Supplementary material. Table of content

Supplementary Table S1. Outcome measurements according to study group and time point (day 0, 21, 28, and 49). Analysis of variance between different participant groups was performed using the Kruskall-Wallis test by ranks.

	naive staff (N=40)	naive resident (N=53)	infected staff (N=66)	infected resident (N=25)	Total (N=184)	p value
Binding RBD-IgG (BAU/mL)						
Day 0						<0.001
GMT	1.3	1.2	47.6	62.8	8.0	
95% CI	1.1, 1.7	1.0, 1.4	33.8, 66.9	28.5, 138.1	5.8, 11.0	
Day 21						<0.001
GMT	207.6	8.8	3938.7	3014.2	340.8	
95% CI	156.9, 274.7	5.6, 13.9	3072.3, 5049.5	1601.2, 5674.3	223.6, 519.4	
Day 28						<0.001
GMT	1804.7	72.3	5633.0	4614.0	1220.7	
95% CI	1403.0, 2321.5	39.3, 132.9	4959.0, 6398.7	2770.0, 7685.3	874.5, 1704.0	
Day 49						<0.001
GMT	1357.8	360.1	6476.3	6695.4	2021.1	
95% CI	1084.0, 1700.9	251.4, 515.9	5519.6, 7598.8	4350.2, 10304.8	1608.1, 2540.1	
Binding S1-IgG (BAU/mL)						
Day 0						<0.001
GMT	1.5	1.4	51.5	62.6	8.8	
95% CI	1.2, 1.9	1.1, 1.8	36.4, 72.9	30.8, 127.2	6.4, 12.0	
Day 21						<0.001
GMT	228.9	9.3	5156.2	3775.2	401.0	
95% CI	177.6, 295.0	6.0, 14.4	3916.0, 6789.1	1879.0, 7585.0	259.6, 619.4	
Day 28						<0.001
GMT	1596.1	65.6	7561.0	5995.5	1331.3	
95% CI	1265.7, 2012.7	35.8, 120.4	6389.2, 8947.5	3066.3, 11723.2	931.1, 1903.5	
Day 49						<0.001
GMT	1591.0	398.6	7627.4	9035.4	2378.1	
95% CI	1276.1, 1983.5	286.9, 553.7	6433.8, 9042.6	5927.5, 13772.6	1888.6, 2994.4	

Table continued on the next page

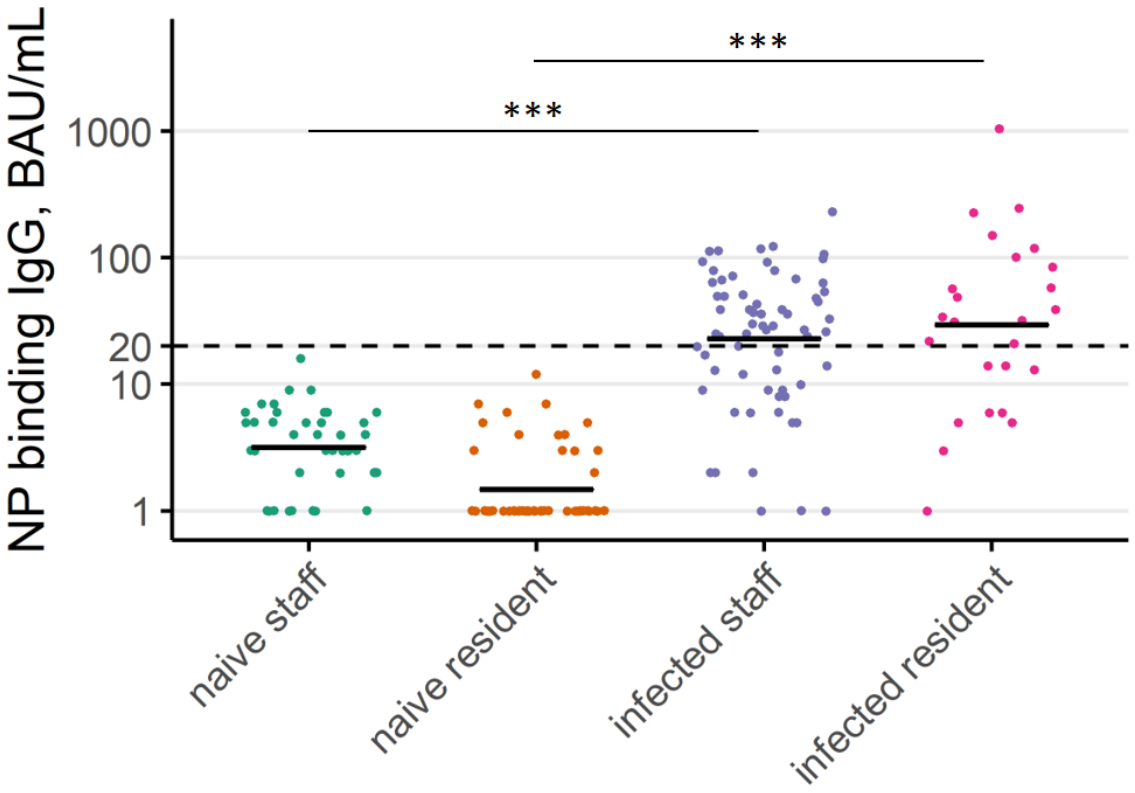
Supplementary Table S1 (continued)

	naive staff (N=40)	naive resident (N=53)	infected staff (N=66)	infected resident (N=25)	Total (N=184)	p value
Binding S2-IgG (BAU/mL)						
Day 0						<0.001
GMT	6.2	4.3	118.8	148.2	25.0	
95% CI	4.1, 9.5	3.1, 5.9	88.8, 158.9	89.9, 244.5	18.6, 33.5	
Day 21						<0.001
GMT	70.5	10.2	1962.7	1250.6	194.2	
95% CI	52.3, 94.9	6.9, 15.0	1485.2, 2593.6	776.2, 2014.9	133.8, 281.9	
Day 28						<0.001
GMT	190.0	22.8	1968.3	1373.0	312.3	
95% CI	140.5, 256.9	15.0, 34.8	1562.9, 2478.9	844.1, 2233.1	226.3, 431.0	
Day 49						<0.001
GMT	112.5	48.4	1259.6	1154.8	288.6	
95% CI	90.9, 139.2	35.7, 65.5	1026.5, 1545.7	774.5, 1722.0	224.4, 371.0	
RBD-IgG avidity K_{off} (1/s)						
Day 0						
Not measured	40	53	23	7	123	
GMT	NA	NA	1.6e-3	1.6e-3	1.6e-3	
95% CI	/	/	1.2e-3, 2.2e-3	9.7e-4, 2.5e-3	1.2e-3, 2.1e-3	
Day 21						<0.001
Not measured	4	43	6	1	54	
GMT	6.2e-4	1.5e-5	4.0e-5	4.2e-5	1.1e-4	
95% CI	5.3e-4, 7.4e-4	7.4e-5, 29e-3	3.1e-5, 5.4e-5	2.2e-5, 8.0e-5	8.5e-5, 1.6e-4	
Day 28						<0.001
Not measured	.	22	4	2	28	
GMT	1.3e-4	7.0e-4	2.5e-5	3.5e-5	7.8e-5	
95% CI	1.1e-4, 1.5e-4	4.7e-4, 1.0e-3	1.9e-5, 3.3e-5	1.6e-5, 7.3e-5	5.9e-5, 1.0e-4	
Day 49						<0.001
Not measured	.	21	3	2	26	
GMT	1.5e-4	3.2e-4	2.2e-5	1.2e-5	5.6e-5	
95% CI	1.2e-4, 1.8e-4	2.5e-4, 4.0e-4	1.4e-5, 3.4e-5	5.0e-6, 2.9e-5	4.1e-5, 7.5e-5	

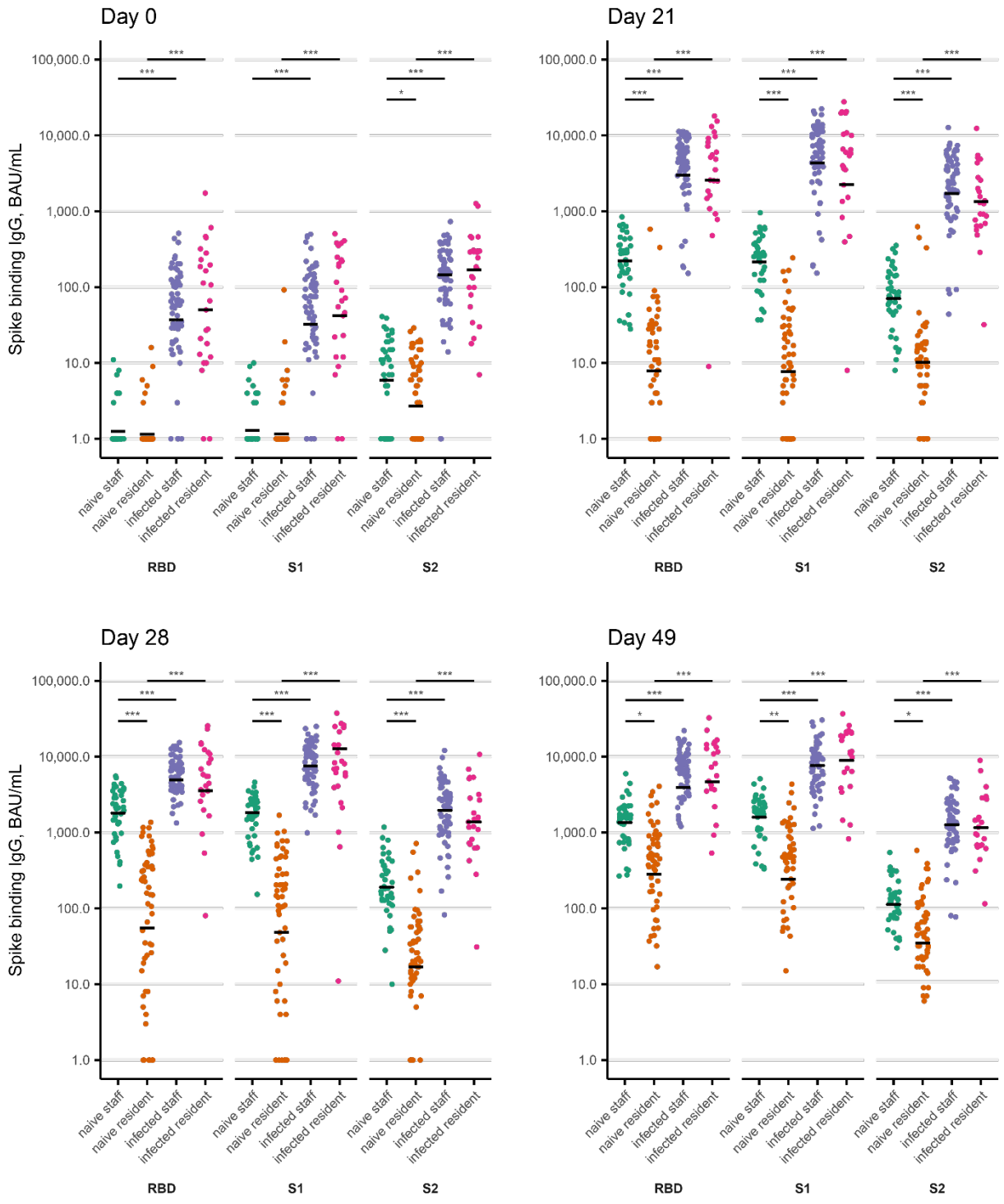
Table continued on the next page

Supplementary Table S1 (continued)

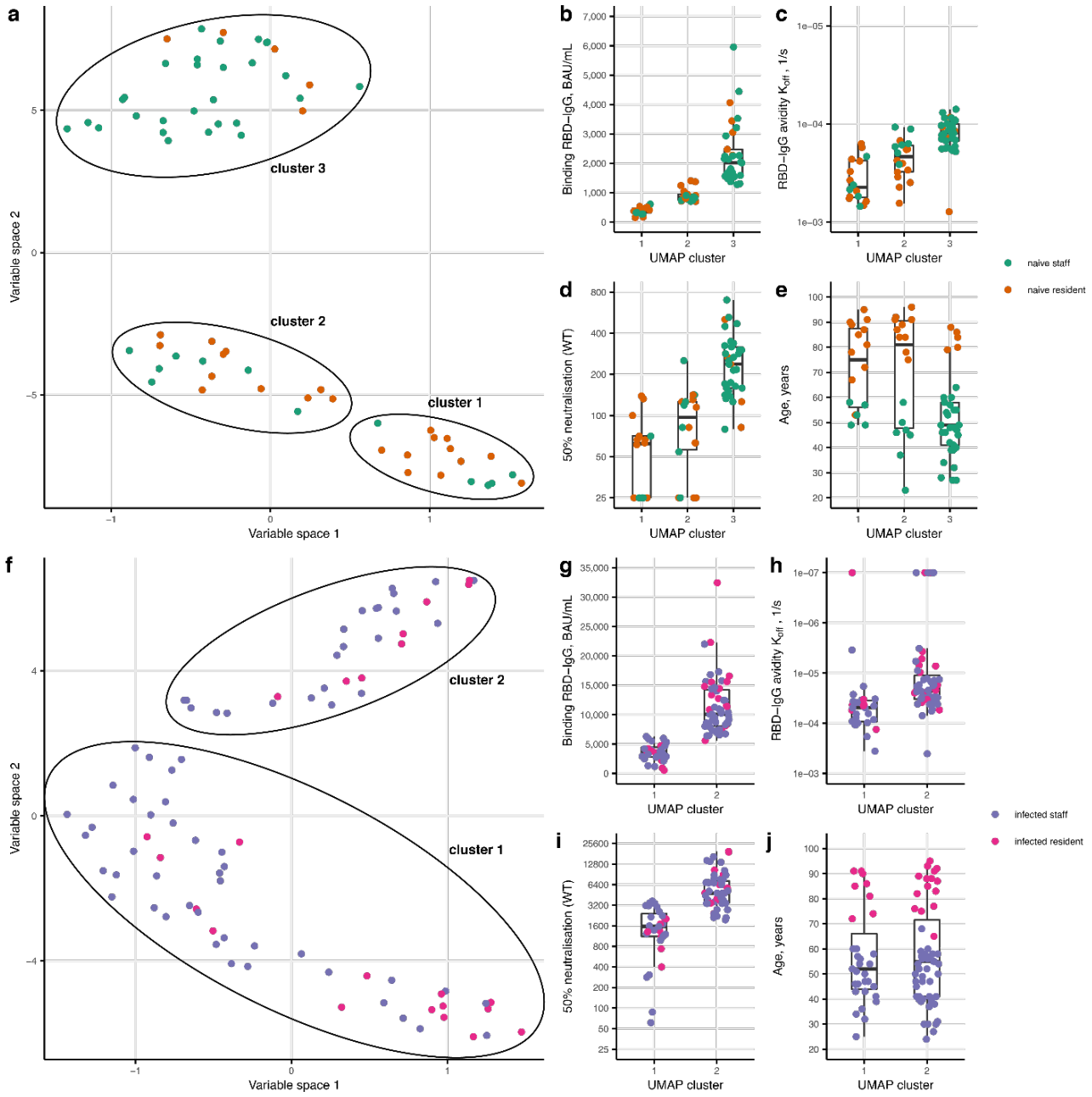
	naive staff (N=40)	naive resident (N=53)	infected staff (N=66)	infected resident (N=25)	Total (N=184)	p value
50% neutralization (wild type)						
Day 0						<0.001
GMT	25.0	25.0	57.1	67.6	38.5	
95% CI	25.0, 25.0	25.0, 25.0	46.4, 70.3	46.3, 98.6	34.5, 42.9	
Day 21						<0.001
GMT	27.7	25.0	2200.4	2409.0	239.9	
95% CI	25.4, 30.2	25.0, 25.0	1521.6, 3182.2	1410.4, 4114.6	167.4, 343.8	
Day 28						<0.001
GMT	252.6	34.3	3677.3	3330.7	527.2	
95% CI	193.8, 329.2	29.0, 40.5	2944.8, 4592.0	1950.4, 5687.9	382.6, 726.5	
Day 49						<0.001
GMT	154.8	48.4	2943.0	2900.6	475.4	
95% CI	117.0, 204.9	38.7, 60.4	2261.3, 3830.1	1817.2, 4629.7	350.6, 644.7	
50% neutralization (B.1.351)						
Day 0						.
Not measured	39	53	21	6	119	
GMT	25.0	NA	26.5	30.8	27.7	
95% CI	/	/	24.8, 28.4	23.4, 40.6	25.3, 30.4	
Day 21						.
Not measured	29	53	5	1	88	
GMT	25.0	NA	531.2	400.8	348.8	
95% CI	25.0, 25.0	/	393.7, 716.8	235.5, 682.0	258.6, 470.4	
Day 28						<0.001
Not measured	.	36	.	1	37	
GMT	37.1	26.0	612.1	523.6	193.1	
95% CI	32.0, 43.0	23.9, 28.3	465.7, 804.6	307.2, 892.3	146.7, 254.2	
Day 49						<0.001
Not measured	.	21	.	1	22	
GMT	27.5	25.0	438.3	478.5	127.3	
95% CI	25.1, 30.2	25.0, 25.0	332.3, 578.2	252.2, 908.1	97.9, 165.6	



Supplementary Figure S1. Levels of SARS-CoV-2 Nucleoprotein (NP) specific IgG before mRNA vaccination. Serum levels of NP-specific IgG were measured on day 0, before Black bars indicate geometric mean titers (GMT). Cut-off concentration is 20 BAU/ml. Statistical comparisons were made between the different study groups using the Kruskall-Wallis test by ranks, and the Mann-Whitney U post-hoc test. Statistical significance is denoted as: * $p<0.05$; ** $p<0.01$; and *** $p<0.001$. 62% (40/65) and 63% (15/24) of infected staff and residents, respectively, had NP-specific IgG titers above the cut-off whereas all naïve participants had NP-specific IgG titers below the cut-off.



Supplementary Figure S2. Multiplex immunoassay results at the different sampling times. Serum levels of spike (RBD, S1, and S2) binding IgG are presented for each sampling day. Black bars indicate geometric mean titers. Cut-off concentrations are 15 BAU/ml, 20 BAU/ml and 20 BAU/ml for anti-RBD IgG, anti-S1 IgG and anti-S2 IgG, respectively. Statistical comparisons were made between the different participant groups using the Kruskall-Wallis test by ranks, and the Mann-Whitney U post-hoc test. Statistical significance is denoted as: * $p<0.05$; ** $p<0.01$; and *** $p<0.001$.



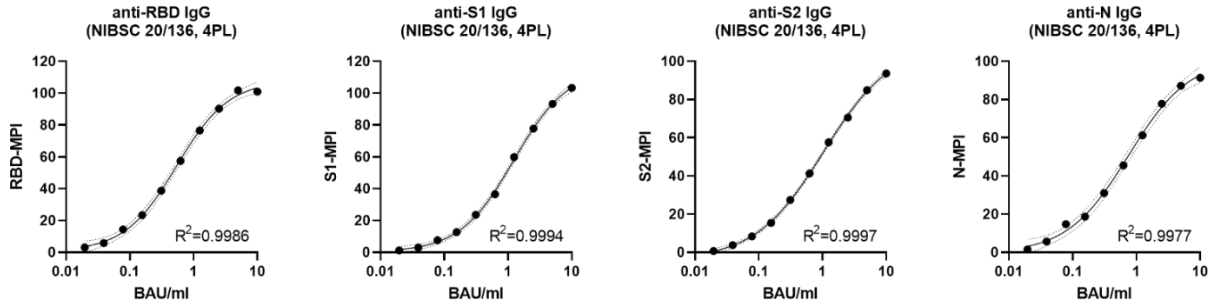
Supplementary Figure S3. Uniform Manifold Approximation and Projection (UMAP) cluster analysis of antibodies in naïve and previously infected subjects. Dimensionality reduction of the following outcomes at day 49: anti-RBD/S1/S2 IgG, anti-RBD IgG avidity, and WT NT50. Stratification occurred for naïve (a-e) and previously infected (f-j) participants. Avidity was log10 and neutralization log2 transformed. The optimal number of clusters was tested via the k-means (range 1:10) and visually identified with an "elbow" in a plot of variance versus number of clusters. DBSCAN identified clusters within the UMAP reduced dimensions.

SUPPLEMENTARY METHODS

A. Assay qualification

1. Calibration curves and accuracy. Verification of the calibration model

Methods: Calibration curves were obtained by serially diluting (duplicates, S1-S10) the WHO International standard NIBSC 20/136 (*https://www.nibsc.org/science_and_research/idd/cfar/covid-19_reagents.aspx*) and measuring net Mean Pixel Intensities (MPI). MPI were plotted against the corresponding units (expressed as Binding Antibody Units (BAU)/ml) and a four-parameter logistic (4PL) standard curve was used for interpolation (GraphPad Prism version 9.0.0 for Windows) (**Fig.S4**). Accuracy was determined by back-calculating the concentrations of the calibration standards using the 4PL-curves, within a single run (within-run accuracy, expressed as recovery (%)), or within 5 runs (between-run accuracy, expressed as mean recovery (%)) (**Tab.S2 and Tab.S3**).



Supplementary Figure S4. Representative sigmoidal 4PL calibration curves used for interpolation.

Sample ID	Nominal conc	Back-calculated results							
		anti-RBD IgG		anti-S1 IgG		anti-S2 IgG		anti-N IgG	
		conc	recovery	conc	recovery	conc	recovery	conc	recovery
	BAU/ml	BAU/ml	%	BAU/ml	%	BAU/ml	%	BAU/ml	%
S1	10.000	8.464	85	9.769	98	9.815	98	9.483	95
S2	5.000	5.525	110	4.959	99	5.103	102	5.511	110
S3	2.500	2.425	97	2.550	102	2.493	100	2.606	104
S4	1.250	1.285	103	1.276	102	1.241	99	1.186	95
S5	0.625	0.621	99	0.611	98	0.627	100	0.625	100
S6	0.313	0.302	97	0.311	100	0.315	101	0.314	101
S7	0.156	0.156	100	0.173	111	0.152	97	0.159	102
S8	0.078	0.083	106	0.088	112	0.084	108	0.083	107
S9	0.039	0.035	88	0.035	89	0.036	91	0.038	98
S10	0.020	0.019	99	0.018	90	0.020	100	0.017	89

Supplementary Table S2. Within-run accuracy.

Sample ID	Nominal conc	Back-calculated results							
		anti-RBD IgG		anti-S1 IgG		anti-S2 IgG		anti-N IgG	
		Mean conc	Mean recovery	Mean conc	Mean recovery	Mean conc	Mean recovery	Mean conc	Mean recovery
	BAU/ml	BAU/ml	%	BAU/ml	%	BAU/ml	%	BAU/ml	%
S1	10.000	7.929	79	9.434	94	10.200	102	9.826	98
S2	5.000	5.296	106	5.226	105	5.064	101	5.013	100
S3	2.500	2.627	105	2.486	99	2.403	96	2.604	104
S4	1.250	1.272	102	1.240	99	1.319	106	1.225	98
S5	0.625	0.615	98	0.621	99	0.620	99	0.643	103
S6	0.313	0.302	97	0.315	101	0.318	102	0.330	106
S7	0.156	0.162	103	0.161	103	0.141	90	0.149	95
S8	0.078	0.083	106	0.082	105	0.084	108	0.075	96
S9	0.039	0.035	90	0.034	87	0.035	89	0.038	98
S10	0.020	0.018	93	0.021	105	0.020	104	0.020	101

Supplementary Table S3. Between-run accuracy (mean of 5 runs).

Conclusion: The MIA-assay is accurate for measuring IgG antibodies directed to RBD, S1, S2 and N. After back-calculation, the % recovery between measured concentrations and their nominal concentrations was within 85-115%, except for anti-RBD IgG at the highest concentration (mean recovery 79%) where a Hook-effect was observed.

2. Precision

Methods: The precision of the MIA-assay was evaluated by repeated measurements of anti-RBD IgG, anti-S1 IgG, anti-S2 IgG and anti-N IgG concentrations (BAU/ml) of 1 negative and 4 positive samples (2 for anti-N IgG). For within-run precision (repeatability), 15 replicates of the same sample were measured within a single run (**Tab. S4**). For between-run precision (intermediate precision), samples were measured in 4 different runs on different days (**Tab. S5**).

anti-RBD IgG	Sample	NEG	POS1	POS2	POS3	POS4
	MEAN (BAU/ml)	0.35	38	726	3361	24471
	STDEV (BAU/ml)	0.04	2	54	305	1610
	% CV		6.4	7.4	9.1	6.6
anti-S1 IgG	Sample	NEG	POS1	POS2	POS3	POS4
	MEAN (BAU/ml)	0.71	68	481	4700	18403
	STDEV (BAU/ml)	0.22	4	46	458	925
	% CV		6.2	9.5	9.7	5.0
anti-S2 IgG	Sample	NEG	POS1	POS2	POS3	POS4
	MEAN (BAU/ml)	1.42	76	456	4196	9622
	STDEV (BAU/ml)	0.17	9	39	306	952
	% CV		11.4	8.5	7.3	9.9
anti-N IgG	Sample	NEG	POS1	POS2		
	MEAN (BAU/ml)	0.58	49	346		
	STDEV (BAU/ml)	0.21	8.67	51		
	% CV		17.7	14.7		

Supplementary Table S4. Within-run precision.

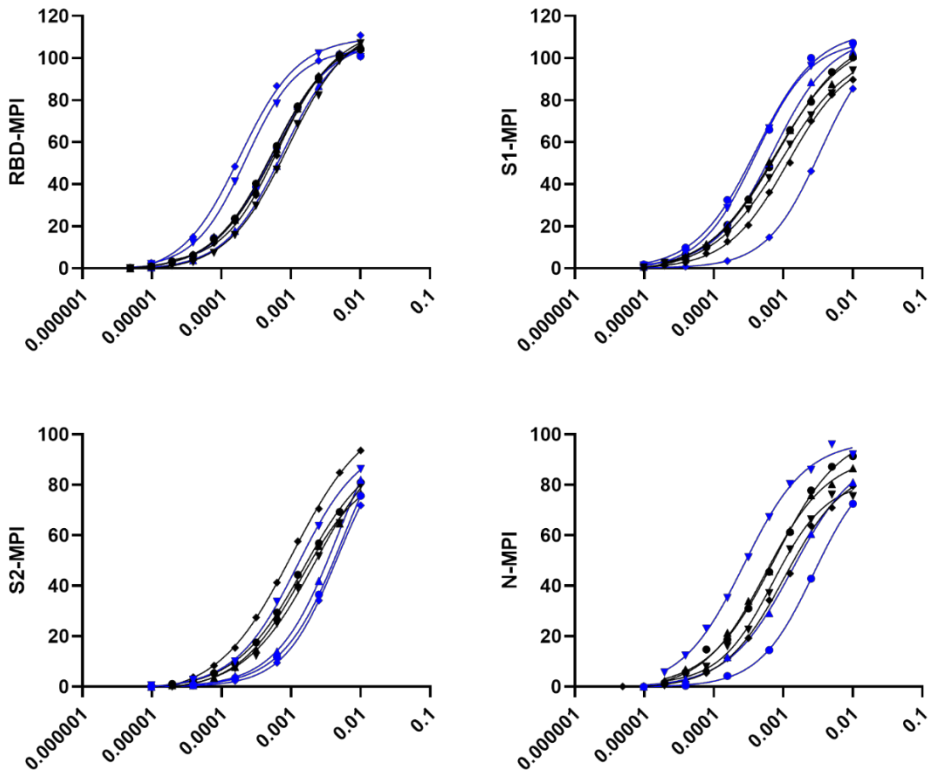
anti-RBD IgG	Sample	NEG	POS1	POS2	POS3	POS4
	run 1	0.36	42	713	3421	24471
	run 2	0.47	36	758	3621	26279
	run 3	0.22	44	720	3441	21480
	run 4	0.35	38	726	3361	24471
	MEAN (BAU/ml)	0.35	40	729	3461	24175
	STDEV (BAU/ml)	0.10	4	20	112	1989
	% CV		9.6	2.7	3.2	8.2
anti-S1 IgG	Sample	NEG	POS1	POS2	POS3	POS4
	run 1	0.78	57	492	4032	19156
	run 2	0.67	58	520	4777	20251
	run 3	0.69	72	571	4230	20198
	run 4	0.71	68	481	4700	18403
	MEAN (BAU/ml)	0.71	64	516	4435	19502
	STDEV (BAU/ml)	0.05	7	40	361	889
	% CV		11.6	7.8	8.1	4.6
anti-S2 IgG	Sample	NEG	POS1	POS2	POS3	POS4
	run 1	0.68	93	490	4267	9423
	run 2	0.74	85	479	3723	7778
	run 3	0.71	79	426	4706	10704
	run 4	1.42	76	456	4196	9622
	MEAN (BAU/ml)	0.89	83	463	4223	9382
	STDEV (BAU/ml)	0.36	8	28	402	1208
	% CV		9.0	6.1	9.5	12.9
anti-N IgG	Sample	NEG	POS1	POS2		
	run 1	0.91	29	452		
	run 2	1.28	36	431		
	run 3	1.79	32	327		
	run 4	0.58	49	346		
	MEAN (BAU/ml)	1.14	37	389		
	STDEV (BAU/ml)	0.52	9	62		
	% CV		24.1	15.9		

Supplementary Table S5. Between-run precision.

Conclusion: The MIA-assay is precise for measuring IgG antibodies directed to RBD, S1 and S2, with % CV for repeated measurements <12%. For repeated anti-N IgG measurement, % CV are slightly higher, but are considered acceptable.

3. Parallelism

Methods: Parallelism of the MIA-assay was evaluated by plotting 4PL curves (measured response (MPI) against log serum dilution) obtained with serial dilutions of the calibrating standard NIBSC 20/136 (4 standardcurves, black) and with serial dilutions of positive test samples (4 curves, blue) (**Fig. S5**).



Supplementary Figure S5. Parallelism of the MIA-assay.

Conclusion: Concentration-response plots of the 4 test samples and calibration standards showed a good parallelism for anti-RBD IgG, anti-S1 IgG, anti-S2 IgG and anti-N IgG.

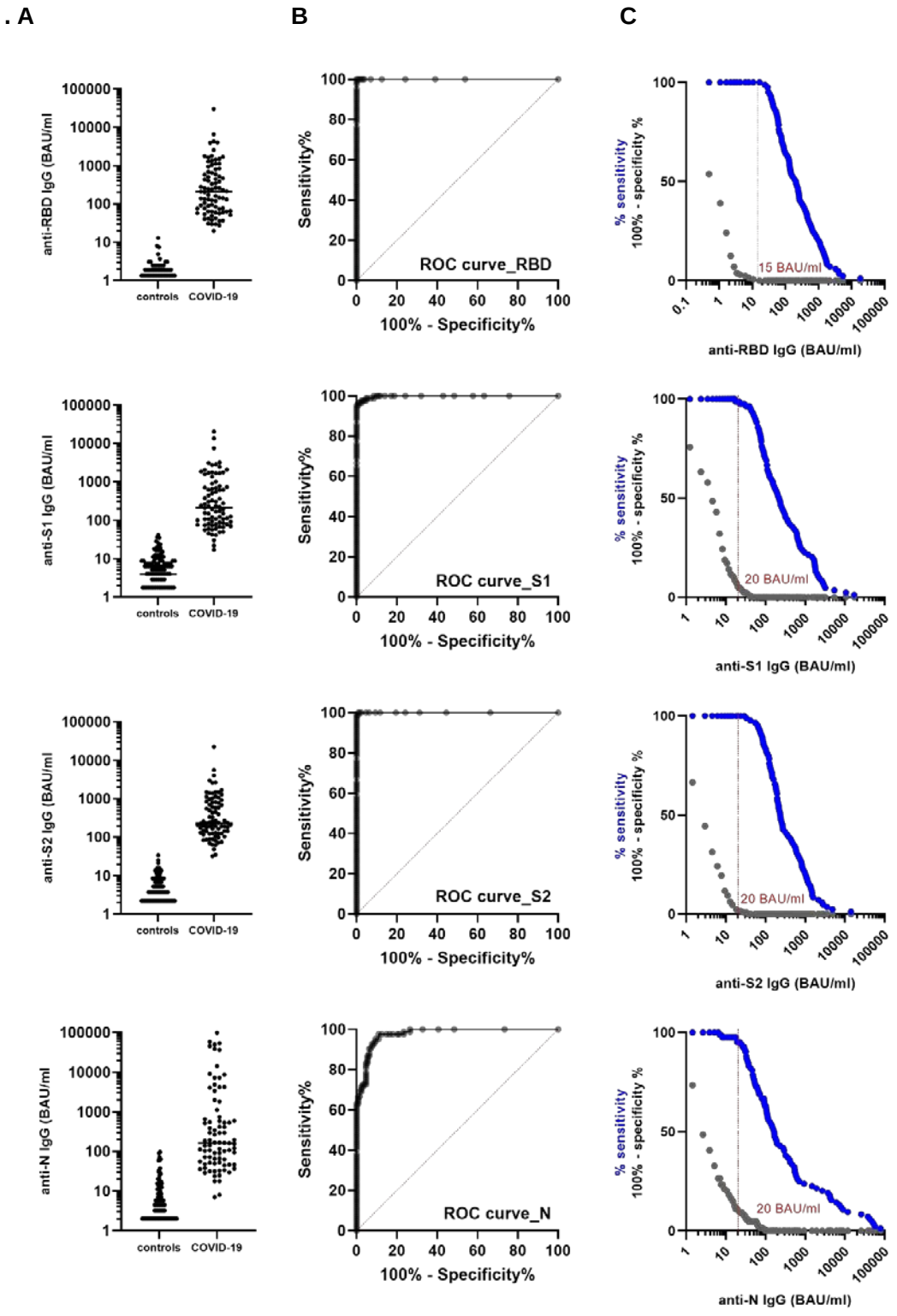
4. Quantitation limit

Methods: The lower limit of quantification (LLOQ) was defined as the lowest concentration of the standard curve with % recovery between measured concentration and nominal concentration within 85-115%. Accuracy-data demonstrate that back-calculated results are acceptable till calibrator concentration 0.02 BAU/ml.

Conclusions: As the lowest test dilution used is 1/100, LLOQ was set at 2 BAU/ml for anti-RBD IgG, anti-S1 IgG, anti-S2 IgG as well as anti-N IgG measurements.

5. Specificity, sensitivity and cut-off determination

Methods: For establishing reliable cut-off values for RBD-, S1-, S2- and N-antibody measurements, sera of 84 PCR-confirmed COVID-19 patients (comprising mild and severe clinical presentation) were tested in the MIA-assay and the results were compared to a control panel of 128 pre-pandemic sera (**Fig.S6A**). Positive samples from PCR-confirmed severe-diseased COVID-19 patients (n=42) were left-over sera from AZ Delta Hospital (Belgium), collected ≥ 15 to 50 days post onset of symptoms. Positive samples from PCR-confirmed mild-diseased COVID-19 patients (n=42) originated from observational clinical trials in health care workers (Duysburgh E *et al*, Lancet Infect Dis 2021; 21(2):163-164 and Triest D *et al*, J Clin Virol 2021; 142:104897) and were collected 4-6 months post PCR-positivity. Negative control samples originated from the Belgian National Reference Center for *Bordetella pertussis* and were collected in 2018. Overlap between both groups for anti-S1 IgG and anti-N IgG can be explained by (i) cross-reactivity due to previous contact with endemic human coronaviruses in the control group, and (ii) low initial titers or waning of antibodies in the mild-diseased group. Assay performance at each individual cut-off was evaluated using ROC (Receiver Operating Characteristic) analyses (**Fig.S6B**) and a specificity-optimized cut-off was determined for each antigen **Fig.S6C, Tab.S6**).



Supplementary Figure S6. Specificity, sensitivity and cut-off determination.

	cut-off	specificity		sensitivity	
	BAU/ml	%	95% CI	%	95%CI
anti-RBD IgG	>15	100	97.09-100	100	95.63-100
anti-S1 IgG	>20	95.31	90.15-97.83	98.81	93.56-99.94
anti-S2 IgG	>20	97.66	93.34-99.36	100	95.63-100
anti-N IgG	>20	89.06	82.48-93.37	95.24	88.39-98.13

Supplementary Table S6. Specificity-optimized cut-off.

Conclusion: The ROC-analyses generated cut-off concentrations of 15 BAU/ml, 20 BAU/ml, 20 BAU/ml and 20 BAU/ml for anti-RBD IgG, anti-S1 IgG, anti-S2 IgG and anti-N IgG, respectively. These cut-offs resulted in a specificity of 100%, 95,3%, 97,7% and 89,1% at a sensitivity of 100%, 98,8%, 100% and 95,2% for anti-RBD IgG, anti-S1 IgG, anti-S2 IgG and anti-N IgG, respectively.

6. Conclusions of the MIA-assay qualification

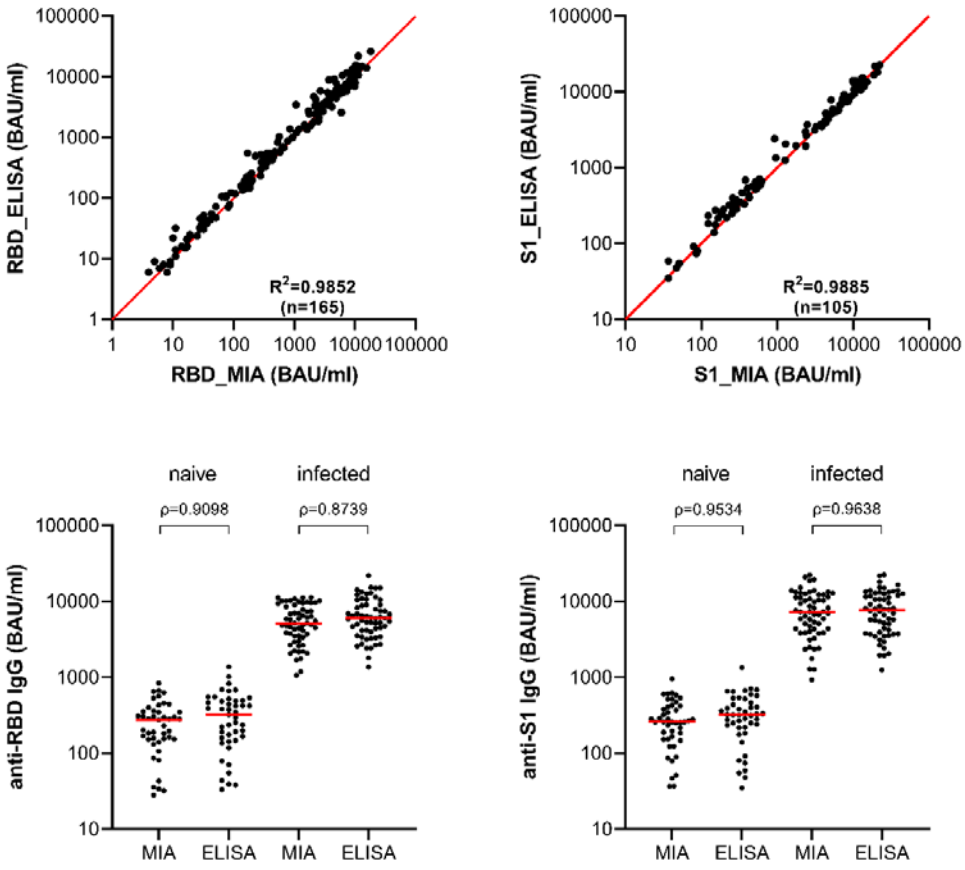
The results obtained from the qualification process demonstrate that the MIA-assay is reliable, reproducible, and suitable for assessing anti-RBD IgG, anti-S1 IgG, anti-S2 IgG and anti-N IgG responses in human serum. The results indicate that the 4PL equation provides an accurate representation of a sigmoidal relationship between the measured response (MPI) and the logarithm of antibody concentrations.

B. Validation with commercially available assays

Methods: To validate the anti-RBD IgG and anti-S1 IgG values obtained with the MIA-assay, a series of samples were quantified using commercial ELISA's, and the results compared. Samples obtained from the PICOV-VAC study (sampled day 21 post dose 1; n=165 for anti-RBD IgG; n= 105 for anti-S1 IgG) were quantified using (i) the WANTAI SARS-CoV-2 IgG ELISA (Quantitative)(Wantai Bio-Pharm, cat n° WS-1396; lot n°NCOGI20210301), detecting IgG antibodies directed to SARS-CoV-2 RBD, and (ii) the Anti-SARS-CoV-2 QuantiVac ELISA (IgG) (Euroimmun; cat n° EI 2606-9601-10 G; lot n° E210225AR), detecting IgG antibodies directed to SARS-CoV-2 S1. Diluted serum samples (1/10 to 1/6400) were tested against an internal standard, calibrated against NIBSC 20/136, control sera and blanks included on each plate. Net OD values were converted to arbitrary IgG units per ml by interpolation from a standard curve obtained by point-to-point plotting of the net OD readings measured from 6 calibration sera against the corresponding units (linear/linear) using GraphPad Prism (version 9.0.0 for Windows), exported to Microsoft Excel and adjusted for any sample dilution. Arbitrary IgG units were multiplied by factor 5.4 (RBD-ELISA) or factor 3.2 (S1-ELISA) to obtain Binding antibody units per ml (BAU/ml). The lower limits of quantification (LLOQ) were 5,4 BAU/ml and 3.2 BAU/ml for RBD- and S1-ELISA, respectively. The NIBSC 20/136 standard (1000 BAU/ml) returned values of 1042 BAU/ml (RBD-ELISA) and 1018 BAU/ml (S1-ELISA).

Correlation between the MIA-assay and the commercial ELISA's were examined (**Fig.S7**, upper panels). Comparisons between the MIA assay and the commercial ELISA's in naïve and in previously infected subjects were performed (**Fig.S7**, lower panels).

Conclusions: The MIA-assay shows a 1:1 correlation with the RBD-ELISA for anti-RBD IgG (R^2=0.9852) and a 1:1 correlation with the S1-ELISA for anti-S1 IgG (R^2=0.9885) (upper panels). Furthermore, Ab titers measured with the MIA-assay are comparable to those obtained with commercial assays in both study groups. The validation supports the reliability of the MIA-assay for the quantification of anti-RBD IgG and anti-S1 IgG in naïve and previously infected subjects.



Supplementary Figure S7. Correlation between the MIA-assay and commercial ELISA's.