

1 A haemagglutination test for rapid detection of antibodies to SARS-CoV-2

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69

70 **ABSTRACT**

71 Serological detection of antibodies to SARS-CoV-2 is essential for establishing rates of
72 seroconversion in populations, detection of seroconversion after vaccination, and for seeking
73 evidence for a level of antibody that may be protective against COVID-19 disease. Several
74 high-performance commercial tests have been described, but these require centralised
75 laboratory facilities that are comparatively expensive, and therefore not available universally.
76 Red cell agglutination tests have a long history in blood typing, and general serology through
77 linkage of reporter molecules to the red cell surface. They do not require special equipment,
78 are read by eye, have short development times, low cost and can be applied as a Point of Care
79 Test (POCT). We describe a red cell agglutination test for the detection of antibodies to the
80 SARS-CoV-2 receptor binding domain (RBD). We show that the Haemagglutination Test
81 ("HAT") has a sensitivity of 90% and specificity of 99% for detection of antibodies after a PCR
82 diagnosed infection. The HAT can be titrated, detects rising titres in the first five days of
83 hospital admission, correlates well with a commercial test that detects antibodies to the RBD,
84 and can be applied as a point of care test. The developing reagent is composed of a previously
85 described nanobody to a conserved glycophorin A epitope on red cells, linked to the RBD from
86 SARS-CoV-2. It can be lyophilised for ease of shipping. We have scaled up production of this
87 reagent to one gram, which is sufficient for ten million tests, at a cost of ~0.27 UK pence per
88 test well. Aliquots of this reagent are ready to be supplied to qualified groups anywhere in
89 the world that need to detect antibodies to SARS-CoV-2, but do not have the facilities for high
90 throughput commercial tests.

91

92 **INTRODUCTION**

93 Red cell agglutination tests have a distinguished history. Since Landsteiner's classic
94 observations in 1901 (Landsteiner, 1961) (English translation), they have been used for the

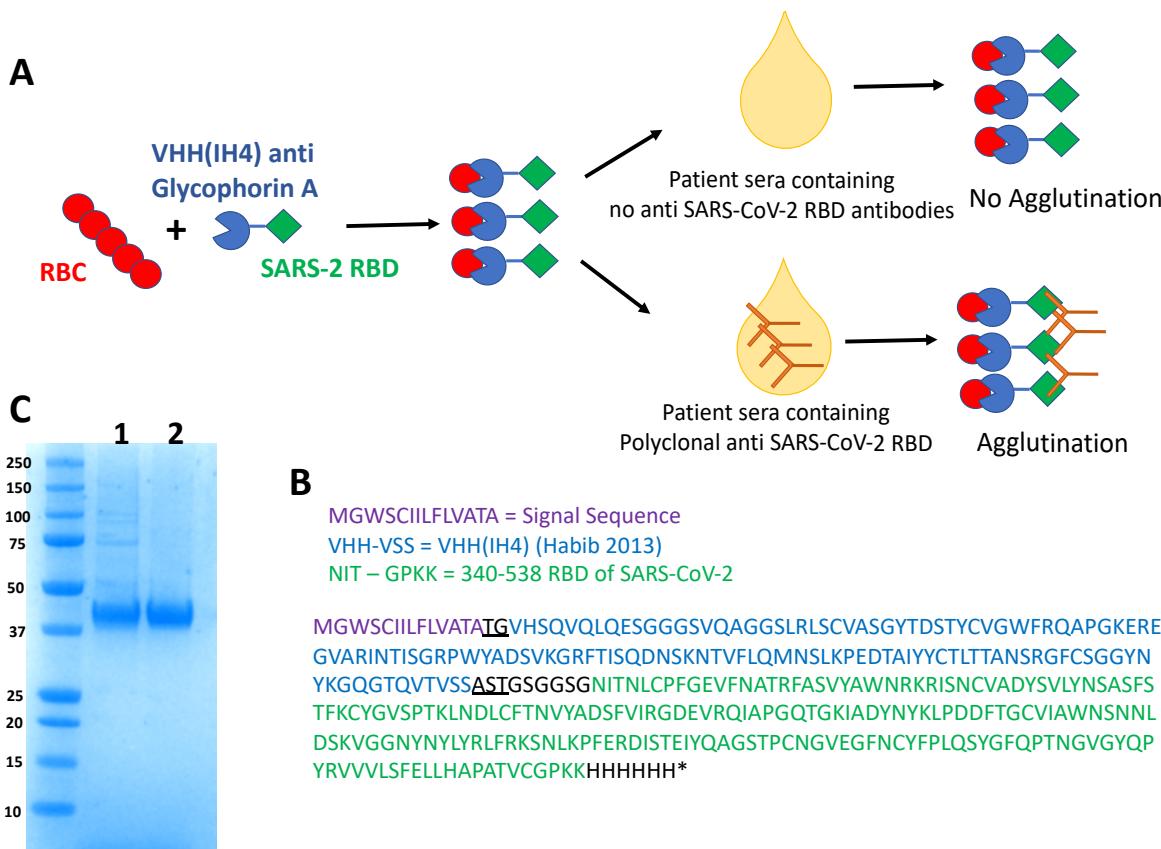
95 determination of blood groups (Schwarz & Dorner, 2003) detection of influenza viruses (Hirst,
96 1941) and in a wide variety of applications championed by Prof. Robin Coombs for the
97 detection of specific antibodies or antigens (Coombs, Mourant, & Race, 1945) (reviewed by
98 (Pamphilon & Scott, 2007)). They have the great advantage of being simple, inexpensive, can
99 be read by eye, and do not require sophisticated technology for their application. In the
100 recent era the linkage of an antigen to the red cell surface has become easier with the
101 possibility of fusing a protein antigen sequence with that of a single domain antibody or
102 nanobody specific for a molecule on the red cell surface (discussed in (Habib et al., 2013)).
103

104 We have applied this concept to provide a simple Haemagglutination Test (“HAT”) for the
105 detection of antibodies to the Receptor Binding Domain (RBD) of the SARS-CoV-2 spike
106 protein. The RBD is a motile subdomain at the tip of the SARS-CoV-2 spike protein that is
107 responsible for binding the virus to its ACE2 receptor. The RBD of betacoronaviruses folds
108 independently of the rest of the spike protein (Lan et al., 2020; Li, Li, Farzan, & Harrison, 2005;
109 Wang et al., 2020; Yan et al., 2020). This useful property provides an Achilles’ heel for the
110 virus and allows many potential applications in vaccine design (Dai et al., 2020; Mulligan et
111 al., 2020; Tan et al., 2020; Walls et al., 2020; Yang et al., 2020; Zha et al., 2020), and serology
112 (Amanat et al., 2020; Piccoli et al., 2020; The National SARS-CoV-2 Serology Assay Evaluation
113 Group, 2020), see also www.gov.uk/government/publications/COVID-19-laboratory-evaluations-of-serological-assays). The majority of neutralising antibodies bind to the RBD
114 (Barnes et al., 2020; Piccoli et al., 2020), and the level of antibody to the RBD detected in
115 ELISA correlates with that of neutralising antibodies (Amanat et al., 2020; Piccoli et al., 2020;
116 Robbiani et al., 2020). We reasoned therefore that a widely applicable and inexpensive test
117 for antibodies to the RBD would be useful for research in settings where high throughput
118 assays were not available.
119

120
121 In order to link the SARS-CoV-2 RBD to red cells we selected the single domain antibody
122 (nanobody) IH4 (Habib et al., 2013), specific for a conserved epitope on glycophorin A.
123 Glycophorin A is expressed at up to 10^6 copies per red cell. The IH4 nanobody has previously
124 been linked to HIV p24 to provide a monomeric reagent that bound p24 to the red cell surface.
125 Antibodies to p24 present in serum cross-linked the p24 and agglutinated the red cells (Habib
126 et al., 2013). We have adapted this approach to detection of antibodies to SARS-CoV-2 by

127 linking the RBD of the SARS-CoV-2 spike protein to IH4 via a short (GSG)2 linker to produce
128 the fusion protein IH4-RBD-6H (Figure 1). Since we embarked on this project, another group
129 has described preliminary results with an approach similar to ours, but using a fusion of the
130 RBD to an ScFV against the H antigen to coat red blood cells with the SARS-2 RBD (Kruse et
131 al., 2020).

132
133



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135

136 **Figure 1. Haemagglutination Test (HAT) for detection of antibodies to SARS-CoV-2 Receptor
137 Binding Domain.**

138 A) Concept of the HAT
139 B) Sequence of VHH(IH4)-RBD fusion protein. Residues underlined are encoded by cloning
140 sites AgeI (TG) and SalI (AST). The codon optimised cDNA sequence is shown in supplementary
141 Information
142 C) SDS-PAGE gel of purified VHH(IH4)-RBD proteins. Three micrograms of protein were run on
143 4-12% Bolt Bis-Tris under reducing conditions. 1: IH4-RBD produced in house in Expi293F cells,
144 2: IH4-RBD produced by Absolute Antibody, Oxford in HEK293 cells.

145

146 RESULTS

147

148 Production of the IH4-RBD Reagent

149 The IH4-RBD sequence (Figure 1B) was codon optimised and expressed in Expi293F cells in a
150 standard expression vector (available on request). One advantage of this mode of production
151 compared to bacterially produced protein as used by Habib et al, is that the reagent will carry
152 the glycosylation moieties found in humans, which may play a role in the antigenicity of the
153 RBD (Pinto et al., 2020). The protein (with a 6xHis tag at the C-terminus for purification) was
154 purified by Ni-NTA chromatography which yielded ~160 mg/L. We later had one gram of the
155 protein synthesised commercially by Absolute Antibody, Oxford. The IH4-RBD protein ran as
156 single band at ~40 kDa on SDS PAGE (Figure 1C).

157

158 **Establishment of the Haemagglutination Test (HAT) with monoclonal antibodies to the RBD**
159 One purpose envisaged for the HAT is for use as an inexpensive Point of Care Test for
160 detection of antibodies in capillary blood samples obtained by a “finger-prick”. We therefore
161 wished to employ human red cells as indicators without the need for cell separation or
162 washes, to mimic this setting. The use of V-bottom microtiter plates to perform simplified
163 hemagglutination tests was first described over 50 years ago (Wegmann & Smithies, 1966).
164 In preliminary tests, we observed that 50 µL of whole blood (K2EDTA sample) diluted 1:40 in
165 phosphate-buffered saline (PBS), placed in V-bottomed wells of a standard 96-well plate,
166 settled in one hour to form a button of red cells at the bottom of the well. The normal
167 hematocrit of blood is ~40% vol/vol, so this dilution provides ~1% red cells. If the plate was
168 then tilted, the red cell button flowed to form a “teardrop” in ~30 seconds (for example Figure
169 2A Row 8).

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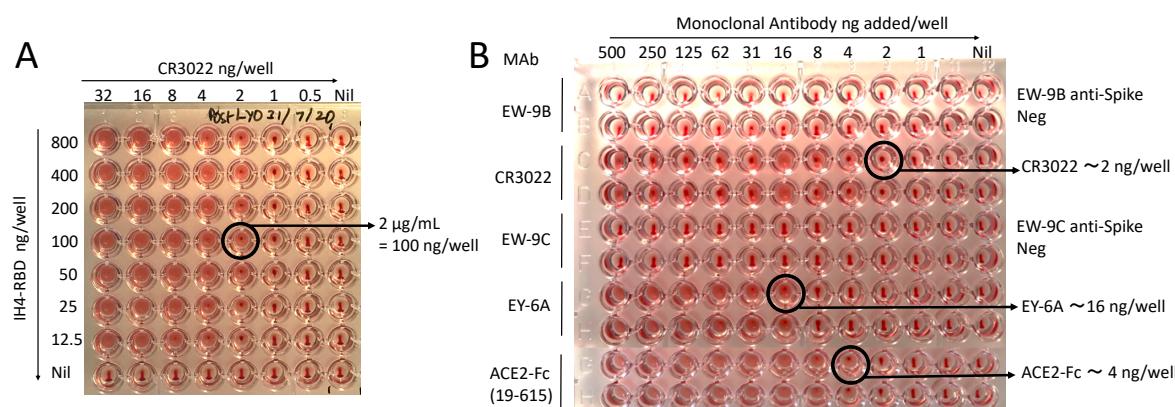
171 If serum or plasma samples are to be tested, a standard collection of 10 mL of Type O Rh-
172 negative (O-ve) blood into a K2EDTA tube will thus provide sufficient red cells for 8,000 test
173 wells.

174

175 A well characterised monoclonal antibody to the RBD, CR3022 (ter Meulen et al., 2006) added
176 to the red cells at between 0.5-32 ng/well in 50 µL, did not agglutinate the cells on its own

177 (Figure 2A Row 8). The addition of the IH4-RBD reagent at between 12.5-800 ng/well (in 50
178 µL PBS) induced a concentration dependent agglutination of the red cells, detected by the
179 formation of a visible mat or plug of agglutinated cells, and the loss of teardrop formation on
180 tilting the plate (Figure 2A). From repeated trials of this experiment we established that a
181 standard addition of 100 ng/well of the IH4-RBD developer (50 µL of a stock solution of 2
182 µg/mL in PBS) induced agglutination of 50 µL of 1:40 human red cells in the presence of as
183 little as 2 ng/well of the CR3022 monoclonal antibody. The standardised protocol used for the
184 subsequent tests were thus performed in 100 or 150 µL final volume, containing 100 ng of
185 the IH4-RBD developer, and 50 µL 1:40 whole blood (~1% v/v red cells ~ 0.5 µL packed red
186 cells per reaction). After 60 minutes incubation at room temperature, we routinely
187 photographed the plates after the 30 s tilt for examination and reading.

188
189 The requirement for 100 ng of the IH4-RBD developer per test well means that the gram of
190 IH4-RBD protein we have had synthesised is sufficient for 10 million test wells at a cost of
191 approximately 0.27 UK pence per test.



193 **Figure 2. Haemagglutination with human monoclonal antibodies or nanobodies to the**
194 **SARS-CoV-2 RBD**

195 A) Titration of IH4-RBD and monoclonal Antibody CR3022 to RBD. Doubling dilutions of
196 CR3022 and IH4-RBD were prepared in separate plates. 50 µL red cells (O-ve whole blood
197 diluted 1:40 in PBS) were added to the CR3022 plate, followed by transfer of 50 µL titrated
198 IH4-RBD. From this titration, 100 ng/well of IH4-RBD was chosen for detection.

199 B) Detection of other anti-RBD monoclonal antibodies and ACE2-Fc. Monoclonal antibodies
200 were prepared in doubling dilutions in 50 µL PBS from left to right, 50 µL of 1:40 O-ve red cells
201 were added, followed by 50 µL of IH4-RBD (2 µg/mL in PBS). The end point was defined as the

202 last dilution without tear drop formation on tilting the plate for ~ 30 s. The binding sites for
203 CR3022, EY6A and ACE2 on RBD have been defined (Huo, Zhao, et al., 2020; Lan et al., 2020;
204 Yan et al., 2020; Zhou et al., 2020). EW-9B and EW-9C are monoclonal antibodies against non-
205 RBD epitopes on the spike protein (Huang et al., 2020). ACE2-Fc has been described (Huang
206 et al., 2020).

207

208 Having established a standard addition of 100 ng/well of the IH4-RBD reagent, we screened a
209 set of twelve human monoclonal antibodies, two divalent nanobodies, and divalent ACE2-Fc,
210 that are known to bind to the RBD (Huang et al., 2020; Huo, Le Bas, et al., 2020; Pinto et al.,
211 2020; Wrapp et al., 2020; Zhou et al., 2020). These reagents bind to at least three independent
212 sites on the RBD, and some are strongly neutralising and capable of profound ACE2 blockade
213 (Huang et al., 2020; Huo, Le Bas, et al., 2020). Twelve of the 15 divalent molecules
214 agglutinated red cells and titrated in the HAT to an end point between 2-125 ng/well, after
215 addition of 100 ng IH4-RBD (Figure 2B and Supplementary Table 1). Two monoclonal
216 antibodies, FD-5D and EZ-7A (Huang et al., 2020) and one divalent nanobody VHH72-Fc
217 (Wrapp et al., 2020), failed to agglutinate red cells in the presence of the IH4-RBD reagent.
218 However, if a monoclonal antibody to human IgG was added to the reaction (50 µL of mouse
219 anti-human IgG, Sigma Clone GG5 1:100), these molecules specifically agglutinated the red
220 cells (Supplementary Figure 1A, B). This result, analogous to the “indirect” Coombs Test
221 (Coombs et al., 1945; Pamphilon & Scott, 2007), suggested that these three molecules had
222 bound to the RBD associated with the red cells but failed to crosslink to RBDs on neighbouring
223 red cells. However, these could be crosslinked by the anti IgG reagent. Monoclonal antibodies
224 to other regions of the spike protein (EW-9B, EW-9C and FJ-1C) failed to agglutinate red cells
225 (Figure 2B, Supplementary Table 1). Finally we looked at the effect of a divalent ACE2-Fc
226 molecule constructed by fusing the peptidase domain of ACE2 (amino acids 19-615) to the
227 hinge and Fc region of human IgG1 (described in (Huo, Le Bas, et al., 2020). ACE2-Fc
228 agglutinated red cells strongly in the presence of 100 ng/well of the IH4-RBD developer,
229 titrating to ~ 4 ng/well (Figure 2B rows 7,8).

230

231 In summary, these results showed that all of the known epitopes bound by characterised
232 monoclonal antibodies were displayed by the IH4-RBD reagent, as well as the ACE2 binding
233 site, and could mediate agglutination by specific antibodies, divalent nanobodies, or ACE2-Fc.

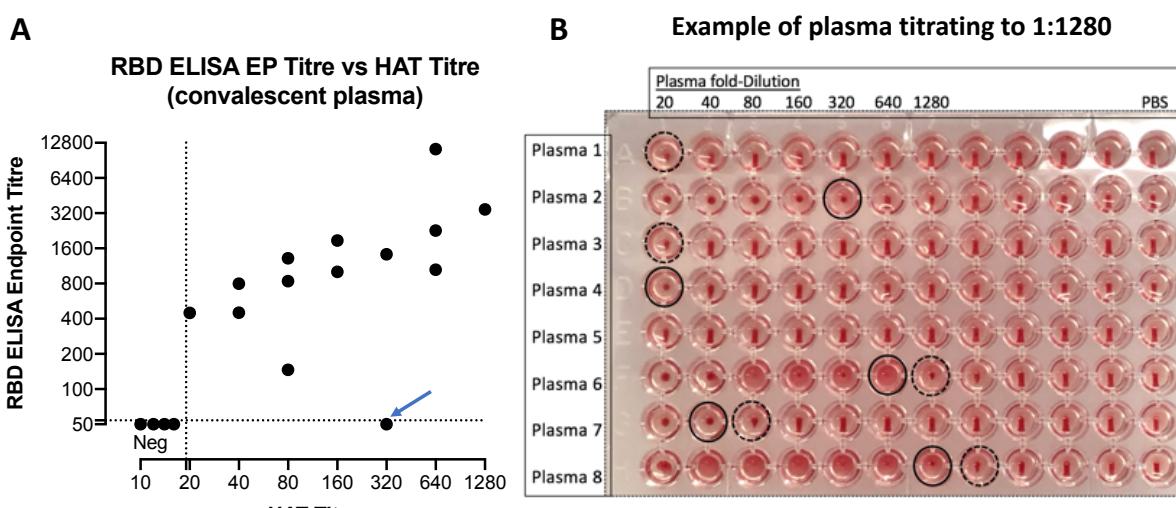
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235 **Agglutination by plasma from donors convalescing from COVID-19**

236 These experiments established the conditions for detection of haemagglutination by
237 monoclonal antibodies to the RBD, in particular the optimum concentration of IH4-RBD of
238 100 ng/well. We then proceeded to look for haemagglutination by characterised plasma from
239 COVID-19 convalescent donors. In the first trial we tested eighteen plasma samples from
240 patients with mostly mild illness, that had been characterised with a quantitative ELISA to
241 detect antibodies to the RBD (Peng et al., 2020). For these experiments we used fresh O-ve
242 blood (K2EDTA sample) diluted to 1:40 as a source of red cells to avoid agglutination by
243 natural agglutinins in the plasma. Plasmas were titrated by doubling dilution from 1:20 in 50
244 µL, then 50 µL of 1:40 O-ve red cells were added, followed by addition of 100 ng of the IH4-
245 RBD in 50 µL PBS. After one-hour incubation, plates were tilted for ~30 seconds,
246 photographed and read. The titre of agglutination was assessed by complete loss of teardrop
247 formation by the red cells, any formation of a teardrop was regarded as negative. Figure 3A
248 shows that the HAT titre matched the RBD ELISA results. Four samples were scored as
249 negative in both assays. The remaining results showed that in general the HAT titre increased
250 with the ELISA end point titre. One sample gave a positive titre of 1:320 in the HAT but was
251 negative in ELISA (indicated with an arrow). We investigated this sample with a developer
252 composed of the IH4 nanobody without the RBD component, which revealed that
253 agglutination was RBD dependent (not shown). This sample was also positive at 1:1123 in an
254 ELISA for full length spike protein (not shown), which suggests that the antibodies contained
255 in this serum recognised epitope(s) present on the RBD exposed in the HAT, but not on the
256 RBD in the RBD-ELISA reference test (Peng et al., 2020). The highest titre detected in these
257 samples by the HAT was 1:1280 (Figure 3B).

258

259 These preliminary results showed that the HAT could detect antibodies to the RBD in plasma
260 samples from convalescent patients in a similar manner to an ELISA test, but were not
261 sufficient to establish the sensitivity and specificity of the HAT.



262

263 **Figure 3. Titration of stored plasma in the agglutination assay.**

264 A) Eighteen plasma samples from mild cases were compared for titration in the HAT with 1:40
265 O-ve whole blood from a seronegative donor, and endpoint titre in an RBD ELISA (Peng et al.,
266 2020)). Four samples were negative in both assays. The data point marked with an arrow on
267 the graph (plasma 2 on the plate, Fig 3B) was checked with a reagent composed of IH4 without
268 RBD and shown to be dependent on antibodies to the RBD. This sample did score positive for
269 antibodies to full length spike in an ELISA (EPT 1:1123).

270 B) An example of titration: positive agglutination endpoints (loss of teardrop) are marked with
271 a black solid-line circle, partial teardrops are marked with a dotted-line circle.

272

273 **Sensitivity and specificity of the Haemagglutination Test**

274 To formally assess the sensitivity and specificity of the HAT we collected a set of 98 “positive”
275 plasma samples from donors diagnosed with COVID-19 by RT-PCR at least 28 days prior to
276 sample collection (NHS Blood and Transplant), and 199 “negative” serum samples from
277 healthy donors from the pre-COVID-19 era (Oxford Biobank). The samples were randomised
278 before plating. The test wells were arranged in duplicate to contain serum/plasma at 1:40
279 dilution, and 1:40 O-ve red cells in 50 µL. 100 ng of IH4-RBD in 50 µL PBS was added to one
280 well of the pair, 50 µL of PBS to the other (as a negative control). The negative control is
281 important because in rare cases, particularly in donors who may have received blood
282 transfusions, the sample may contain antibodies to non-ABO or Rhesus D antigens. After
283 development, the plates were photographed, and read by two independent masked
284 observers. Complete loss of teardrop was scored as positive, any flow in the teardrop as

285 negative. These rules were established before setting up the tests. Disagreements (7%
286 overall) were resolved by accepting the weaker interpretation – i.e. if one observer scored
287 positive but the other negative, the well was scored as negative. Having completed the
288 scoring the columns of samples were re-randomised and the test and scoring repeated.

289

290 Examples of test wells and scoring are shown in Figure 4. The red cells in the negative control
291 (PBS) wells formed a clear teardrop. Red cells in positive wells (indicated with a solid ring)
292 settled either into a mat or a button that failed to form any teardrop on tilting for 30 seconds.
293 Occasional wells (15 of 297) formed a “partial” teardrop (shown by a dashed ring). These were
294 scored as negative by prior agreement.

295

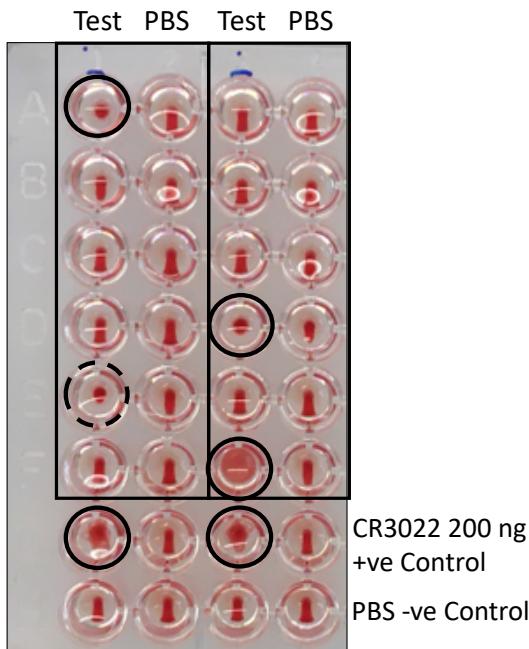
296 With these rules in place we obtained in the first run sensitivity 88%, specificity 99%, and in
297 the second run sensitivity 93%, specificity 99% (Figure 4). The Siemens Atellica
298 Chemiluminescence assay for detection of IgG antibodies to the RBD was run in parallel on
299 293 of these 297 samples and gave sensitivity 100%, specificity 100% for this sample set.

300

301 We decided prior to this formal assessment to score wells with partial teardrop formation as
302 negative, as these wells tended to give rise to disagreements between scorers and were not
303 very helpful. Fifteen of 297 wells gave a partial teardrop. Six of these fifteen were from PCR-
304 ve donors and scored negative on the Siemens assay, 9/16 were from PCR+ve donors and
305 were Siemens positive. If partial teardrops were scored as positive, the sensitivity of the two
306 assays increased to 97% and 99% (from 88% and 93%), but specificity was reduced to 96%
307 and 98% (from 99%). This small loss of specificity would be unacceptable in sero-surveys
308 where the expected prevalence of previous SARS-CoV-2 infection was low.

309

A Examples from screen of 297 test samples



B Operating Characteristics of the Haemagglutination Test

98 PCR +ve Samples >=28 days post diagnosis

199 –ve samples from The Oxford Biobank Pre Covid-19

| 11 June | | | |
|--|---------|---------|-----|
| Sensitivity and Specificity Calculator | | | |
| +VE = No Teardrop | | | |
| | HAT +VE | HAT -VE | |
| PCR +VE | 86 | 12 | 98 |
| PCR -VE | 1 | 198 | 199 |
| | 87 | 210 | 297 |
| Sensitivity % | 88 | | |
| Specificity % | 99 | | |
| Likelihood Ratio | 174.6 | | |

| 13 June REPEAT | | | |
|--|---------|---------|-----|
| Sensitivity and Specificity Calculator | | | |
| +VE = No Teardrop | | | |
| | HAT +VE | HAT -VE | |
| PCR +VE | 91 | 7 | 98 |
| PCR -VE | 1 | 198 | 199 |
| | 92 | 205 | 297 |
| Sensitivity % | 93 | | |
| Specificity % | 99 | | |
| Likelihood Ratio | 184.8 | | |

310

Figure 4. Operating characteristics of the HAT.

311 A) The test set of 297 randomised plasma samples were diluted 1:40 mixed with 1:40 O-ve
312 blood in two columns. IH4-RBD (100 ng in 50 µL) was added to the test samples, and PBS to
313 negative control wells. The plates were incubated at room temperature for one hour to allow
314 the red cell pellet to form, then tilted for ~30 seconds to allow a teardrop to form. Complete
315 loss of teardrop was scored as positive agglutination (marked with a black solid-line circle).
316 Full teardrop or partial teardrop (marked with a dotted-line circle) were scored negative. The
317 samples in columns were re-randomised and tested for a second time two days later.
318

319 B) Contingency table showing the operating characteristics of the HAT.

320

HAT in the Hospital Setting

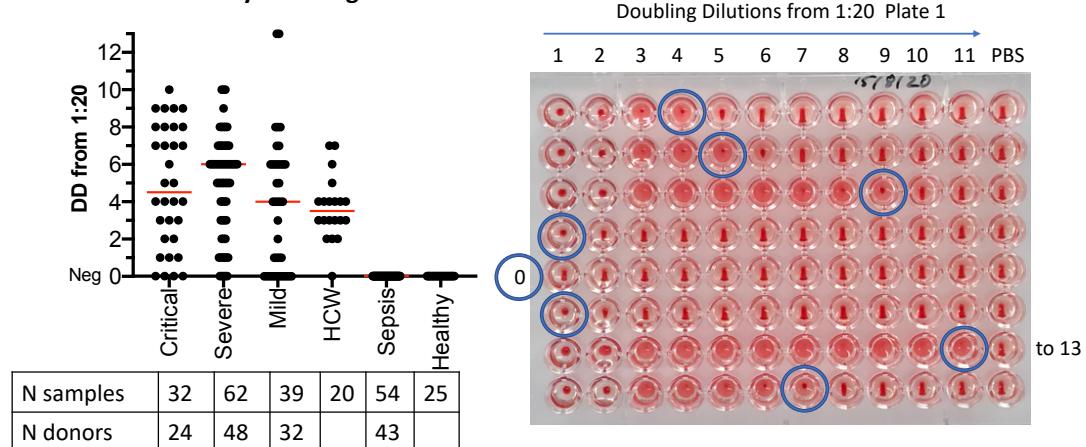
321 We next assessed the HAT in the setting of patients recently admitted to hospital (the first
322 five days) through access to the COMBAT collection of samples (see methods). This set
323 comprised 153 plasma samples from donors diagnosed with COVID-19 by PCR, with clinical
324 syndromes classified as « Critical », « Severe », « Mild », and «PCR positive Health Care
325 Workers ». Seventy-nine control plasma samples donated in the pre-COVID-19 era were

327 obtained either from patients with bacterial sepsis (54 samples), or healthy volunteers (25
328 samples). Samples were titrated in 11 doubling dilutions of 50 µL from 1:40 – 1:40,096
329 (columns 1-11). Column 12 contained 50 µL PBS as a negative control. 50 µL of 1:40 O-ve
330 whole blood was added, followed by 50 µL of 2 µg/mL IH4-RBD (100 ng/well). In parallel, all
331 of the 153 samples from PCR positive donors were assessed by the Siemens Atellica
332 Chemiluminescence test for antibodies to the RBD of the spike protein. The HAT scores (as
333 the number of doubling dilutions of the sample required to reach the endpoint of complete
334 loss of teardrop), and representative agglutination results are shown in figure 5A. In Figure
335 5B the HAT scores are plotted with their related Siemens test scores.

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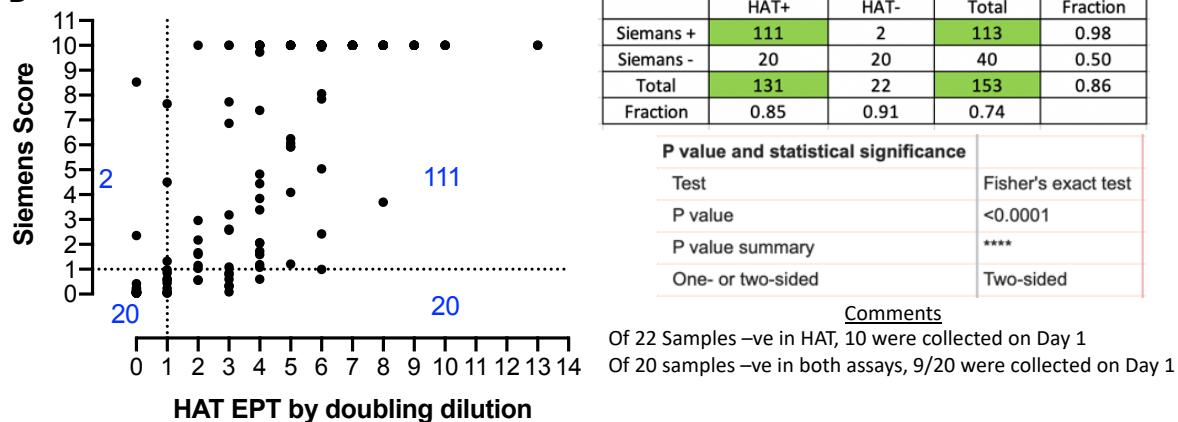
337 None of the seventy-nine negative control samples scored as positive in the HAT at a dilution
338 of 1:40, thus providing 100% specificity in this set of samples. The HAT detected 131/153 (86%
339 sensitivity) of the samples from PCR-diagnosed donors within the first five days of hospital
340 admission, whereas the Siemens test detected 113/153 (74%). On day 5 the HAT detected
341 41/45 (91% sensitivity). Two samples had an endpoint greater than 11 doubling dilutions in
342 the HAT, and required a repeat measurement spanning two plates. These two samples
343 titrated to 13 doubling dilutions (1: 163,840). Unmasking the samples revealed that both were
344 acquired from an elderly lady with mild disease on days 3 and 5 of her admission. The range
345 of positive titres detected by HAT was broad: 1 to 13 doubling dilutions (1:40 – 1:163,840). A
346 correlation coefficient with the Siemens test could not be calculated as the latter has a ceiling
347 score of 10 (Figure 5B). A comparison of the two tests in a contingency table with cut-off of
348 1:40 (first doubling dilution) for HAT, and a score ≥ 1 for the Siemens test (as defined by the
349 manufacturer), showed a strong correlation between the two tests for detection of antibodies
350 to the RBD ($P < 0.0001$; two-tailed Fisher's exact test, Figure 5B). Fifty-two of the 153 samples
351 were from 24 donors with COVID-19 from whom repeated samples were taken on days 1, 3,
352 or 5 of admission. The HAT detected a rise in agglutination titre over the first five days of
353 admission in 16/24 (67%) of these patients (Table 1). Reductions in titre were not detected.

A Titration in HAT by doubling dilution



Donors were grouped according to clinical criteria; HCW = Front Line Health Care Workers; Sepsis = Samples with patients with Sepsis prior to Covid-19 pandemic; Healthy = Healthy volunteers prior to Covid-19; DD 1 = 1:40. Actual Titre = $20 \times 2^{\text{DD}}$.

B



354

355 Figure 5. Titration of the set of 232 samples in the HAT

356 A) The collection included 32 samples from 24 Critical patients, 62 samples from 48 Severe,
 357 39 samples from 32 Mild, 20 single samples from health care workers (HCW), 54 samples from
 358 43 patients with unrelated sepsis in the pre-COVID-19 era, 25 samples from healthy
 359 unexposed controls. Median is indicated by a red line. DD: doubling dilutions.

360 B) Comparison to Siemens Result (anti RBD) with HAT titre by doubling dilution for 153
 361 samples from Critical, Severe, Mild and HCW SARS-CoV-2 PCR positive donors.

362

363 These results showed that in the setting of hospital admission in the UK for suspected COVID-
 364 19 disease, the HAT has an overall sensitivity of 86% and specificity of 100% by day five, and
 365 frequently (67%) detected a rise in HAT titre during the first five days of admission. In this
 366 context the HAT performed at least as well as the commercially available Siemens Atellica
 367 Chemiluminescence assay (74%) for the detection of antibodies to the RBD of SARS-CoV-2
 368 spike protein. Twenty samples were negative in both tests, but nine of these were taken on

369 day 1 of admission, which suggests that both tests have lower detection levels early in the
370 course of hospital admission, before the antibody response has fully developed.

371

372 The O-ve blood used as indicator for this experiment was collected into a heparin tube, and
373 then transferred to a K2EDTA tube. In order to be sure that the presence of heparin in the red
374 cells had not altered the behaviour of the test, and to confirm the robustness of the results,
375 we repeated the titrations on all of the 232 samples 34 days later, with fresh O-ve red cells
376 from a different donor collected as usual into a K2EDTA tube. The results are shown in
377 supplementary figures 2A-C. Specificity of the HAT remained at 100% (none of the 79 control
378 samples were detected as positive at 1:40). The correlation with the previous assay was
379 strong ($R^2 = 0.975$), and 99% of the 232 titrations were within one doubling dilution of the
380 matched earlier measurement. The slope of the correlation was 0.94 (95% CI 0.92-0.96),
381 significantly less than 1. This was due to a proportion of results titrating to one doubling
382 dilution lower titre. However, this had only a small impact on sensitivity (81% from 86%),
383 which was still an improvement on the Siemens test (74%) in this context of the first five days
384 of hospital admission. A rise in HAT titre in 16/24 during the first five days of admission was
385 confirmed.

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401 **Table 1. Fifty-two samples from 24 donors who were sampled repeatedly during the first**
 402 **five days in hospital.**

| No | Case | Day | | | | |
|----|------|------|---|--------|---|--------|
| | | 1 | 2 | 3 | 4 | 5 |
| 1 | C2 | | | 40 | | 160 |
| 2 | C6 | | | 40 | | 80 |
| 3 | C7 | 0 | | | | 0 |
| 4 | C9 | 40 | | | | 2560 |
| 5 | C10 | 320 | | 320 | | |
| 6 | C23 | 0 | | 160 | | 640 |
| 7 | S5 | 80 | | 160 | | |
| 8 | S6 | 1280 | | 1280 | | 2560 |
| 9 | S7 | 80 | | | | 2560 |
| 10 | S12 | 640 | | 1280 | | |
| 11 | S13 | 0 | | 40 | | 320 |
| 12 | S14 | | | 640 | | 640 |
| 13 | S17 | | | 2560 | | 2560 |
| 14 | S20 | 160 | | 320 | | |
| 15 | S31 | 40 | | | | 1280 |
| 16 | S41 | 640 | | 1280 | | |
| 17 | S43 | | | 40 | | 160 |
| 18 | S48 | | | 1280 | | 2560 |
| 19 | M9 | | | 0 | | 160 |
| 20 | M11 | 0 | | 0 | | 0 |
| 21 | M13 | | | 0 | | 0 |
| 22 | M14 | | | 163840 | | 163840 |
| 23 | M25 | 40 | | | | 640 |
| 24 | M30 | | | 320 | | 320 |

403

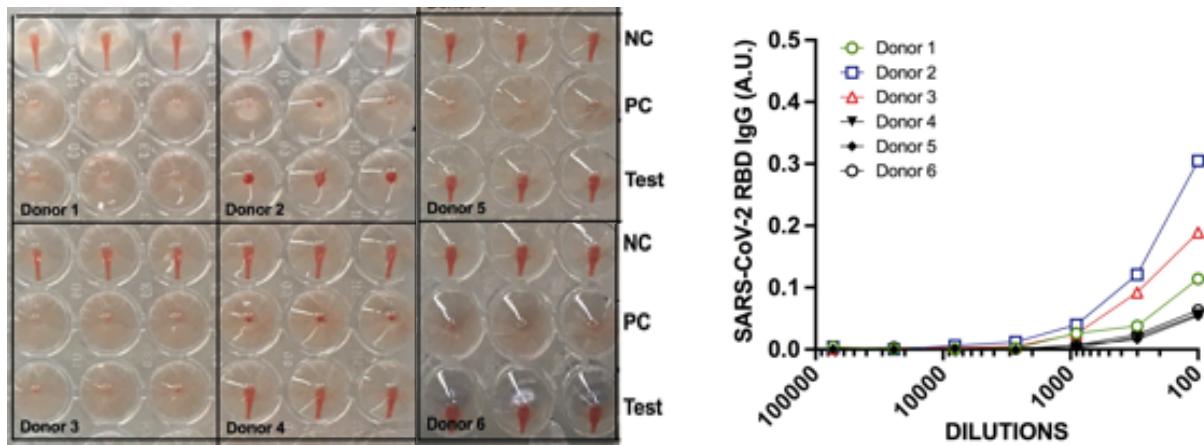
404 C: Critical, S: Severe, M: Mild

405

406 **HAT as a Point of Care Test on Capillary Samples**

407 The HAT is designed to detect antibodies to the RBD starting at a serum dilution of 1:40, and
 408 we have found that that 50 µL of 1:40 dilution of whole blood provides an optimal
 409 concentration of red blood cells for detection by agglutination in V-bottomed 96 well plates.
 410 We have not completed an extensive analysis of the HAT as a Point of Care Test. However,
 411 we have preliminary evidence that lyophilised IH4-RBD sent to the National Institute of
 412 Immunology, New Delhi, functions as a Point of Care Test on capillary blood obtained by

413 finger-prick. In Figure 6, three positive (donors 1, 2 and 3) and three negative (donors 4, 5 and
414 6) HAT results are compared to a standard ELISA for detection of antibodies to the RBD.
415



416
417 **Figure 6. HAT as a point of care test.**
418 Capillary blood samples were obtained by lancet. Antibodies to the RBD were detected by
419 HAT on autologous red cells in the sample in “Test” wells (plasma at 1:40) after addition of
420 100 ng/well IH4-RBD (see methods). NC, Negative Control (PBS replaces IH4-RBD); PC Positive
421 Control (20 ng/well CR3022, an anti-RBD monoclonal antibody added). In parallel, after
422 removal of red cells, the plasma was tested in a standard ELISA for detection of antibodies to
423 the RBD. Low levels of antibody detected in the ELISA were sufficient to give a positive result
424 in the HAT.

425
426 Further work is needed to establish the operating characteristics of the HAT as a Point of Care
427 Test on capillary samples. We provide a suggested operating procedure for capillary samples
428 in methods.

429

430 **Distribution of the IH4-RBD as lyophilised protein**

431 In order to ship the IH4-RBD reagent efficiently we have examined the effects of lyophilisation
432 and reconstitution with water. IH4-RBD synthesised for the purpose of distribution was
433 provided at 5 mg/mL in PBS by Absolute Antibody Ltd, Oxford. Two hundred microlitre
434 aliquots (1 mg, enough for 10,000 test wells) were lyophilised overnight and stored at -20 °C.
435 Aliquots were thawed, reconstituted with 1 ml double distilled water and titrated against the
436 pre-lyophilisation material. No change in the titration occurred. We have synthesised one

437 gram of IH4-RBD, sufficient for 10 million test wells. This is available free of charge for any
438 qualified group anywhere in the world in aliquots of 1 mg (20,000 test wells).

439

440 **DISCUSSION**

441 The COVID-19 pandemic has had a particularly gruelling influence on the world economy, and
442 on most populations of the world. The appearance of such a new highly contagious virus will
443 probably not be a unique occurrence in the decades ahead. One of the lessons learned is the
444 importance of developing affordable serological tests for detection of immune responses to
445 SARS-CoV-2. Commercial antibody tests are not widely available to low- and middle-income
446 countries, and lateral flow assays, while offering early promise as a near-patient test, have
447 failed to deliver in terms of performance metrics, are expensive, and there are concerns about
448 significant batch-to-batch variation (Adams et al., 2020). By contrast, the advantages of the
449 HAT are the low cost of production of its single reagent (~0.27 UK pence per test well), better
450 performance than most lateral flow devices (Adams et al., 2020), and versatility in not
451 requiring anything other than a source of O-ve blood (10 mL of K2EDTA blood provides
452 enough for 8,000 tests), an adjustable pipette, PBS, and a standard 96 well V-bottomed plate.

453

454 We have demonstrated that the HAT functions as a viable test for the presence of antibodies
455 to the RBD of the SARS-CoV-2 spike protein in stored serum/plasma samples, using O-ve red
456 cells as indicators. In the formal assessment of sensitivity and specificity we recorded an
457 average 90% sensitivity and 99% specificity (Likelihood Ratio ~175), compared to 100%
458 sensitivity and 100% specificity for the Siemens Atellica Chemiluminescence test on the same
459 set of samples. The “positive” samples were selected to have been taken at least 28 days after
460 a positive PCR test. These conditions are optimal for serological tests by allowing time for a
461 rise in antibodies. The sensitivity of ~90% and specificity of 99% did not reach the level of
462 98% for both recommended by the UK MHRA (The National SARS-CoV-2 Serology Assay
463 Evaluation Group, 2020), or 99.5% for both recommended by the Infectious Disease Society
464 of America (Hanson et al., 2020). However, these values are still consistent with a useful test
465 in appropriate contexts, provided that users are fully aware of the operating characteristics
466 and interpret the results correctly.

467

468 The sensitivity of the single point HAT can be enhanced (to ~98%) if wells with partial teardrop
469 formation are scored as positive. However, this improvement in sensitivity is gained at the
470 expense of a reduced specificity (to ~97%). If partial teardrops are to be scored as positive in
471 the spot test at 1:40, we recommend obtaining confirmation for these by ELISA. Improvement
472 in the operating characteristics of the HAT may be possible by a systematic analysis of buffer
473 composition and experimental conditions, and is being investigated. We have deliberately
474 kept complexity to a minimum, and thus all dilutions were made in standard PBS, and for the
475 moment we recommend scoring wells with partial teardrop formation as negative.

476

477 It is interesting that the HAT titrations actually performed a little better than the Siemens test
478 on 153 stored plasma samples from donors during the first five days of their hospital
479 admission (note that symptom onset may have been several days earlier), in whom it
480 detected 86% (81% in the repeat) of samples from PCR-diagnosed donors, compared to 74%
481 for the Siemens test, and gave 100% specificity for the sample set containing control plasma
482 from patients with sepsis and healthy controls. We speculate that at this early period of the
483 COVID-19 illness the immune response may be dominated by IgM that would be expected to
484 be particularly efficient at crosslinking the IH4-RBD labelled red cells. In addition, of the
485 twenty-four donors who were tested more than once in the first five days in hospital, the HAT
486 detected a rise in titre in sixteen (67%). A fixed high titre was detected in a further four to
487 provide a sensitivity of 83% in these twenty-four cases. In situations of high clinical suspicion,
488 the HAT could potentially have a place as a helpful test to support the diagnosis of COVID-19
489 by detecting a rising titre of antibodies to the RBD during hospital admission. In patients with
490 a prior probability of a diagnosis of COVID-19 of ~10%, the likelihood ratio of ~175 for the HAT
491 provides a posterior probability of ~95% for this diagnosis. However, it is essential that if
492 clinicians use a rising titre in the HAT as a diagnostic aid, they should be aware of the relatively
493 low sensitivity (~67%) in this context.

494

495 With a specificity of 99%, the HAT could be employed for epidemiological surveys of the
496 seropositive rate in stored serum/plasma samples from populations with a moderate
497 expected prevalence ~10%, which would entail a negative predictive value of ~90%. The HAT
498 may be useful to detect seroconversion after vaccination, and for the identification of
499 potential donors of high titre plasma for therapy, if the clinical trials that are in progress

500 demonstrate a benefit. In the absence of knowledge about the level of antibody that indicates
501 protection, the HAT should not be used to provide personal results to individuals, as discussed
502 by the UK Royal College of Pathologists ([https://www.rcpath.org/profession/on-the-](https://www.rcpath.org/profession/on-the-agenda/COVID-19-testing-a-national-strategy.html)
503 [agenda/COVID-19-testing-a-national-strategy.html](https://www.rcpath.org/profession/on-the-agenda/COVID-19-testing-a-national-strategy.html)). Finally, we show that the lyophilised
504 IH4-RBD reagent sent to New Delhi functioned as expected in preliminary point of care testing
505 on capillary samples obtained by finger-prick. However, additional evidence is needed to
506 show that the sensitivity and specificity of the HAT, applied as a Point of Care Test in this way,
507 are comparable to the tests on stored plasma samples, as stressed by the IDSA guideline on
508 serological testing (Hanson et al., 2020). This will need to be done in field conditions, which is
509 planned.

510

511 The technique required for applying the HAT can be learned in a day by a trained laboratory
512 technician, paramedic, nurse or doctor. We have produced one gram of the developing IH4-
513 RBD reagent (enough for ten million test wells) and offer to ship lyophilised aliquots of this
514 material (sufficient for 10,000 tests) anywhere in the world, free of charge, for use as a
515 research reagent for serological studies of COVID-19.

516

517 METHODS

518

519 Sample Collection and Ethics

520 **Figures 1,2:** Control whole blood (K2EDTA) as a source of red cells was collected from a
521 healthy donor after informed consent.

522

523 **Figure 3:** Pre-pandemic negative controls: these samples were collected from healthy adults
524 in the Oxfordshire region of the UK between 2014 and 2016, ethics approval: Oxfordshire
525 Clinical Research Ethics Committee 08/H0606/107+5. Positive sample set: these were
526 convalescent plasma donors recruited by NHS Blood and Transplant (NHSBT), ethics approval
527 (NHSBT; RECOVERY [Cambridge East REC (ref: 20/EE/0101)] and REMAP-CAP [EudraCT 2015-
528 002340-14] studies).

529

530 **Figures 4, 5 and Table 1:** Known COVID-19 positive samples were collated from three ethically
531 approved studies: Gastro-intestinal illness in Oxford: COVID substudy [Sheffield REC,

532 reference: 16/YH/0247]ISARIC/WHO, Clinical Characterisation Protocol for Severe Emerging
533 Infections [Oxford REC C, reference 13/SC/0149], the Sepsis Immunomics project [Oxford REC
534 C, reference:19/SC/0296]) and by the Scotland A Research Ethics Committee (Ref:
535 20/SS/0028). Patients were recruited from the John Radcliffe Hospital in Oxford, UK, between
536 March and May 2020 by identification of patients hospitalised during the SARS-CoV-2
537 pandemic and recruited into the Sepsis Immunomics and ISARIC Clinical Characterisation
538 Protocols. Time between onset of symptoms and sampling were known for all patients and if
539 labelled as convalescent patients were sampled at least 28 days from the start of their
540 symptoms. Written informed consent was obtained from all patients. All patients were
541 confirmed to have a test positive for SARS-CoV-2 using reverse transcriptase polymerase
542 chain reaction (RT-PCR) from an upper respiratory tract (nose/throat) swab tested in
543 accredited laboratories. The degree of severity was identified as mild, severe or critical
544 infection according to recommendations from the World Health Organisation. Severe
545 infection was defined as COVID-19 confirmed patients with one of the following conditions:
546 respiratory distress with RR>30/min; blood oxygen saturation<93%; arterial oxygen partial
547 pressure (PaO₂) / fraction of inspired O₂ (FiO₂) <300 mmHg; and critical infection was defined
548 as respiratory failure requiring mechanical ventilation or shock; or other organ failures
549 requiring admission to ICU. Comparator samples from healthcare workers with confirmed
550 SARS-CoV-2 infection who all had mild non-hospitalised disease were collected under the
551 Gastro-intestinal illness in Oxford: COVID sub-study, and samples from patients with
552 equivalently severe disease from non-COVID infection were available from the Sepsis
553 Immunomics study where patients presenting with significantly abnormal physiological
554 markers in the pre-pandemic timeframe had samples collected using the same methodology
555 as that applied during the COVID pandemic. Blood samples were collected in K2EDTA
556 vacutainers and PBMCs were separated from plasma using Sepmate isolation tubes
557 (STEMCELL Technologies) and plasma was used in the downstream HAT assay.

558

559 **Figure 6:** Capillary samples were collected from members of institute staff with informed
560 consent (New Delhi, India). The study is a part of the COVID-19 project 'IPA/2020/000077'.
561 The project has been approved by the Institutional Human Ethics Committee; Ref. no. -
562 IHEC#128/20.

563

564 **Cloning, Expression and Purification of VHH(IH4)-RBD**

565 The codon-optimised gene encoding IH4-RBD sequence (Figure 1B and supplementary for the
566 cDNA sequence) was synthesized by Integrated DNA Technologies. The gene was cloned in
567 to the AbVec plasmid (Genbank FJ475055) using the restriction sites AgeI and HindIII (the
568 vector supplied the signal sequence). **This expression plasmid for IH4-RBD is available on
569 request.** Protein was expressed in Expi293F™ cells using the manufacturer's protocol
570 (Thermo Fisher). Protein supernatant was harvested on day 5/6 after transfection, spun and
571 0.22 µm filtered. The protein was affinity-purified using a His-Trap HP column (Cytiva). Binding
572 buffer consisted of 20 mM Sodium Phosphate, 150 mM NaCl and 20 mM Imidazole at pH 7.4,
573 and the elution buffer of 500 mM Imidazole in 1 x binding buffer. Protein was concentrated
574 using 15 ml Vivaspin 30 kDa MWCO filter and then buffer exchanged to PBS using a 10 ml 7
575 kDa Zebaspin column (Thermo Fisher).

576

577 For large scale production, the protein was synthesized by Absolute Antibody Ltd, Oxford
578 using the same plasmid construct in HEK293 cells.

579

580 **Lyophilisation of IH4-RBD and CR3022 monoclonal antibody**

581 For lyophilisation, 200 µL (1 mg) of IH4-RBD (5 mg/mL) and 100 µL (200 µg) CR3022 mAb (2
582 mg/mL) in PBS buffer prepared in Protein Lo-Bind microcentrifuge tube (Fisher Scientific)
583 were frozen at -80 °C and further cooled down to - 196 °C using liquid nitrogen. Pre-cooled
584 samples were transferred to Benchtop K freeze-dryer (VirTis) with chamber at 49 µbar and
585 condenser pre-cooled to -72.5 °C. The samples were freeze-dried for a minimum of 24 h,
586 wrapped in Parafilm (Merck) and stored at -20 °C. Lyophilised sample was reconstituted in
587 the same original volume of MilliQ water.

588

589 **Indirect ELISA to detect SARS-CoV-2 specific IgG (Figure 6)**

590 A standard indirect ELISA was used to determine the SARS-CoV-2 specific IgG levels in plasma
591 samples. A highly purified RBD protein from SARS-CoV-2 Wuhan strain (NR-52306, BEI
592 Resources, USA), expressed in mammalian cells, was used to capture IgG in the plasma
593 samples. Briefly, ELISA plates (Nunc, Maxisorp) were coated with 100 µL/well of RBD antigen
594 diluted in PBS (pH 7.4) at the final concentration of 1 µg/mL and incubated overnight at 4°C.
595 Plates were washed three times with washing buffer (0.05% Tween-20 in PBS) followed by

596 the incubation with blocking buffer (3% Skim milk and 0.05% Tween-20 in PBS). The 3-fold
597 serially diluted heat inactivated plasma samples in dilution buffer (1% Skim milk and 0.05%
598 Tween-20 in PBS) were added into the respective wells, followed by incubation at room
599 temperature for 1 hour. After incubation, plates were washed, and anti-human IgG
600 conjugated with Horseradish Peroxidase (HRP) (Southern Biotech) was added in each well.
601 After 1 h incubation, plates were washed and developed by OPD-substrate (Sigma-Aldrich) in
602 dark at room temperature. The reaction was stopped using 2N HCl and the optical density
603 (OD) was measured at 492 nm. The RBD-antigen coated wells that were added with sample
604 diluent alone were used as the blank. The OD values from sample wells were plotted after
605 subtracting the mean of OD values obtained in the blank wells.

606

607 **Equipment and Reagents for HAT**

- 608 • O-ve blood as a source of red cells collected in K2EDTA tube, diluted **in PBS to 1:20 or**
609 **1:40 as needed.** Resuspend by inverting gently ~12 times.
- 610 • BD Contact Activated Lancet Cat. No. 366594 (2 mm x 1.5 mm)
- 611 • 100 µL, 20 µL pipettes, Multichannel pipettes
- 612 • V-bottomed 96-well plates (Greiner Bio-One, Cat. No. 651101, Microplate 96-well, PS,
613 V-bottom, Clear, 10 pieces/bag)
- 614 • Eppendorf Tubes
- 615 • K2EDTA solution (add 5 mL PBS to 10 mL K2EDTA blood collection tube = 3.6 mg
616 K2EDTA/mL, store at 4 °C)
- 617 • Phosphate Buffered Saline Tablets (OXOID Cat. No. BR0014G)
- 618 • IH4-RBD Reagent diluted 2 µg/mL in PBS. This remains active for at least 1-2 weeks
619 stored at 4 °C
- 620 • V-bottomed 96-well plates, numbered, dated, timed (helps when timing many plates)
- 621 • Positive control monoclonal antibody CR3022 diluted to 2 µg/mL in PBS

622

623 **Other Reagents**

624 Monoclonal antibody to human IgG (Gamma chain specific) Clone GG-5 Sigma Cat. No. I5885

625

626 **1. Spot test on Stored Serum/Plasma samples (Figure 4).**

- 627 1. Plate out 50 µL of **1:20** serum/plasma in alternate columns 1,3,5,7,9,11 (add 2.5 µL
628 sample to 47.5 µL PBS).
- 629 2. Add 50 µL **1:20** O-ve blood collected in (**so that now sample is diluted to 1:40 and red**
630 **cells at ~1% v/v**)
- 631 3. Mix and transfer 50/100 µL to neighbouring columns 2,4,6,8,10,12 for -ve controls.
632 The negative control is important because in rare cases, particularly in donors who
633 have received blood transfusions, the sample in principle may contain antibodies to
634 non-ABO or Rhesus D antigens.
- 635 4. Add 50 µL IH4-RBD reagent (2 µg/mL in PBS = 100 ng/well) to Columns 1,3,5,7,9,11
- 636 5. Add 50 µL PBS to columns 2,4,6,8,10,12.
- 637 6. Inc 1 hr RT
- 638 7. Tilt for 30 seconds
- 639 8. Photograph: with mobile phone use the zoom function to obtain a complete field
- 640 9. Read as Positive = No teardrop, Negative <1:40 = partial teardrop, Neg = complete
641 teardrop.
- 642 10. Two readers should read the plates independently, and disagreements resolved by
643 taking the lesser reading.
- 644 11. For each batch of samples set up positive control wells containing 20-100 ng
645 monoclonal antibody CR3022 (as in Finger-Prick test below). This establishes that all
646 of the reagents are working.
- 647
- 648 **2. Titration of Stored Serum/Plasma Samples.**
- 649 1. Dilute samples to **1:20** in 50 µL PBS (2.5 µL to 47.5 µL) in V-bottomed plate in Rows A-
650 H, column 1.
- 651 2. Prepare doubling dilutions with PBS across the plate columns 1-11 (1:40 to 1:40,960),
652 PBS control in column 12. Eight samples can be titrated per 96-well plate.
- 653 3. Add 50 µL 1:40 O-ve red cells (1% v/v or 1:40 fresh EDTA O-ve blood sample) to all
654 wells
- 655 4. Add 50 µL IH4-RBD (2 µg/mL, = 100 ng/well). [Note: the red cells and IH4-RBD can be
656 pre-mixed and added together in either 50 µL or 100 µL volume, to save a step. This
657 variation in technique does not alter the measured titres.]
- 658 5. Allow red cells to settle for 1 hr

659 6. Tilt plate for at least 30 s and photograph. The titre is defined by the last well in which
660 the tear drop fails to form. Partial teardrop regarded as negative.

661

662 **3. Finger-prick test on capillary blood as a Point of Care Test**

663 1. Preparation: Clean Hands, warm digit. Prepare a plate (96-well V-bottomed) labelled
664 with Date and Time.

665 2. Prick skin on outer finger pulp with disposable, single use BD or another Lancet.

666 3. Wipe away first drop of blood with sterile towel/swab

667 4. Massage second drop

668 5. Take a minimum of 5 µL blood with 20 µL pipette, mix immediately into 20 µL K2EDTA
669 (3.6 mg/mL/PBS) in Eppendorf. If possible, take 20 µL of blood and mix into 80 µL
670 K2EDTA solution. Another approach is collection of blood drops into a BD Microtainer
671 K2E EDTA lavender vials that take 250-500 µL.

672 6. For 5 µL sample dilute to 200 µL with PBS (add 175 µL PBS), for 20 µL sample dilute to
673 800 µL (add 700 µL PBS). ***Sample is now at 1:40, and the red cells are at the correct
674 density (~1% v/v assuming a haematocrit of 40%) to give a clear tear drop.***

675 7. Plate 50 µL x 3 in V bottomed microtitre wells labelled T (Test), + (PC, positive Control),
676 - (NC, negative control) – see figure 6.

677 8. Add 10 µL of control anti RBD Mab CR3022 (2 µg/mL stock in PBS, 20 ng/well) to “+”
678 well

679 9. Add 50 µL IH4-RBD (2 µg/mL in PBS) to “T” (Test) and “+ve” wells, 50 µL PBS to “-ve”
680 well.

681 10. Incubate 1 hour at RT for Red Cells to form a pellet in the “-ve” well

682 11. Tilt plate against a well-lit white background for ~30 seconds to allow Tear drop to
683 form in “-ve” well.

684 12. The presence of antibodies to RBD is shown by loss of Tear Drop formation in the “T”
685 and “+ve” wells. Occasionally a partial tear drop forms – these wells are counted as
686 Negative.

687 13. **Photograph the plate to record the results with the date and time.** Results can be
688 reviewed and tabulated later. Taking picture from a distance and using the zoom
689 function helps to take a clear picture of all wells in a 96-well plate.

690 14. The negative (PBS) control should be done on every sample for comparison. The
691 Positive control induced by CR3022 is used to check that all the reagents are working,
692 and that the glycophorin epitope recognised by VHH(IH4) is present on the red cells.
693 Absence of the IH4 epitope should be *very rare* (Habib et al., 2013). For setting up
694 cohorts a positive control on every sample is therefore not necessary but should be
695 included in every *batch* of samples.

696 15. If a 20 µL sample of blood was taken from the finger prick there should be 650 µL of
697 the 1:40 diluted blood left. The red cells can be removed and a preparation of 1:40 O-
698 ve red cells used as above to titrate the sample. In principle the autologous red cells
699 could be washed repeatedly, resuspended in the same volume of PBS, and used as
700 indicators for the titration, however we have not attempted to do this. The s/n is 1:40
701 plasma that can be used in confirmatory ELISA or other tests.

702
703 **Author Contributions**

704 **Conceived, initiated and followed the project, documented portability and robustness of**
705 **HAT method in a separate laboratory, recruited collaborators:** Etienne Joly.

706 **Designed and produced the IH4-RBD reagent, established conditions for lyophilisation,**
707 **isolated and expanded human monoclonal antibodies to the RBD and ACE2-Fc, performed**
708 **the standard HAT assays, wrote the paper:** Alain Townsend, Pramila Rijal, Julie Xiao, Tiong
709 Kit Tan, Lisa Schimanski, Jiangdong Huo, Rolle Rahikainen, Kuan-Ying A Huang.

710 **Contributed examples of HAT as a Point of Care Test:** Nimesh Gupta.

711 **Provision of Serum/Plasma Sample Sets for Figure 4: Project management for Oxford**
712 **serology work for sensitivity and specificity measurement, assessment of preliminary data:**
713 Philippa Matthews, Derrick Crook, Sarah Hoosdally, Nicole Stoesser; **collection and**
714 **processing of samples, coordination of sample banks and running Siemens assay, ethics,**
715 **storage of pre-pandemic samples, coordination of provision of pre-pandemic samples from**
716 **Oxford BioBank, and sero-positive donors through NHSBT :** Teresa Street, Justine Rudkin,
717 Fredrik Karpe, Matthew Neville, Rutger Ploeg, David J Roberts, Abbie Bown, Richard Vipond,
718 Marta Oliveira, Abigail A Lamikanra, Hoi Pat Tsang.

719 **Provision of Serum Sample Sets for Figure 5 and Table 1 (COMBAT samples):** Alexander J
720 Mentzer, Julian C Knight, Andrew Kwok, Paul Klenerman, Christina Dold; **ISARIC4C**
721 **Investigators:** J. Kenneth Baillie, Shona C Moore, Peter JM Openshaw, Malcolm G Semple,

722 Lance CW Turtle; **Oxford Immunology Network Covid-19 Response Clinical Sample**
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727

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738

739 The Spike Glycoprotein Receptor Binding Domain (RBD) from SARS-Related Coronavirus 2
740 Wuhan-Hu-1 (Figure 6), Recombinant from HEK293 Cells, NR-52306 was produced under
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742

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745

746 **DECLARATION**

747 Competing Interests none.

748 The views expressed are those of the author(s) and not necessarily those of the NHS, the
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750

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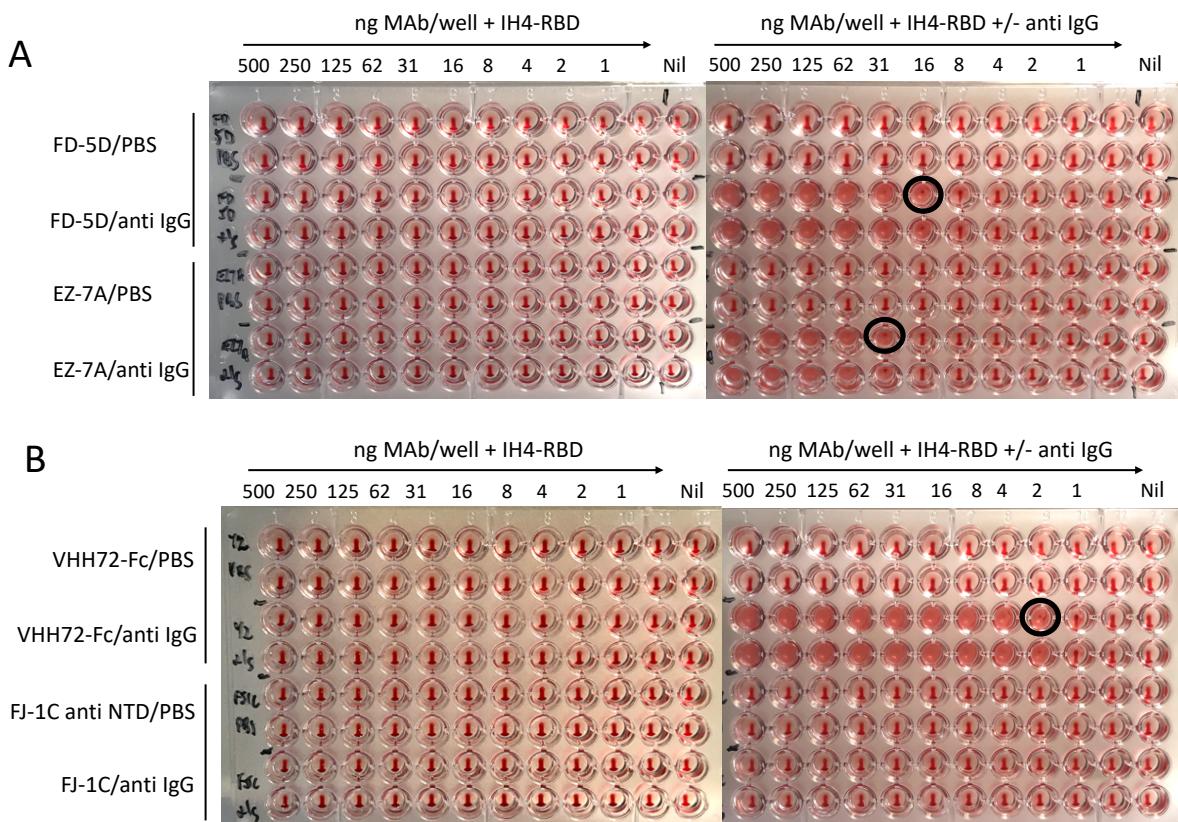
852 **SUPPLEMENTARY DATA**

853

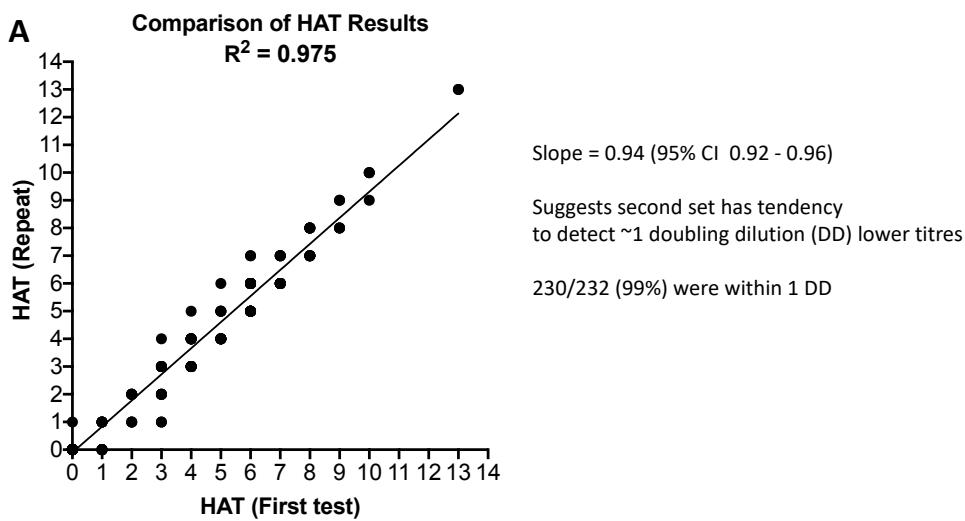
854 Supplementary Table 1. Fifteen human monoclonal antibodies or nanobodies with endpoint
 855 titres for detection in the HA Test. One divalent nanobody VHH72-Fc and two monoclonal
 856 antibodies FD-5D and EZ-7A specific for the RBD failed to agglutinate red cells with the IH4-
 857 RBD reagent. Three monoclonal antibodies to other regions of the spike protein EW-8B, EW-
 858 9C and FJ-1C acted as negative controls.

859

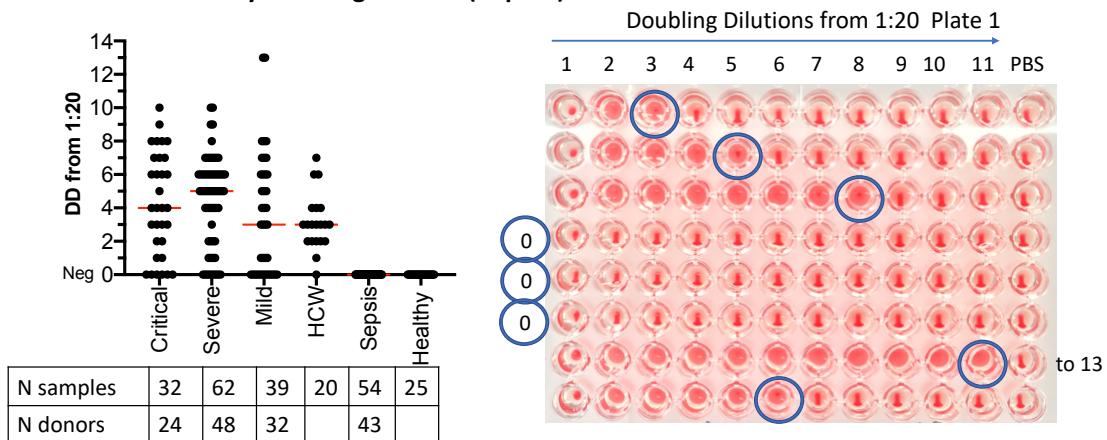
| Mab or divalent Nanobody | HAT endpoint titre (ng/well) | Reference |
|--------------------------|------------------------------|--|
| CR3022 | 2 | (Huo, Zhao, et al., 2020; ter Meulen et al., 2006) |
| EY-6A | 16 | (Zhou et al., 2020) |
| VHH-72-Fc | Negative | (Wrapp et al., 2020) |
| FI-4A | 16 | (Huang et al., 2020) |
| ACE2-Fc | 4 | (Huo, Le Bas, et al., 2020) |
| FI-3A | 8 | (Huang et al., 2020) |
| FI-1C | 4 | (Huang et al., 2020) |
| H11-H4-Fc | 16 | (Huo, Le Bas, et al., 2020) |
| FD-5D | Negative | (Huang et al., 2020) |
| FD-11A | 31 | (Huang et al., 2020) |
| FN-12A | 31 | (Huang et al., 2020) |
| FJ-10B | 62 | (Huang et al., 2020) |
| FM-7B | 62 | (Huang et al., 2020) |
| EZ-7A | Negative | (Huang et al., 2020) |
| S309 | 125 | (Pinto et al., 2020) |
| EW-8B non-RBD anti Spike | Neg Control | (Huang et al., 2020) |
| EW-9C non-RBD anti Spike | Neg Control | (Huang et al., 2020) |
| FJ-1C anti NTD | Neg Control | (Huang et al., 2020) |



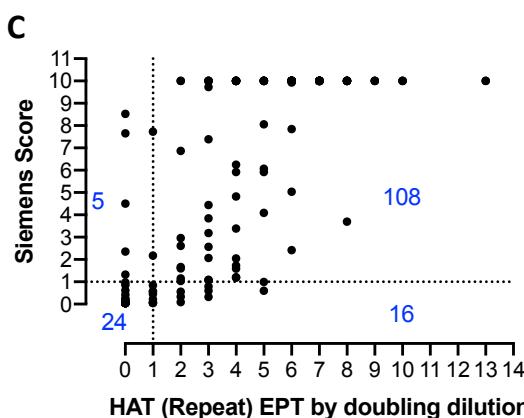
861
862 Supplementary Figure 1 A, B) Three MAbs or Nanobody-Fc reagents of 15 tested specific for
863 RBD failed to cross-link red cells but could be developed with an anti IgG reagent. Antibodies
864 were titrated from 500 ng/well to 1 ng/well in 50 µL, 1:40 red cells were added in 50 µL,
865 followed by 100 ng/well IH4-RBD in 50 µL, and incubated for 1 hour. The labelled red cells
866 were then resuspended, and either 50 µL PBS (L hand plates), or Sigma Clone GG5 anti human
867 IgG 1:100 in 50 µL (R hand plates). Red cells were allowed to settle for one hour, the plate
868 tilted for 30s and photographed.



B Titration in HAT by doubling dilution (Repeat)



Donors were grouped according to clinical criteria; HCW = Front Line Health Care Workers; Sepsis = Samples with patients with Sepsis prior to Covid-19 pandemic; Healthy = Healthy volunteers prior to Covid-19; DD 1 = 1:40. Actual Titre = 20 x 2^{DD}.



| | HAT +ve | HAT -ve | Total | Fraction |
|-------------|---------|---------|-------|----------|
| Siemens +ve | 108 | 5 | 113 | 0.96 |
| Siemens -ve | 16 | 24 | 40 | 0.40 |
| Total | 124 | 29 | 153 | 0.81 |
| Fraction | 0.87 | 0.83 | 0.74 | |

| P value and statistical significance | |
|--------------------------------------|---------------------|
| Test | Fisher's exact test |
| P value | <0.0001 |
| P value summary | *** |
| One- or two-sided | Two-sided |

Sensitivity (Sn) HAT 81%, Sn Siemens 74%
 HAT still more sensitive than Siemens

Specificity 100% for HAT in these samples

871

872 Supplementary Figure 2. A) The 232 samples from figure 5 in the main paper were titrated a
 873 second time 34 days after the first measurement, as described for figure 4 (Methods). The
 874 values for the end point doubling dilution were compared and a correlation coefficient, and
 875 slope calculated (Prism v8).

876 B) The collection included 32 samples from 24 Critical patients, 62 samples from 48 Severe,
877 39 samples from 32 Mild, 20 single samples from health care workers (HCW), 54 samples from
878 43 patients with unrelated sepsis in the pre-Covid-19 era, 25 samples from healthy unexposed
879 controls. Median is shown.

880 C) Comparison to Siemens Result (anti RBD) with HAT Titre by Doubling Dilution for 153
881 samples from Critical, Severe, Mild and HCW PCR+ve donors. The sensitivity of the HAT in this
882 repeat was 81%, v 86% in the first test.

883

884 Supplementary Table 2. Fifty-two samples from 24 donors who were sampled repeatedly
885 during the first five days in hospital; assay repeated 34 days after the measurements in Table
886 1. Sixteen of 24 showed a rise in titre.

| No | Case | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
|----|------|-------|-------|--------|-------|--------|
| 1 | C2 | | | 40 | | 160 |
| 2 | C6 | | | 0 | | 80 |
| 3 | C7 | 0 | | | | 0 |
| 4 | C9 | 0 | | | | 1280 |
| 5 | C10 | 160 | | 320 | | |
| 6 | C23 | 0 | | 80 | | 320 |
| 7 | S5 | 40 | | 40 | | |
| 8 | S6 | 1280 | | 2560 | | 2560 |
| 9 | S7 | 80 | | | | 1280 |
| 10 | S12 | 320 | | 640 | | |
| 11 | S13 | 0 | | 0 | | 160 |
| 12 | S14 | | | 640 | | 1280 |
| 13 | S17 | | | 1280 | | 1280 |
| 14 | S20 | 80 | | 320 | | |
| 15 | S31 | 0 | | | | 640 |
| 16 | S41 | 640 | | 1280 | | |
| 17 | S43 | | | 40 | | 80 |
| 18 | S48 | | | 1280 | | 1280 |
| 19 | M9 | | | 40 | | 160 |
| 20 | M11 | 0 | | 0 | | 0 |
| 21 | M13 | | | 0 | | 0 |
| 22 | M14 | | | 163840 | | 163840 |
| 23 | M25 | 40 | | | | 320 |
| 24 | M30 | | | 160 | | 160 |

887

888

889 **DNA sequence of codon optimised cDNA encoding IH4-RBD**

890 ATGGGATGGTCATGTATCATCCTTTCTAGTAGCAACTGCAACC GG TGTTCATAGCCAGGTC
891 CAGCTGCAAGAGTCTGGCGGAGGATCTGTT CAGGCTGGCGGAAGCCTGAGACTGAGCTGTGT
892 GGCCAGCGGCTACACCGATA TAGCACATACTGCGTCGGCTGGTT CAGACAGGCCCTGGCAAAG
893 AGAGAGAGGGCGTCGCCAGAAATCAACACC ATCAGCGGCAGAC CCTGGTACGCCGACTCTGTG
894 AAGGGCAGATTACAATCAGCCAGGACA ACAGCAAGAACACCGTGTTCTGCAGATGAACA
895 GCCTGAAGCCAGAGGACACCGCCATCTACTACTG CACCCTGACCACGCCAACAGCAGAGGC
896 TTTTGTCCGGCGGCTACA ACTACAAAGGCCAGGGCACCCAAAGTGACCGTGTCTAGCGCGTC
GACCGGCTCTGGCGGCAGCGGCAACATC ACCAATCTGTGCCCTTCGGCGAGGTGTTCAACG
CCACCAGATTGCCAGCGTGTACGCCCTGGAACCGGAAGAGAAATCAGCAACTGCGTGGCCGAC
TACAGCGTGCTGTACAATAGGCCAGCTTCAGCACCTCAAGTGCTACGGCGTGTCCCCTACC
AAGCTGAACGACCTGTGCTTCACCAATGTGTACGCCGACAGCTCGTGTACAGAGGCGACGA
AGTTCGGCAGATCGCTCCTGGACAGACAGGCAAGATGCCGATTACA ACTACAAGCTGCCCG
ACGACTTCACCGCTGCGTGATGCCCTGGAAATAGCAACAAACCTGGACAGCAAAGTCGGCGC
AACTACA ACTACCTGTACCGGCTGTTCCGGAAAGTCCAACCTGAAGCCTTCGAGCGGGACAT
CAGCACCGAGATCTATCAGGCCGGCAGCACCCCTGTAATGGCGTGGAAAGGCTTCAACTGCT
ACTTCCC ACTGCAGTCCTACGGCTTCAGCCTACAAACGGCGTGGCTACCAGCCTTATAGAG
TGGTGGTGCTGAGCTCGAACATGCTGCATGCCCTGCTACC GTGTGCGGCCCTAAAAAACACC
ATCACCACCA CATTGA