assays also provide data that offer insights into the kinetics of antibody responses to SARS-CoV-2 following infection or vaccination and the potential duration of protection. Antibody levels from infections in convalescent individuals or breakthrough cases in immunised individuals in clinical trials can help determine a correlate of protection. All the assay parameters met the acceptance criteria, and the assay

reference standard (AZ reference standard) has been calibrated to the WHO International and MSD reference standards. The multiplex SARS-CoV-2 ECL serology assay is considered validated and fit for its intended purpose of measuring SARS-CoV-2-specific IgG antibodies in registrational clinical trials and sero-epidemiology studies.

METHODS

Multiplex SARS-CoV-2 ECL serology assay components

All experiments utilised Meso Scale Discovery (Rockville, MD, USA; MSD) custom SARS-CoV-2 MULTI-SPOT[®] plates (Lots Z0056737 and Z0056738), which were directly coated with antigen proteins, including S, RBD and N of SARS-CoV-2. Additional components of the assay included MSD[®] Diluent 100 (Catalogue # R50AA), MSD[®] Blocker A Solution (Catalogue # R93BA), MSD[®] GOLD[™] Read Buffer B (Catalogue # R60AM), MSD[®] SULFO-TAG[™]-labelled IgG detection antibody (mouse anti-human IgG; Lot # D00V0003) and MSD[®] Wash Buffer (20X; Catalogue # R61AA). All components listed above are now part of the MSD V-PLEX[®] SARS-CoV-2 Panel 2 (IgG) Kit (Catalog # K15383U). At the time of validation, the kit was not available, and MSD provided custom components for the work. Prior to the work described here, assay components including antigens, reference standard, plates, diluents and detection antibody were characterised, and assay optimisation was performed by MSD. Additionally, because the MSD Reference Standard 1 was not available, the AZ reference standard (not provided by MSD) was created (see below).

Serum samples for reference serum, controls and validation panel

The AstraZeneca reference standard was made by pooling sera from 10 convalescent donors containing antibodies specific for the SARS-CoV-2 S, RBD and N antigens. Four different quality control samples (QCS) were made by pooling sera from 4 or 5 convalescent donors and selected to be at approximately 20%, 50% and 80% of the maximum assay signal. Additionally, a set of individual convalescent serum samples was used during validation. Sera used for specificity analyses pre-dated November 2019 and were deemed prepandemic. These prepandemic serum samples ($n = 195$) were derived from anonymised male donors aged 18–80 years (with a median age of 40.5 years) and were prescreened to have low or negative SARS-CoV-2 antibody concentrations as measured in the multiplex ECL serology assay. All serum samples were obtained from a commercial vendor (BioIVT, Westbury, NY, USA).

Serum samples for cut point analysis

Serum samples confirmed as SARS-CoV-2 PCR-positive were obtained from a commercial vendor (BioIVT). These confirmed SARS-CoV-2 PCR-positive serum samples ($n = 86$)

were obtained from 46 female and 40 male donors aged 18–82 years (with a median age of 40.5 years). Serum samples were collected 14–30 days after a SARS-CoV-2-positive PCR result for 29 donors and 30–60 days after a SARS-CoV-2-positive PCR result for 57 donors. SARS-CoV-2 prepandemic samples were obtained from a commercial vendor (BioIVT) and were collected prior to November 2019.

Assay protocol

A MULTI-SPOT® 96-well plate was coated with SARS-CoV-2 S, RBD and N antigens (MSD® SARS-CoV-2 Plate 2). To measure IgG antibodies to SARS-CoV-2 antigens, plates were blocked with MSD Blocker A for 1 h and washed prior to the addition of reference standard, controls and samples. After incubation for 2 h, the plates were washed and detection antibody was added (MSD® SULFO-TAG™ Anti-Human IgG Antibody). Plates were incubated for 1 h and washed three times. MSD GOLD™ Read Buffer B was added, and the plates were read using a MESO® SECTOR S 600 Reader. The AstraZeneca reference standard and four QCS, as previously described, were included on each plate in routine operation of the assay, and the plate was repeated if two or more QCS exceeded their respective 2σ limits.

Assay validation and characterisation

Assay validation and characterisation were conducted by PPD® Laboratories (Richmond, VA, USA). A series of studies, as described below, were performed to determine the precision, linearity, ruggedness, sensitivity and specificity of the multiplex SARS-CoV-2 ECL serology assay in detecting three SARS-CoV-2 antigen-specific antibodies (S, RBD and N) in serum samples.

Standard curve characterisation

Standard curve modelling was performed using an 11-point and 2.5-fold dilution series of the reference standard on each assay plate. The associated standard curve starting concentration was arbitrarily assigned to 100 000 AU mL⁻¹ for each antigen, and a 4-parameter logistic function was used to model the 2.5-fold standard dilution series for each plate. ECL signals were quantified in relative light units (RLU). Based on RLU values and variability estimates for each standard curve point, RLU values < 200 were not included in the fit for the standard curve because of high variability. The standard curve criteria permitted dropping ≤ 2 points with RLU values < 200 for an appropriate and adequate fit to the model.

Assay precision, dilutional linearity and ruggedness

Assay precision, dilutional linearity and ruggedness were assessed by using 21 samples prescreened to have antibody concentrations that span the quantifiable range analysed across 15 runs by five analysts utilising two plate lots at three dilutions (1:500, 1:5000 and 1:50 000). Variability estimates were obtained using variance component analysis and expressed as the per cent geometric coefficient of

variation (% GCV) calculated as $\% \text{ GCV} = 100\% \times [e^{\sqrt{\hat{\sigma}^2}} - 1]$ for assay precision where $\hat{\sigma}^2$ represents the variance of the natural log- (\ln) transformed antibody concentrations. Intra-assay precision was evaluated using four quality controls on each plate. For dilutional linearity, dilution bias per 10-fold dilution was estimated across all dilutions tested, and the assay was considered to be dilutable if the dilution bias per 10-fold dilution was < 2-fold difference. Assay ruggedness was assessed by comparing fold differences in antibody concentrations between analysts and plate lots and considered to be acceptable if within ± 1.3 -fold for each antigen tested. Meaningful fold-rise was calculated as $e^{3 \times \sqrt{2 \times \hat{\sigma}^2}}$, where $\hat{\sigma}^2$ represents the variance of the natural log- (\ln) transformed antibody concentrations.

Analytical sensitivity and quantifiable range

The LOD was determined using mock samples prepared with increasing antibody concentrations. Differences in RLU values between the mock samples and the blank samples containing no added antibody were compared. The differences were determined separately within each plate, run in combination and then averaged across plates/runs for each spike level. The standard deviations of the differences at each antibody level were calculated. The LOD was set at the antibody level that provided a statistically significant increase in RLU above that in the blank sample, where significance was based on a *t*-distribution at the 5% significance level and determined using the mean and standard deviation of the individual differences.

The LLOQ and ULOQ for a single dilution for each of the three SARS-CoV-2 antigen-specific antibodies were determined by evaluating the precision profiles of the antibody concentrations and the relative accuracy of the assay. The dynamic range of the assay was evaluated by testing spiked serum samples at three different dilutions for each antigen, S, RBD and N.

Analytical specificity

Analytical specificity of the assay was evaluated using SARS-CoV-2 S, RBD and N antigens for homologous competition, and seasonal coronavirus OC43 S and H3 Hong Kong influenza hemagglutinin antigens for heterologous competition. Competition experiments were performed by spiking homologous or heterologous antigens into eight human serum samples with mid-to-high range antibody concentrations. Assay specificity was determined by comparing antibody concentration of serum spiked with antigens to the antibody concentration of the serum samples that were not spiked with antigens.

During development of the assay, MSD performed competition experiments with additional heterologous antigens, including SARS CoV-1, Middle East respiratory syndrome coronavirus (MERS-CoV) and human coronavirus HKU1 (hCoV-HKU1) spike proteins. Although this analysis was not completed using the final method, observed cross-reactivity to these heterologous antigens was comparable to OC43 S (signals within two-fold of 'unspiked' serum), confirming the assays are specific (data not shown).

Serostatus cut points

The antigen cut point values were established by running 585 measurements from 195 pre-pandemic serum samples run in triplicate in the assay. Cut points for each antigen were determined using the 99th percentile on the natural log-transformed sample antibody concentrations.

Clinical sensitivity and specificity

The 99th percentile cut point based on the SARS-CoV-2 known negative pre-pandemic samples was used to calculate clinical sensitivity and specificity.

Concordance with FDA-authorized Elecsys[®] assay and a SARS-CoV-2 neutralisation assay

Serostatus cut points established for the multiplex SARS-CoV-2 ECL serology assay were applied to a subset of 150 samples with known results using the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay (i.e. a diagnostic assay for detecting antibodies to the SARS-CoV-2 N antigen). This subset of samples was also assessed for neutralisation activity using the BBRC SARS-CoV-2 live-virus MN assay. The Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay is an ECL immunoassay that uses a recombinant protein representing nucleocapsid (N) antigen for determination of antibodies against SARS-CoV-2. The Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay was performed by PPD[®] Laboratories (Highland Heights, KY, USA). The BBRC SARS-CoV-2 live-virus MN assay was developed, validated and performed at BBRC (Columbus, OH, USA). The BBRC assay is a cell-based, wild-type live-virus MN assay with an *in situ* ELISA readout that detects SARS-CoV-2 viral protein in a fixed VERO E6 monolayer. The reciprocal of the highest dilution of test sample with an optical density less than the neutralising plate cut off (50% of the viral signal) is reported as the endpoint titre. The half-maximal inhibitory concentration (IC₅₀) is determined by logistic regression analysis. Assay concordance was determined by assessing percentage agreement between the positive and negative samples between the multiplex SARS-CoV-2 ECL serology assay and the Roche Elecsys[®] Anti-SARS-CoV-2 immunoassay and also by calculating Pearson correlation between the multiplex SARS-CoV-2 ECL serology S assay (AU mL⁻¹) and BBRC MN assay (IC₅₀). Assay results were log-transformed prior to calculating Pearson correlation.

Assay calibration to the WHO international and MSD reference standards

WHO international reference standard (NIBSC 20/136)

The First WHO International Standard of anti-SARS-CoV-2 IgG NIBSC 20/136 Version 2.0 was used in the study.¹⁶ It consists of pooled plasma samples obtained from individuals recovered from SARS-CoV-2 infection and was evaluated in a WHO international collaborative study.³⁷ The

intended use of the WHO International Standard is for the calibration and harmonisation of serological assays detecting anti-SARS-CoV-2 neutralising antibodies. Per product instructions, the assigned potency of the WHO International Standard for SARS-CoV-2 (NIBSC 20/136) for binding antibody assays is an arbitrary unitage of 1000 BAU mL⁻¹ and can be used to assist in the comparison of assays detecting the same class of IgGs with the same specificity (e.g. anti-RBD IgG or anti-N IgG). To anchor the multiplex SARS-CoV-2 ECL serology assay unitage to the WHO International Standard NIBSC 20/136, a series of calibration experiments were performed to interpolate the WHO International Standard RLU from the AZ reference standard for the SARS-CoV-2 S, RBD and N antigens to derive conversion factors for AU mL⁻¹ to BAU mL⁻¹.

MSD reference standard 1

A series of calibration experiments were also performed to interpolate the MSD Reference Standard 1 (component of the V-PLEX[®] SARS-CoV-2 Panel 2 Kit, Catalog Number K15383U, Lot A00V0004) RLU to the AZ reference standard used in the development and validation of the multiplex SARS-CoV-2 ECL serology assay and to derive conversion factors for AZ AU mL⁻¹ to MSD AU mL⁻¹.

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CONFLICT OF INTEREST

DW, AAA, AR, KMT, MTE and EJK are employees of AstraZeneca and may hold stock or stock options. TG, RG, BF and CJB are employees of PPD[®] Laboratories and may hold stock or stock options.

AUTHOR CONTRIBUTIONS

Deidre Wilkins: Conceptualization; Formal analysis; Methodology; Supervision; Validation; Writing – original draft; Writing – review & editing. **Anastasia A Aksyuk:** Conceptualization; Formal analysis; Methodology; Validation; Writing – original draft; Writing – review & editing. **Alexey**

Ruzin: Conceptualization; Formal analysis; Writing – original draft; Writing – review & editing. **Kevin M Tuffy:** Formal analysis; Writing – review & editing. **Tina Green:** Formal analysis; Validation; Writing – review & editing. **Rebecca Greway:** Validation; Writing – review & editing. **Brittany Fikes:** Data curation; Methodology; Validation; Writing – review & editing. **Cyrille J Bonhomme:** Data curation; Methodology; Validation; Writing – review & editing. **Mark T Esser:** Conceptualization; Supervision; Writing – review & editing. **Elizabeth J Kelly:** Conceptualization; Supervision; Validation; Writing – review & editing.

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