1   Title: Evaluation of a novel multiplexed assay for determining IgG levels and functional

2   activity to SARS-CoV-2.

3   Running title: Measuring SARS-CoV-2 immunity

4

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28   Abstract

29   Background

30   The emergence of SARS-CoV-2 has led to the development of new serological assays

31   that could aid in diagnosis and evaluation of seroprevalence to inform an

32   understanding of the burden of COVID-19 disease. Many available tests lack rigorous

33   evaluation and therefore results may be misleading.

34   Objectives

35   The aim of this study was to assess the performance of a novel multiplexed

36   immunoassay for the simultaneous detection of antibodies against SARS-CoV-2

37   trimeric spike (S), spike receptor binding domain (RBD), spike N terminal domain and

38   nucleocapsid antigen and a novel pseudo-neutralisation assay.

39   Methods

40   A multiplexed solid-phase chemiluminescence assay (Meso Scale Discovery) was

41   evaluated for the simultaneous detection of IgG binding to four SARS-CoV-2 antigens

42   and the quantification of antibody-induced ACE-2 binding inhibition (pseudo-

43   neutralisation assay). Sensitivity was evaluated with a total of 196 COVID-19 serum

44   samples (169 confirmed PCR positive and 27 anti-nucleocapsid IgG positive) from

45   individuals with mild symptomatic or asymptomatic disease. Specificity was evaluated

46   with 194 control serum samples collected from adults prior to December 2019.

47   Results

48   The specificity and sensitivity of the binding IgG assay was highest for S protein with

49   a specificity of 97.4% and sensitivity of 96.2% for samples taken 14 days and 97.9%

50   for samples taken 21 days following the onset of symptoms. IgG concentration to S

51   and RBD correlated strongly with percentage inhibition measured by the pseudo-

52   neutralisation assay.

53   Conclusion

54   Excellent sensitivity for IgG detection was obtained over 14 days since onset of

55   symptoms for three SARS-CoV-2 antigens (S, RBD and N) in this multiplexed assay

56   which can also measure antibody functionality.

4

Introduction

Severe acute respiratory syndrome-related coronavirus-2 (SARS-CoV-2) was first recognised in January 2020 and rapidly spread world-wide with the WHO declaring a COVID-19 pandemic on March 11th, 2020 (1). Soon after the identification and genetic sequencing of the virus, diagnostic tests became available for the detection of live virus in human secretions followed rapidly by tests designed to measure antibodies to SARS-CoV-2 antigens. Antibody tests have a variety of uses including supporting diagnosis and informing individual risk of future disease and thereby determining correlates of and duration of protection. With further potential for understanding exposure to virus which in turn could help inform disease burden estimates, studies of transmission dynamics and modelling of the epidemic. Antibody tests are particularly important in the context of mild or asymptomatic disease where a swab reverse transcriptase polymerase chain reaction (RT-PCR) test may be negative. For this reason, an understanding of the sensitivity and specificity of the tests being used is critical.

The trimeric spike (S) protein of SARS-CoV-2 is a large molecule that is critical to virus dissemination and pathogenesis. It is densely glycosylated and present on the viral surface and in most cases is cleaved by host proteases into the S1 and S2 subunits, which are responsible for receptor recognition and membrane fusion respectively. S1 uses a region of the molecule, known as the receptor binding domain (RBD) to bind to host ACE-2 receptor and thereby gain entry to the cell (2). Due to this critical function in host-cell entry, the S protein is a major target for vaccine research. The N terminal domain (NTD) of the spike protein does not interact with the receptor but contains the functional elements required for membrane fusion of the virion. The nucleocapsid (N) protein plays an important role in transcription enhancement and viral assembly (3).

82   Specific immunoglobulin-G (IgG) and IgM antibody responses to SARS-CoV-2 S, N

83   and RBD of the spike protein develop between 6-15 days following disease-onset (4).

84   Despite a rapid increase in the number and availability of serologic assays that can

85   detect antibodies against SARS-CoV-2, most have undergone minimal external

86   evaluation and validation (5). The high sensitivity and specificity for commercially

87   obtainable kits are often not reproduced when appropriate samples are used for

88   evaluation. A recent large scale Spanish seroprevalence study used a point of care

89   IgG test with a stated sensitivity of 97.2% but on verification found it to have a

90   sensitivity of either 82.1%, 89.7%, 99.6% or 100% depending on the sample sets used

91   for evaluation (6). All assays currently suffer from the absence of a defined standard

92   serum so results are reported as positive or negative or as optical density readouts

93   complicating the comparison between assays and studies. Furthermore, most assays

94   measure responses to a single antigen, usually nucleocapsid or spike/spike derived

95   proteins, which may not capture the breadth of antibody responses to SARS-CoV-2.

96   Finally, for many binding assays, the relationship between the concentration of

97   antibody detected and their function is unclear and few available assays permit the

98   measurement of both binding and function on the same testing platform.

99   We have evaluated a novel assay designed to simultaneously measure IgG to four

100   SARS-CoV-2 antigens; full-length trimeric S, RBD and NTD of spike as well as N

101   protein. The assay, based on Meso Scale Discovery (MSD) technology, utilises a 96-

102   well based solid-phase antigen printed plate and an electrochemiluminescent

103   detection system. In addition, unlike most binding assays, this assay can be adapted

104   to measure the ability of serum to inhibit the interaction between spike protein

105   components and soluble ACE-2, also called a pseudo-neutralisation assay (7). To

106   evaluate the sensitivity and specificity of the MSD assay, we were able to utilise a

107   relatively large number of samples obtained from SARS-CoV-2 RT-PCR positive

108   health care workers or patients as well as antibody positive health care staff enrolling

109   in a large SARS-CoV-2 cohort study.

110

111   Materials and Methods

112   *Serum Samples*

113   Serum samples for sensitivity analyses were obtained from Great Ormond Street

114   Children's Hospital NHS Foundation Trust (GOSH) and came from three sources; (i)

115   healthcare workers who tested SARS-CoV-2 RT-PCR positive following signs or

116   symptoms of COVID-19 and who gave written consent for participation in the service

117   evaluation of SARS-CoV-2 serological assays, (ii) staff enrolling in a prospective

118   longitudinal cohort study of SARS-CoV-Serology (COSTARS, IRAS 282713,

119   ClinicalTrials.gov Identifier: NCT04380896) who tested positive in a commercial

120   screening assay for anti-Nucleocapsid IgG (Epitope Diagnostics Inc, San Diego, USA)

121   (iii) a small number of RT-PCR positive sera from hospitalised children (n=10).

122   Serum samples for the analysis of specificity were collected prior to December 2019

123   and derived from anonymised samples in assay development or quality control sera

124   developed for other assays or residual, anonymised samples from healthy adults

125   enrolled in previous studies.

126   Serum from two individuals with high convalescent antibody levels were pooled to

127   create an interim standard serum. This serum was calibrated against research

128   reagents NIBSC 20/130 and NIBSC 20/124 distributed by the National Institute for

129   Standards and Biological Control (NIBSC, Potters Bar, UK, https://www.nibsc.org/) for

130   the purpose of development and evaluation of serological assays for the detection of

131   antibodies against SARS-CoV-2. These two plasma samples were obtained from

132   COVID-19 recovered patients and were distributed with known end-point titres to

133   trimeric S, S1 and N as well as antibody functionality measured by live virus

134   neutralisation, pseudo-neutralisation and plaque reduction neutralisation.

135   *Serological assays*

136   Samples were screened for IgG to SARS-CoV-2 N protein using a commercially

137   available kit (Epitope Diagnostics Inc, San Diego, USA) as previously described (8).

138   *Meso Scale Discovery coronavirus panel for COVID-19 serology*

139   A multiplexed MSD immunoassay (MSD, Rockville, MD) was used to measure the

140   antigen-specific response to SARS-CoV-2 infection and other respiratory pathogens.

141   A MULTI-SPOT® 96-well, 10 Spot Plate was coated with four SARS CoV-2 antigens

142   (S, RBD, NTD and N), SARS-CoV-1 and MERS spike trimers, spike proteins from

143   seasonal coronaviruses OCV43S and HKU1, influenza A antigen derived from

144   H3/HongKong and Bovine Serum Antigen. Antigens were spotted at 200-400 µg/mL

145   in a proprietary buffer, washed, dried and packaged for further use (MSD®

146   Coronavirus Plate 1). Proteins were expressed in a mammalian cell expression

147   system (Expi 293F), purified by ion exchange chromatography, affinity purification,

148   and size exclusion chromatography. They were characterized by reducing SDS Page

149   chromatography, mass spectrometry, size-exclusion chromatography and multi-angle

150   light scattering (SEC-MALS). All protein constructs were produced with His6 and/or

151   Strept-TAG affinity tags to support affinity purification; the spike proteins were

152   produced as trimers in the pre-fusion form. These assays were developed by MSD in

153   collaboration with the Vaccine Research Center at NIAID (A. McDermott).

154 Internal quality controls and reference standard reagents were developed from pooled

155 human serum. To measure IgG antibodies, plates were blocked with MSD Blocker A

156 for between 30 minutes and 2 hours then washed three times prior to the addition of

157 reference standard, controls and samples diluted 1:500 in diluent buffer. After

158 incubation for 2 hours with shaking at 700rpm, the plates were washed three times

159 and detection antibody was added at 2 µg/mL (MSD SULFO-TAG™ Anti-Human IgG

160 Antibody). Plates were incubated for 1 hour with shaking and washed three times.

161 MSD GOLD™ Read Buffer B was added and the plates were read using a MESO®

162 SECTOR S 600 Reader.

163 *Meso Scale Discovery* pseudo-neutralisation assay

164 Plates were blocked and washed as above, assay calibrator (COVID-19 neutralising

165 antibody; monoclonal antibody against S protein; 200µg/ml), control sera and test sera

166 samples diluted 1 in 10 in assay diluent were added to the plates. Plates were

167 incubated for 1 hour with shaking at 700rpm. A 0.25µg/ml solution of MSD SULFO-

168 TAG™ conjugated ACE-2 was added to unwashed plates followed by incubation for 1

169 hour with shaking, plates were washed and read as above. Percentage inhibition was

170 calculated relative to the assay calibrator; the maximum inhibition reached with

171 calibrator was set as 100% inhibition, minimum at 0.01%.

172

173 *Statistical analysis*

174 Statistical analysis was performed using MSD Discovery Workbench and GraphPad

175 Prism version 8.0 (GraphPad, San Diego, CA). Antibody concentration in arbitrary

176 units (AU) was interpolated from the ECL signal of the internal standard sample using

177 a 4-parameter logistic curve fit. ROC curves showing the sensitivity and specificity

178   (plotted as 100%-specificity %) calculated using each value in the data as a cut-off

179   were plotted for each antigen. A cut-off antibody concentration was chosen based on

180   the lowest value leading to a positive likelihood ratio (LR) of >10, in order to maximise

181   sensitivity while providing strong evidence to rule-in infection (9). For S antigen

182   binding, all LR's were above 10, therefore the LLOD was used as the cut-off for this

183   antigen. Positive predictive value (PPV) was calculated as

184    $$PPV = \frac{sensitivity \times prevalence}{sensitivity \times prevalence + (1-specificity) \times (1-prevalence)},$$

185   negative predictive value (NPV) was calculated as

186    $$NPV = \frac{specificity \times (1-prevalence)}{(1-sensitivity) \times prevalence + specificity \times (1-prevalence)}.$$

187   Comparisons between groups were performed by Kruskal-Wallis one-way ANOVA

188   with Dunn's correction for multiple comparisons. Correlation analysis was performed

189   using Spearman correlation. P values of <0.05 were considered as significant. Latent

190   class models with two classes were fitted with the binary antibody responses as

191   outcome variables, using the poLCA package in the R statistical environment. The

192   code used for the latent class analysis is available on request.

193 Results

194 *Participants and samples*

195 SARS-CoV-2 positive samples (COVID-19 cohort) comprised 169 PCR positive and

196 27 anti-N IgG positive serum samples from mild symptomatic or asymptomatic cases

197 (total n=196). The cohort comprised of 138 females, 56 males (2 not recorded) with a

198 median age of 37 years (range 1-66y). Recorded symptoms included abnormal taste

199 and smell, cough, fatigue and fever. The date of symptom onset was established and

200 verified for 168 subjects, time between symptom onset and sampling ranged from 4 to

201 63 days. Of the 169 individuals with documented RT-PCR testing, 37 samples were

202 negative for nucleocapsid IgG on the EDI screening ELISA and 11 were equivocal.

203 Serum samples were collected between 26th March and 18th May 2020 and analysed

204 between 1st June and 10th July 2020.

205 Control serum samples for the analysis of specificity comprised 194 anonymised

206 legacy samples obtained from healthy adults, aged predominantly over 50 years.

207

208 *Standard serum assignment*

209 An internal standard serum was assigned values for S, RBD and N by calibration

210 against the NIBSC control sera. The ECL signal obtained for NIBSC 20/130 was used

211 as a binding curve to assign arbitrary unit (AU) values for S and RBD while NIBSC

212 20/124 was used to assign a value for N (Supplementary Figure S1). Binding of pooled

213 standard serum to NTD produced low ECL signals and no endpoint titre corresponding

214 to NTD antigen was available for standard serum assignment. The interim values

215 assigned were S 2154 AU, RBD 1837 AU and N 3549 AU. NTD and the remaining

11

216   antigens were assigned a value of 1000 AU. The focus of this study was the evaluation

217   of the four SARS-CoV-2 antigens only.

218

219   *Evaluation of the coronavirus panel for COVID-19 serology*

220   The lower limit of detection (LLOD) was assigned as 1% of the standard value in AU,

221   for statistical purposes, values below LLOD were reported as half LLOD (Table 1).

222   The upper limit of detection (ULOD) was assigned for NTD and RBD only as the S and

223   N antigen did not reach an upper limit (Table 1). For statistical purposes, ULOD was

224   assigned the highest calculated concentration plus 20%.

225   The coefficient of variation (CV) between duplicates was assessed by analysing 390

226   samples run on 11 plates on 3 different days. All antigens produced a mean CV of

227   <15%, with only NTD falling above the accepted CV of 15% at 17.4% (data not shown).

228   Intra-assay (within plate) and inter-assay (between plate) variation of the assay was

229   assessed by running four samples of varying antibody levels in four replicates on the

230   same plate and across 4 different runs on different days (Supplementary Table 1). The

231   mean intra-assay CV was 6.2% and inter-assay variation <15% across all SARS-CoV-

232   2 antigens except NTD (19.0%) on one of four samples.

233   To control day to day performance of the assay, a QC sample was run on each plate

234   and an acceptable performance range was set as within 3 SD of the mean. This was

235   determined by running the sample on 8 different plates on 8 different days (average

236   CV 10.3%) (Table 1).

237

238   *Assay sensitivity and specificity*

12

239 Figure 1A-D shows the concentration of IgG to each SARS-CoV-2 antigen in the

240 COVID-19 cohort and the controls. Receiver Operating Characteristic (ROC) curves

241 were plotted to visualise the trade-off between sensitivity and specificity for each

242 antigen (Figure 2A-D). The high area under the curve (AUC) values for S (0.95%;

243 95%CI 0.93 to 0.97), RBD (0.92%, 0.89-0.95) and N (0.90%, 0.87-0.94) indicates the

244 high accuracy of these tests. Table 1 shows the cut-off values selected using our rule

245 of choosing the lowest cut-off with LR>10. For S all LRs were above 10, therefore the

246 LLOD was used as the cut-off for this antigen. NTD data was less consistent than the

247 other SARS-CoV-2 antigens and demonstrated lower sensitivity and specificity (Figure

248 2D), so this antigen was not evaluated further.

249 The specificity for S, RBD and N assays calculated from the control sera were 97.4%

250 (95%CI 94.1 to 98.9), 92.3% (95%CI 87.6 to 95.3) and 92.8% (95%CI 88.2 to 95.7)

251 respectively (Table 2). Assay sensitivity was initially calculated on the entire COVID-

252 19 cohort; S antigen had the highest AUC and was the most sensitive and specific at

253 90.8% and 97.4% respectively.

254 Using the calculated specificity and sensitivity, the positive and negative predictive

255 values (PPV and NPV) for each antigen at a range of prevalence estimates between

256 0.01 and 0.5 were calculated (Supplementary Figure 3A-B). The PPV and NPV were

257 best for S antigen; for an overall prevalence of 10% the assay has a PPV of 80.4%

258 and NPV of 99.6% for samples taken over 14 days since onset of symptoms, this

259 increased to 92.5% and 98.7% for an overall prevalence of 25%.

260

261 *Evaluation of sensitivity according to time since onset of symptoms*

262 Figure 3 shows the anti-S, RBD and N IgG concentration split into time since onset of

263 symptom intervals of 0-7 days, 8-14 days, 15-21 days and over 21 days. For all three

264 antigens, the median antibody concentration increased significantly between 8-14

265 days and over 21 days and all interval groups were significantly (p=<0.0001) higher

266 than the control cohort (Figure 3A-C). There was a significant association between

267 antibody concentration and time since onset of symptoms (SARS-CoV-2 S, Spearman

268 correlation (r)=0.453; SARS-CoV-2 RBD, r=0.478; SARS-CoV-2 N, r=0.392, all

269 p=<0.0001) (Supplementary Figure 2A-C).

270 The assay cut-off determined above was applied and sensitivity and specificity were

271 calculated for groups 0-7 days, over 7 days, over 14 days and over 21 since the onset

272 of symptom for (Table 2). The S antigen was the most sensitive of the three, with a

273 sensitivity of 96.2% and 97.9% >14 days and >21 days since onset of symptoms

274 respectively.

275

276 *Antibody concentration relationship between antigens*

277 The concentration of anti-S, RBD and N antibody all correlated significantly with each

278 other (p<0.0001; Figure 4A-C), the strongest association was between S and RBD

279 (r=0.882) (Figure 4A). Our two-class latent class model built using binary S, RBD and

280 N antigen results predicted known status with 81.1% (95%CI 74.8-86.2) sensitivity and

281 99.0% (95%CI 95.9-99.8) specificity. It therefore had lower sensitivity and no

282 meaningful improvement in specificity, compared to using the concentration of S

283 antibody alone, with the 21.54 AU cut-off.

284

285 *Pseudo-neutralisation*

14

286 183 COVID-19 cohort samples with sufficient volume and 194 control group samples

287 were evaluated in the pseudo-neutralisation assay. The percentage inhibition of ACE-

288 2 receptor binding to the S and RBD antigens was calculated for the COVID-19 and

289 control group (Figure 5A-B). The percentage inhibition for the COVID-19 cohort was

290 significantly higher than the controls for both antigens (S, median 1.94% (95%CI 1.36-

291 2.25) vs 0.063% (95%CI 0.053-0.073), p=<0.0001 by Mann-Whitney U test; RBD,

292 1.50% (95%CI 1.064-2.11) vs 0.38% (95%CI 0.36-0.39); p=<0.0001). In the COVID-

293 19 cohort, there was a significant association between percentage inhibition and IgG

294 concentration for both S and RBD antigens (Spearman correlation (r)=0.805 and

295 r=0.834 respectively, p=<0.0001) (Figure 5C-D).

296 ROCs were plotted to visualise the trade-off between sensitivity and specificity for S

297 and RBD neutralisation. Cut-offs (LR>10) were 0.162% for S and 0.524% for RBD

298 (shown by the dotted line on Figure 5A-B). Sensitivity and specificity for S were 97.8%

299 and 97.9% respectively but lower for RBD (77.2% and 92.8% respectively). In the

300 COVID-19 cohort there were some IgG positive sera that did not demonstrate

301 neutralisation (below cut-off, n= 4 for S and 36 for RBD). These sera were

302 predominantly those taken soon after the onset of symptoms; 22 between 0-7 days, 9

303 over 14 days and 5 over 21 days.

304 Discussion

305 Accurate tests of SARS-CoV-2 antibodies are critical for reliably evaluating exposure

306 to the virus causing COVID-19. Despite a large number of assays rapidly becoming

307 available, many have not undergone rigorous evaluation. In this study we describe a

308 novel assay that can measure antibody to several SARS-CoV-2 antigens

309 simultaneously as well as evaluating the functional capacity of anti-Spike antibodies.

310 The assay we used is based on existing technology developed by Meso Scale

311 Discovery that uses high binding carbon electrodes in the bottom of 96-well

312 microplates. Each well contains up to 10 antigens bound in discrete spots and bound

313 serum-derived IgG is detected by electro-chemiluminescent labelled (SULFO-TAG)

314 anti-human IgG. Electricity is applied to the plate electrodes leading to light emission

315 by the SULFO-TAG labelled detection antibody and light intensity is measured to

316 quantify analytes in the sample. We decided to evaluate IgG only as the kinetics of

317 IgM responses appear to mimic those of IgG and thus add little value (4).

318 Unlike the majority of studies published to date, we were able to utilise a panel of

319 COVID-19 convalescent plasma recently distributed by WHO to calibrate an internal

320 standard made from pooled convalescent serum. This allowed us to express titres in

321 arbitrary units that can then be compared to other assays that report values calibrated

322 against the WHO panel. The assays performed reliably and consistently over the

323 period of study and passed all the performance criteria expected for a solid-phase

324 based assay with acceptably low inter- and intra-assay coefficients of variation. A QC

325 range established for a medium titre serum gave consistent results throughout the

326 study indicating the stability and repeatability of the platform.

327 Using a carefully defined cohort of known SARS-CoV-2 exposed individuals and

328 relevant controls we were able to show the sensitivity and specificity of the assay for

329   the four antigens of interest. While all antigens had good specificity, the full-length

330   trimeric spike protein had the highest sensitivity, particularly for serum taken more than

331   14 days following the onset of symptoms. Comparing our data for the S and RBD

332   antigens to data in a recently published systematic review and metanalysis of the

333   diagnostic accuracy of serological tests for COVID-19 (10) the trimeric spike assay we

334   evaluated had superior sensitivity to all of the assays included in the review while the

335   RBD antigen performance was superior to most. The reason for this could be related

336   to the technical aspects of the assay itself including the integrity of the antigen used

337   and the sensitivity of the detection platform but also the use of a well-defined cohort

338   of individuals with known exposure to SARS-CoV-2. Only one of the four SARS-CoV-

339   2 antigens, the N terminal domain of the spike protein, did not perform well in this

340   assay with poor sensitivity due to the overlap in antibody titres between the COVID-

341   19 cohort and controls.

342   The ability to simultaneously measure responses to various SARS-CoV-2 antigens

343   could be seen as an advantage in this type of assay although we did not show an

344   advantage of combined analysis of responses to three antigens compared to using S

345   antigen results alone to predict exposure correctly to the virus. The assay format also

346   permitted the measurement of antibody against spike protein derived from SARS-1,

347   MERS and two seasonal coronaviruses, but the results of antibody binding to these

348   antigens could not be assessed in the same way as for the SARS-CoV-2 antigens due

349   to the absence of defined negative and positive serum sets.

350   A further advantage of this assay is the ability to adapt it for measuring antibody

351   induced inhibition of the interaction between the spike antigen and soluble ACE-2

352   receptor, without the use of live virus and category 3 facilities. This is important as it

353   is thought to be the major mechanism by which SARS viruses, including SARS-CoV-

354 2 attach to host cell surfaces (11, 12). In the COVID-19 group, there was a good

355 correlation between the concentration of anti-S and anti-RBD IgG and the inhibitory

356 capacity of serum measured in the pseudo-neutralisaton assay, although a few sera

357 bound antigen but did not neutralize ACE-2 binding. Recently, a study of convalescent

358 serum by Sedoux *et al*. identified that the majority of antibodies against spike that were

359 generated during the first weeks of COVID-19 infection were non-neutralising and

360 target epitopes outside the RBD (13) which may account for our results. Few of the

361 control cohort sera had any pseudo-neutralisation activity suggesting that pre-existing

362 IgG directed against seasonal Coronavirus spike proteins are unlikely to modify

363 interaction with SARS-CoV-2 although other cross reactive immunological

364 mechanisms (eg T cells) cannot be ruled out and may explain the varied clinical

365 response following exposure to SARS-CoV-2 (14). This pseudo-neutralisation assay

366 has been shown to correlate well with neutralisation assays using live SARS-CoV-2

367 (MSD, personal communication). While plaque reduction neutralisation assays are

368 currently standard for determining host antibody induced viral inhibition, they must be

369 performed in a biosafety level 3 laboratory which limits their widespread use.

370 In summary, the MSD multiplexed coronavirus panel assay evaluated in this study is

371 highly reproducible, specific and sensitive for the detection of anti-SARS-CoV-2

372 antibody over 14 days since the onset of COVID-19 symptoms. The assay can be

373 adapted to measure antibody function which corelated well with spike protein antibody

374 concentration.

375

376

385   Conflicts of interest

386   The authors declare no conflicts of interest.

387

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References

389 References

390 1.   World Health Organisation. 2020. Coronavrius disease 2019 (COVID-19)

391   Situation Report - 51.

392 2.   Salazar E, Kuchipudi SV, Christensen PA, Eagar TN, Yi X, Zhao P, Jin Z,

393   Long SW, Olsen RJ, Chen J, Castillo B, Leveque C, Towers DM, Lavinder J,

394   Gollihar JD, Cardona J, Ippolito GC, Nissly RH, Bird IM, Greenawalt D, Rossi

395   RM, Gontu A, Srinivasan S, Poojary IB, Cattadori IM, Hudson PJ, Joselyn N,

396   Prugar L, Huie K, Herbert A, Bernard DW, Dye J, Kapur V, Musser JM. 2020.

397   Relationship between Anti-Spike Protein Antibody Titers and SARS-CoV-2 In

398   Vitro Virus Neutralization in Convalescent Plasma. bioRxiv

399   doi:10.1101/2020.06.08.138990.

400 3.   Satarker S, Nampoothiri M. 2020. Structural Proteins in Severe Acute

401   Respiratory Syndrome Coronavirus-2. Arch Med Res

402   doi:10.1016/j.arcmed.2020.05.012.

403 4.   To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, Yip CC, Cai JP,

404   Chan JM, Chik TS, Lau DP, Choi CY, Chen LL, Chan WM, Chan KH, Ip JD,

405   Ng AC, Poon RW, Luo CT, Cheng VC, Chan JF, Hung IF, Chen Z, Chen H,

406   Yuen KY. 2020. Temporal profiles of viral load in posterior oropharyngeal

407   saliva samples and serum antibody responses during infection by SARS-CoV-

408   2: an observational cohort study. Lancet Infect Dis 20:565-574.

409 5.   Cheng MP, Yansouni CP, Basta NE, Desjardins M, Kanjilal S, Paquette K,

410   Caya C, Semret M, Quach C, Libman M, Mazzola L, Sacks JA, Dittrich S,

411   Papenburg J. 2020. Serodiagnostics for Severe Acute Respiratory Syndrome-

412   Related Coronavirus-2: A Narrative Review. Ann Intern Med doi:10.7326/M20-

413   2854.

414   6.   Pollan M, Perez-Gomez B, Pastor-Barriuso R, Oteo J, Hernan MA, Perez-

415   Olmeda M, Sanmartin JL, Fernandez-Garcia A, Cruz I, Fernandez de Larrea

416   N, Molina M, Rodriguez-Cabrera F, Martin M, Merino-Amador P, Leon

417   Paniagua J, Munoz-Montalvo JF, Blanco F, Yotti R, Group E-CS. 2020.

418   Prevalence of SARS-CoV-2 in Spain (ENE-COVID): a nationwide, population-

419   based seroepidemiological study. Lancet doi:10.1016/S0140-6736(20)31483-

420   5.

421   7.   Byrnes JR, Zhou XX, Lui I, Elledge SK, Glasgow JE, Lim SA, Loudermilk R,

422   Chiu CY, Wilson MR, Leung KK, Wells JA. 2020. A SARS-CoV-2 serological

423   assay to determine the presence of blocking antibodies that compete for

424   human ACE2 binding. medRxiv doi:10.1101/2020.05.27.20114652.

425   8.   Kruttgen A, Cornelissen CG, Dreher M, Hornef M, Imohl M, Kleines M. 2020.

426   Comparison of four new commercial serologic assays for determination of

427   SARS-CoV-2 IgG. J Clin Virol 128:104394.

428   9.   Deeks JJ, Altman DG. 2004. Diagnostic tests 4: likelihood ratios. BMJ

429   329:168-9.

430   10.   Lisboa Bastos M, Tavaziva G, Abidi SK, Campbell JR, Haraoui LP, Johnston

431   JC, Lan Z, Law S, MacLean E, Trajman A, Menzies D, Benedetti A, Ahmad

432   Khan F. 2020. Diagnostic accuracy of serological tests for covid-19:

433   systematic review and meta-analysis. BMJ 370:m2516.

434   11.   Kuhn JH, Li W, Choe H, Farzan M. 2004. Angiotensin-converting enzyme 2: a

435   functional receptor for SARS coronavirus. Cell Mol Life Sci 61:2738-43.

436   12.   Wang Q, Zhang Y, Wu L, Niu S, Song C, Zhang Z, Lu G, Qiao C, Hu Y, Yuen

437   KY, Wang Q, Zhou H, Yan J, Qi J. 2020. Structural and Functional Basis of

438   SARS-CoV-2 Entry by Using Human ACE2. Cell 181:894-904 e9.

439   13.   Seydoux E, Homad LJ, MacCamy AJ, Parks KR, Hurlburt NK, Jennewein MF,

440   Akins NR, Stuart AB, Wan YH, Feng J, Nelson RE, Singh S, Cohen KW,

441   McElrath MJ, Englund JA, Chu HY, Pancera M, McGuire AT, Stamatatos L.

442   2020. Characterization of neutralizing antibodies from a SARS-CoV-2 infected

443   individual. bioRxiv doi:10.1101/2020.05.12.091298.

444   14.   Wiersinga WJ, Rhodes A, Cheng AC, Peacock SJ, Prescott HC. 2020.

445   Pathophysiology, Transmission, Diagnosis, and Treatment of Coronavirus

446   Disease 2019 (COVID-19): A Review. JAMA doi:10.1001/jama.2020.12839.

447

448   Figure Legends

449   **Figure 1: Anti-SARS-CoV-2 IgG concentration.**

450   The concentration of SARS-CoV-2 antibody against (a) spike (S), (b) receptor binding

451   domain (RBD), (c) nucleocapsid (N) and (d) N terminal domain (NTD) was measured

452   using the MSD coronavirus panel. Graphs show data in arbitrary units (AU) (based on

453   the calibrated internal standard serum) in the COVID-19 cohort (n=196) and controls

454   (n=194, pre-December 2019). Line shows positive/negative discrimination cut-off.

455

456   **Figure 2: Receiver Operating Characteristic (ROC) curves for each SARS-CoV-2**

457   **antigen.**

458   Sensitivity and specificity were calculated using each value in the data table as a cut-

459   off value (n=390). Graphs show the sensitivity vs 100%-specificity of SARS-CoV-2

460   antigen (a) spike (S), (b) receptor binding domain (RBD), (c) nucleocapsid (N) and (d)

461   N terminal domain (NTD). The area under curve (AUC) and 95% CI is also shown for

462   each antigen.

463

464   **Figure 3: Anti-SARS-CoV-2 IgG concentration according to time since onset of**

465   **symptoms.**

466   Graphs show the concentration of SARS-CoV-2 antibody against (a) spike (S), (b)

467   receptor binding domain (RBD) and (c) nucleocapsid (N) in arbitrary units (AU) (based

468   on the calibrated internal standard serum) of the COVID-19 cohort split in to intervals

469   of 0-7 days, 8-14 days, 15-21 days and over 21 (>21) days since symptom onset (to

470   sample collection). Error bars show geometric mean with 95% CI, line shows

471   positive/negative discrimination cut-off, *p<0.05, ** p<0.01 determined by Dunn's

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472   multiple comparisons test. Comparisons across interval groups had $p < 0.0001$ by one-

473   way ANOVA Kruskal-Wallis test. The assay sensitivity at each time point is shown in

474   Table 3.

475

476   **Figure 4: IgG concentration relationship between antigens.**

477   Correlation between anti-SARS-CoV-2 antibody concentration of all COVID-19 group

478   samples (n=196) (a) S vs RBD, (b) S vs N and (c) N vs RBD. r and p value were

479   determined by Spearman correlation. p values of <0.05 were considered as significant.

480

481   **Figure 5: Percentage inhibition by anti-SARS-CoV-2 S and RBD antibody**

482   **measured by MSD pseudo-neutralisation assay.**

483   Inhibition of ACE-2 binding by SARS-CoV-2 antibody against (a) spike (S) and (b)

484   receptor binding domain (RBD) was measured using the MSD coronavirus pseudo-

485   neutralisation assay. 183 COVID-19 cohort samples and 194 control samples were

486   analysed. Graphs show median and 95% CI with a line showing neutralisation assay

487   positive/negative discrimination cut-off determined by ROC. The correlation between

488   antibody concentration and percentage inhibition of (c) S and (d) RBD antigens in all

489   positive group samples was assessed and r and p was determined by Spearman

490   correlation, line shows binding assay positive/negative discrimination cut-off.

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493   Tables

494   Table 1: The lower limit of detection (LLOD), upper limit of detection (ULOD), quality

495   control (QC) sample range in arbitrary units (AU) and positive/negative cut-off for each

496   SARS-CoV-2 antigen analysed.

Antigen	LLOD (max.) (AU)	ULOD (min.) (AU)	QC sample range (AU)	Positive/ negative cut-off
CoV-2 S	21.54	NA	1092-1478	21.5
CoV-2 RBD	18.37	125477	2176-2944	201.7
CoV-2 N	35.49	NA	3627-4907	185.4
CoV-2 NTD	10.00	19452	1004-1359	1924

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499   Table 2: Assay specificity calculated for each SARS-CoV-2 antigen from the control

500   cohort.

Antigen	n	Positive	Negative	Specificity (95% CI) (%)
CoV-2 S	194	5	189	97.4% (94.1 to 98.9)
CoV-2 RBD	194	15	179	92.3% (87.6 to 95.3)
CoV-2 N	194	14	180	92.8% (88.2 to 95.7)

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502   Table 3: Assay sensitivity by time since onset of symptoms for each SARS-CoV-2

503   antigen calculated using the COVID-19 cohort with verified time between onset of

504   symptoms and blood sampling. Time was divided into 0-7 days, over 7 days, over 14

505   days and over 21 days since the onset of symptoms.

Antigen	Group		n	Positive	Negative	Sensitivity (95% CI) (%)
CoV-2 S	Total		196	178	18	90.8% (86.0 to 94.1)
	Time since onset of symptoms	0-7 days	20	15	5	75.0% (53.1 to 88.8)
		Over 7 days	148	138	10	93.2% (88.0 to 96.3)
		Over 14 days	78	75	3	96.2% (89.3 to 99.0)
		Over 21 days	47	46	1	97.9% (88.8 to 99.9)
CoV-2 RBD	Total		196	153	43	78.1% (71.8 to 83.3)
	Time since onset of symptoms	0-7 days	20	12	8	60.0% (38.7 to 78.1)
		Over 7 days	148	119	29	80.4% (73.3 to 86.0)
		Over 14 days	78	71	7	91.0% (82.6 to 95.6)
		Over 21 days	47	44	3	93.6% (82.8 to 97.8)
CoV-2 N	Total		196	143	53	73.0% (66.3 to 78.7)
	Time since onset of symptoms	0-7 days	20	12	8	60.0% (38.7 to 78.1)
		Over 7 days	148	106	42	71.6% (63.9 to 78.3)
		Over 14 days	78	66	12	84.6% (75.0 to 91.0)
		Over 21 days	47	41	6	87.2% (74.8 to 94.0)

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507   Supplementary Tables

508   Table S1: Intra and inter-assay variability. Within plate (intra) and between plate (inter)

509   assay repeatability was assessed by running four samples (1-4) of varying antibody

510   levels in four replicates on the same plate and across 4 different runs on different days

Antigen	Control serum	Average conc. (AU)	Average intra-assay %CV	Average inter-run %CV
CoV-2 S	1	2063.8	3.5%	1.5%
	2	2579.8	7.2%	1.8%
	3	<LLOD	NA	NA
	4	1282.3	5.7%	8.9%
CoV-2 RBD	1	1811.0	4.8%	2.1%
	2	2290.2	6.5%	2.2%
	3	144.7	8.3%	NA
	4	2301.3	3.4%	7.6%
CoV-2 N	1	3380.2	10.9%	0.4%
	2	5934.2	2.4%	6.3%
	3	<LLOD	6.9%	2.1%
	4	3557.3	7.5%	7.6%

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Figure 1: Anti-SARS-CoV-2 IgG concentration.

The concentration of SARS-CoV-2 antibody against (a) spike (S), (b) receptor binding domain (RBD), (c) nucleocapsid (N) and (d) N terminal domain (NTD) was measured using the MSD coronavirus panel. Graphs show data in arbitrary units (AU) (based on the calibrated internal standard serum) in the COVID-19 cohort (n=196) and controls (n=194, pre-December 2019). Line shows positive/negative discrimination cut-off.

Figure 2: Receiver Operating Characteristic (ROC) curves for each SARS-CoV-2 antigen.

Sensitivity and specificity were calculated using each value in the data table as a cut-off value (n=390). Graphs show the sensitivity vs 100%-specificity of SARS-CoV-2 antigen (a) spike (S), (b) receptor binding domain (RBD), (c) nucleocapsid (N) and (d) N terminal domain (NTD). The area under curve (AUC) and 95% CI is also shown for each antigen.

Figure 3: Anti-SARS-CoV-2 IgG concentration according to time since onset of symptoms.

Graphs show the concentration of SARS-CoV-2 antibody against (a) spike (S), (b) receptor binding domain (RBD) and (c) nucleocapsid (N) in arbitrary units (AU) (based on the calibrated internal standard serum) of the COVID-19 cohort split in to intervals of 0-7 days, 8-14 days, 15-21 days and over 21 (>21) days since symptom onset (to sample collection). Error bars show geometric mean with 95% CI, line shows positive/negative discrimination cut-off, *$p<0.05$, ** $p<0.01$ determined by Dunn's multiple comparisons test. Comparisons across interval groups had $p<0.0001$ by one-way ANOVA Kruskal-Wallis test. The assay sensitivity at each time point is shown in Table 3.

Figure 4: IgG concentration relationship between antigens.
Correlation between anti-SARS-CoV-2 antibody concentration of all COVID-19 group samples (n=196) (a) S vs RBD, (b) S vs N and (c) N vs RBD. r and p value were determined by Spearman correlation. p values of <0.05 were considered as significant.
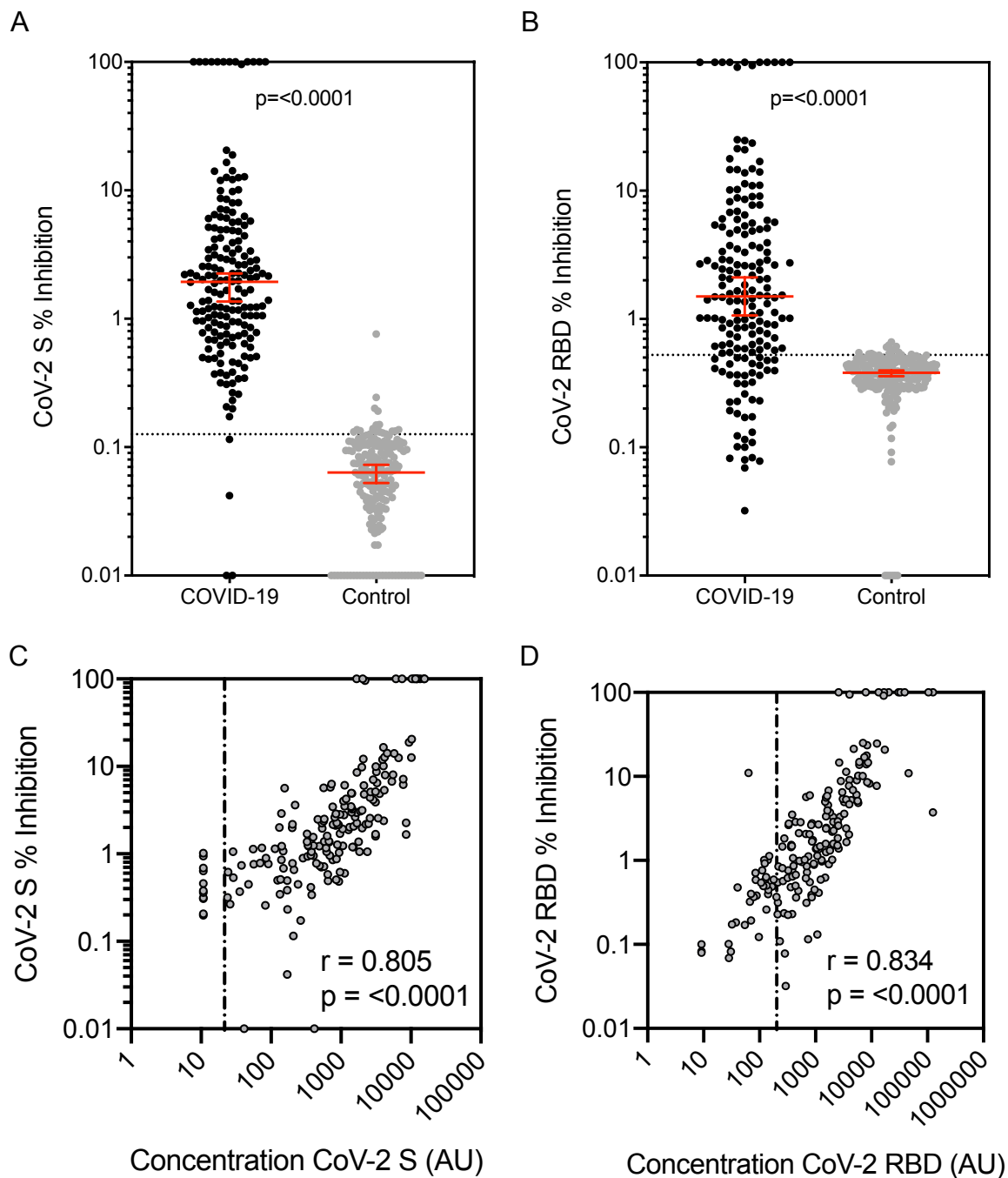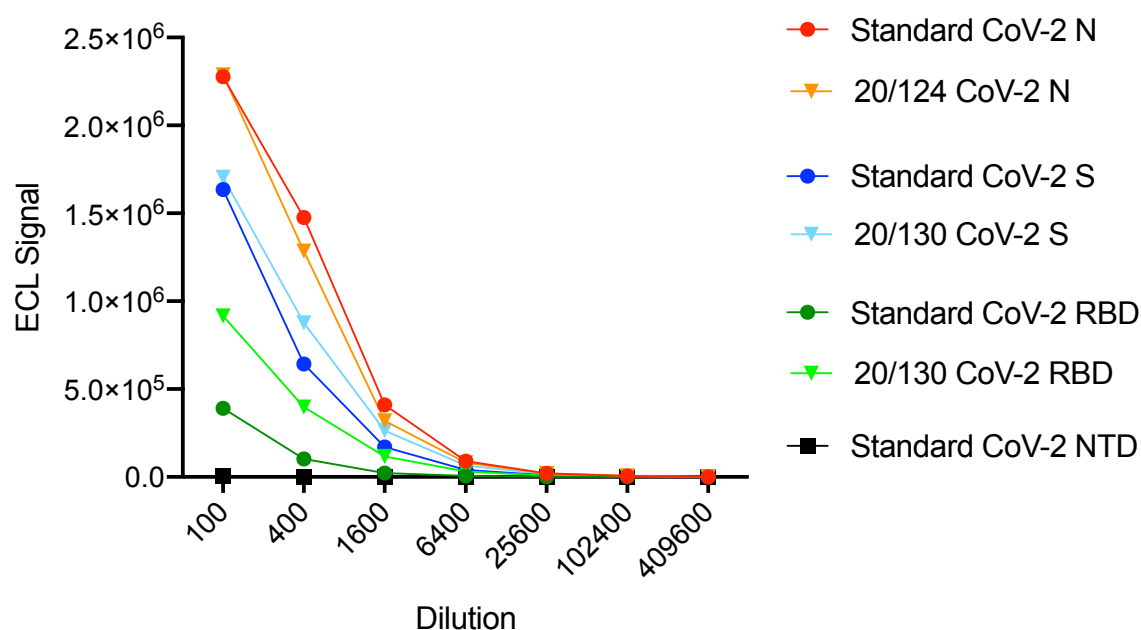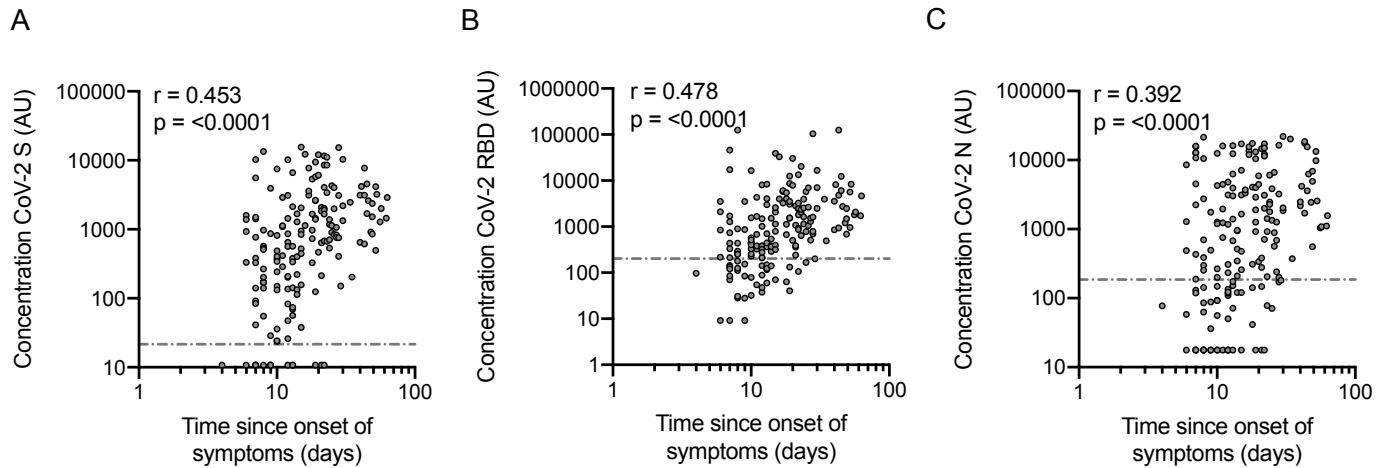
Figure 5: Percentage inhibition by anti-SARS-CoV-2 S and RBD antibody measured by MSD pseudo-neutralisation assay.

Inhibition of ACE-2 binding by SARS-CoV-2 antibody against (a) spike (S) and (b) receptor binding domain (RBD) was measured using the MSD coronavirus pseudo-neutralisation assay. 183 COVID-19 cohort samples and 194 control samples were analysed. Graphs show median and 95% CI with a line showing neutralisation assay positive/negative discrimination cut-off determined by ROC. The correlation between antibody concentration and percentage inhibition of (c) S and (d) RBD antigens in all positive group samples was assessed and r and p was determined by Spearman correlation, line shows binding assay positive/negative discrimination cut-off.
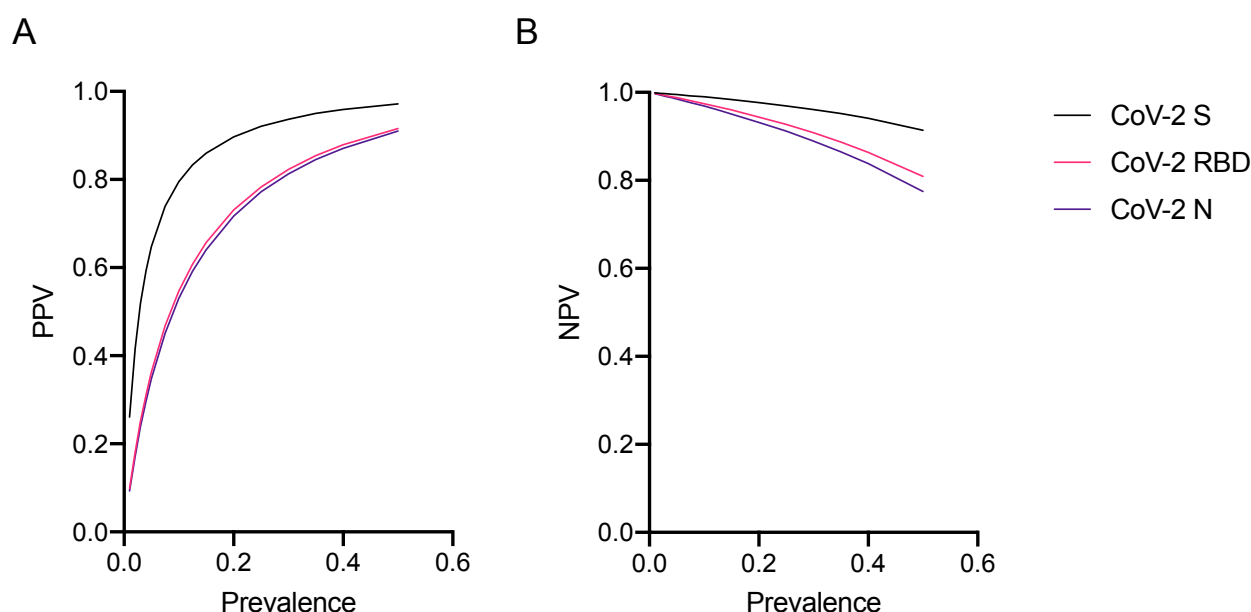
Supplementary Figure 1: Assignment of standard values to internal standard serum and standard curves for each antigen.

Graph shows ECL signal obtained from a serial dilution series (1 in 100, then 1 in 4 serial dilution) of standard serum and NIBSC control sera 20/130 and 10/124. NIBSC control serum 20/130 was used to assign values to standard serum for SARS-CoV-2 spike (S) and receptor binding domain (RBD) and NIBSC control serum 20/124 was used to assign a value to SARS-CoV-2 nucleocapsid (N). No endpoint titre corresponding to NTD antigen was available for standard serum assignment.

Supplementary Figure 2: Relationship with time since onset of symptoms.

Graphs show the relationship between antibody concentration against against (a) spike (S), (b) receptor binding domain (RBD) and (c) nucleocapsid (N) for all samples with known and verified time since onset of symptoms to sampling (n=176). Correlation analysis was performed using Spearman correlation. P values of <0.05 were considered as significant.

Supplementary Figure 3: Positive and negative predictive values (PPV and NPV).

Graphs show the positive (PPV) and negative (NPV) predictive values for each antigen at a range of prevalence estimates between 0.01 and 0.5 based on fixed specificity and sensitivity values calculated for the whole COVID-19 and control groups (97.4%, 92.3% and 92.8% specificity; 90.8%, 78.1% and 73.0% specificity for S, RBD and N respectively).