1   A serological assay to detect SARS-CoV-2 seroconversion in humans

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3   Fatima Amanat[1,2], Thi H.O. Nguyen[3], Veronika Chromikova[1], Shirin Strohmeier[1,4], Daniel Stadlbauer[1],

4   Andres Javier[1], Kaijun Jiang[1], Guha Asthagiri Arunkumar[1,2], Jose Polanco[1,5], Maria Bermudez-Gonzalez[1],

5   Daniel Caplivski[6], Allen Cheng[7], Katherine Kedzierska[3], Olli Vapalahti[8,9,10], Jussi M. Hepojoki[8,11], Viviana

6   Simon[1,12,13] and Florian Krammer[1]*

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8   *[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

9   *[2]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

10   *[3]Department of Microbiology & Immunology, University of Melbourne, The Peter Doherty Institute for*

11   *Infection & Immunity, Melbourne, Victoria, Australia*

12   *[4]Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria*

13   *[5]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY,*

14   *USA*

15   *[6]Travel Medicine Program, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New*

16   *York, NY, USA*

17   *[7]School of Public Health and Preventive Medicine, Monash University; Infection Prevention and*

18   *Healthcare Epidemiology Unit, Alfred Health*

19   *[8]Department of Virology, Medicum, University of Helsinki, Helsinki, Finland*

20   *[9]Veterinary Biosciences, Veterinary Faculty, University of Helsinki, Helsinki, Finland*

21   *[10]Department of Virology and Immunology, Helsinki University Hospital (HUSLAB), Helsinki, Finland*

22   *[11]Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland*

23   *[12]Global Health Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, NY, USA*

24   *[13]Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New*

25   *York, NY, USA*

26

27   *To whom correspondence should be addressed: florian.krammer@mssm.edu

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29

30   Abstract

31   *Introduction:* SARS-Cov-2 (severe acute respiratory disease coronavirus 2), which causes Coronavirus

32   Disease 2019 (COVID19) was first detected in China in late 2019 and has since then caused a global

33   pandemic. While molecular assays to directly detect the viral genetic material are available for the

34   diagnosis of acute infection, we currently lack serological assays suitable to specifically detect SARS-CoV-

35   2 antibodies.

36   *Methods*: Here we describe serological enzyme-linked immunosorbent assays (ELISA) that we developed

37   using recombinant antigens derived from the spike protein of SARS-CoV-2. These assays were developed

38   with negative control samples representing pre-COVID 19 background immunity in the general

39   population and samples from COVID19 patients.

40 *Results*: The assays are sensitive and specific, allowing for screening and identification of COVID19
41 seroconverters using human plasma/serum as early as 3 days post symptom onset. Importantly, these
42 assays do not require handling of infectious virus, can be adjusted to detect different antibody types and
43 are amendable to scaling.

44 *Conclusion*: Serological assays are of critical importance to determine seroprevalence in a given
45 population, define previous exposure and identify highly reactive human donors for the generation of
46 convalescent serum as therapeutic. Sensitive and specific identification of Coronavirus SARS-Cov-2
47 antibody titers will also support screening of health care workers to identify those who are already
48 immune and can be deployed to care for infected patients minimizing the risk of viral spread to
49 colleagues and other patients.

50

51 **Introduction**

52 On December 31st, 2019 China reported first cases of atypical pneumonia in Wuhan, the capital of Hubei
53 province. The causative virus was found to be a betacoronavirus, closely related to the severe acute
54 respiratory syndrome coronavirus (SARS-CoV-1) from 2003 and similar to *Sarbecoviruses* isolated from
55 bats.[1,2] It was therefore termed SARS-CoV-2 and the disease it causes was named COVID19 (COronaVIrus
56 Disease 2019).[3] The outbreak in Wuhan expanded quickly and led to the lockdown of Wuhan, the Hubei
57 province and other parts of China. While the lockdown, at least temporarily, brought the situation under
58 control in China, SARS-CoV-2 spread globally causing a pandemic with, so far, 150,000 infections and
59 5,500 fatalities (as of March 16th, 2020).

60 Nucleic acid tests that detect the SARS-CoV-2 RNA genome were quickly developed and are now widely
61 employed to diagnose COVID19 disease.[4,5] However, there remains a great need for laboratory assays
62 that measure antibody responses and determine seroconversion. While such serological assays are not
63 well suited to detect acute infections, they are support a number of highly relevant applications. First,
64 serological assays allow us to study the immune response(s) to SARS-CoV-2 in dynamic qualitative and
65 quantitative manner. Second, serosurveys are needed to determine the precise rate of infection in an
66 affected area, which is an essential variable to accurately determine the infection fatality rate. Third,
67 serological assays will allow for the identification of individuals who mounted strong antibody responses
68 and who could serve as donors for the generation of convalescent serum therapeutics. Lastly, serological
69 assays will permit to determine who is immune and who is not. This would be very useful for deploying
70 immune healthcare workers in a strategic manner as to limit the risk of exposure and spread of the virus
71 inadvertently.

72 *Sarbecoviruses* express a large (approximately 140 kDa) glycoprotein termed spike protein (S, a
73 homotrimer), which mediates binding to host cells via interactions with the human receptor angiotensin
74 converting enzyme 2 (ACE2).[6-8] The S protein is very immunogenic with the receptor-binding domain
75 (RBD) being the target of many neutralizing antibodies.[9] Individuals infected with coronaviruses typically
76 mount neutralizing antibodies, which might be associated with some level of protection for a period of
77 months to years, and neutralizing response has demonstrated for SARS-CoV-2 in an individual case from
78 day 9 onwards.[10-13] Serum neutralization can be measured using live virus but the process requires
79 several days and must be conducted under biosafety level 3 laboratory containment for SARS-CoV-2
80 Potentially, pseudotyped viral particle entry assays based on lentiviruses or vesicular stomatitis virus can

81   be used as well, but these reagents are not trivial to produce. A simple solution is the use of a binding
82   assay, e.g. an enzyme linked immunosorbent assays (ELISA), with recombinantly expressed antigen as
83   substrate. Here we report the development of such an assay and provide a protocol for both
84   recombinant antigen production as well as the ELISA.

85

86   **Results**

87   **Expression constructs and generation of recombinant SARS-Cov-2 proteins**

88   We generated two different versions of the spike protein. The first construct expresses a full length
89   trimeric and stabilized version of the spike protein and the second only the much smaller receptor
90   binding domain (RBD). The sequence used for both proteins is based on the genomic sequence of the
91   first virus isolate, Wuhan-Hu-1, which was released on January 10th 2020.[1] Sequences were codon
92   optimized for mammalian cell expression. The full-length spike protein sequence was modified to
93   remove the polybasic cleavage site, which is recognized by furin and to add a pair of stabilizing
94   mutations (**Figure 1**).[7,14,15] These two modifications were included to enhance the stability of the protein
95   based on published literature.[7,14] At amino acid P1213 the sequence was fused to a thrombin cleavage
96   site, a T4 foldon sequence for proper trimerization and a C-terminal hexahistidine tag for purification
97   (**Figure 1**).[16,17] The sequence was cloned into a pCAGGS vector for expression in mammalian cells and
98   into a modified pFastBac Dual vector for generation of baculoviruses and expression in insect cells. For
99   expression of the RBD, the natural N-terminal signal peptide of S was fused to the RBD sequence (amino
100 acid 419 to 541) and joined with a C-terminal hexahistidine tag.[18] The same vectors as for the full length
101 S protein were used to express the RBD. In mammalian cells, the RBD domain gave outstanding yields
102 (approximately 25 mg/liter culture), but expression was lower in insect cells (approximately 0.4 mg/liter
103 culture). Clear single bands were visible when the recombinant RBD proteins were analyzed on a
104 reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), with the insect cell
105 derived protein (iRBD) running slightly lower than the mammalian cell derived protein (mRBD) (**Figure
106 1**). The size difference likely reflects differences in glycan sizes between insect cells and mammalian
107 cells. The full-length S protein was expressed in both systems with slightly higher yields in mammalian
108 cells (mSpike) than in insect cells (iSpike) (approximately 2 mg/liter cultures versus 0.5 mg/liter culture).
109 The full-length protein appeared as a double band on a reducing SDS-PAGE, the higher species likely
110 being the full-length protein and the slightly lower species likely a cleavage product.

111

112 **ELISA development**

113 We used a panel of 59 banked human serum samples collected from study participants including
114 participants with confirmed previous viral infections (e.g., hantavirus, dengue virus, coronavirus NL63 –
115 sample take 30 days post symptom onset) to establish an ELISA with these proteins. These human sera
116 were used to test the background reactivity to the SARS-CoV-2 spike in the general US population
117 covering an age range from approximately 20 to 65+ years. Four plasma/serum samples from three
118 COVID19 patients were used to determine the reactivity of SARS-CoV-2 infected individuals to the RBD
119 and the full length spike.

120 ELISAs were performed by doing serial dilution of the individual serum samples. Values from the dilution
121 curves were used to determine the area under the curve (AUC), which was graphed. All COVID10
122 plasma/serum samples reacted strongly to both RBD and full-length spike protein while reactivity of the
123 other serum samples only yielded background reactivity (**Figure 2**). Reactivity of COVID19 sera was, in
124 general, stronger against the full-length S protein than against the RBD, likely reflecting the higher
125 number of epitopes found on the much larger spike protein. For the RBD the difference between
126 control sera and convalescent sera was larger when the insect cell derived protein was used as
127 compared to the mammalian cell derived RBD. The same was true for the full-length spike protein. The
128 assay allowed to clearly distinguish the convalescent sera from the banked control sera.

129

130 **Antibody isotyping and subtyping**

131 For the four COVID19 patient plasma/sera, we also performed an isotyping and subtyping ELISA using
132 the insect cell and mammalian cell expressed S proteins. Strong reactivity was found for all samples for
133 IgG3, IgM and IgA (**Figure 3**). An IgG1 signal was also detected for three out of the four samples, while
134 one sample showed no reactivity. No signal was detected for IgG4 and reagents for IgG2 were
135 unavailable.

136

137 **Discussion**

138 Here we describe a serological method to detect seroconversion upon SARS-CoV-2 infection. The
139 method is based on reactivity to the immunogenic S protein of the virus. It is relatively simple and quick
140 in its execution and can be performed at biosafety level 2 level as it does not involve life virus. We have
141 tested these methods using banked serum samples obtained from study participants in 2019 and early
142 2020 when this virus was not widely circulating in the US. These serum samples produced low, close to
143 baseline signals in our ELISA. Since the age range of the participants was broad, ranging from to 65+
144 years of age, it is likely that most had experienced infections with human coronaviruses including the
145 alphacoronaviruses NL63 and 229E as well as the betacoronaviruses OC43 and HKU1. We included
146 paired serum samples (acute and convalescent) from a participant with a laboratory confirmed
147 coronvirus NL63 infection. Our data show that there is no or only negligible cross-reactivity from human
148 coronaviruses to SARS-CoV-2. Of note, even infection with the human alphacoronavirus NL63, which
149 also uses ACE2 as receptor[19], did not induce cross-reactivity. This is of great importance because it
150 suggests that humans are completely naïve to SARS-CoV-2, which may explain the relatively high R_0 of
151 SARS-CoV-2 compared to other respiratory viruses such as influenza virus.[20] It might also suggest that
152 antibody-dependent enhancement from human coronavirus induced cross-reactive antibodies targeted
153 at the S protein is unlikely to be the cause of the high pathogenicity of the virus in humans.[21]

154 The plasma/sera used in this study from patients with COVID19 were obtained at day 20 (SARS-CoV2
155 #1), at day 4 (SARS-CoV-2 #2), days 2 and 6 (SARS-CoV-2 #3A and B) post symptom onset. Our data
156 shows significant seroconversion after natural infection with SARS-CoV-2. Our results suggest that
157 antibodies mounted upon infection target the full length S protein as well as the RBD, which is the major
158 target for neutralizing antibodies for related viruses coronaviruses.[9] In fact, sample SARS-CoV2 #1 was
159 tested in another study in neutralization assays and showed a neutralizing titer of 1:160.[13] Thus,

160   seroconversion may lead to protection at a minimum for a limited time. Interestingly, the IgG3 response
161   was stronger than the IgG1 response which is in contrast to e.g. the immune response to influenza
162   where usually IgG1 responses dominates.[22,23] Lastly, we also detected strong IgA and IgM responses in
163   the blood compartment. Of note, level of reactivity and antibody isotypes matched expected patterns
164   based on time since symptom onset very well.

165   We believe that our ELISA method will be key for serosurveys aimed at determining the real attack rate
166   and infection fatality rate in different human populations and to map the kinetics of the antibody
167   response to SARS-CoV-2. In addition, clinical trials with convalescent serum as therapeutic have been
168   initiated in China (e.g. NCT04264858) and anecdotal evidence from the epidemic in Wuhan suggests that
169   compassionate use of these interventions was successful. China has recently shipped convalescent sera
170   to Italy for use in patients and efforts to produce convalescent serum batches are ongoing in the US as
171   well. Screening sera using our assay would be faster and easier than performing standard neutralization
172   assays in BSL3 containment laboratories. Patients recovering from COVID19 disease could be screened
173   for strong antibody responses using the assays described here, especially the one using the RBD as
174   substrate since anti-RBD antibodies likely correlate with virus neutralization. In addition, the assay could
175   be used to screen health care workers to allow selective deployment of immune medical personnel to
176   care for patients with COVID19. Such a strategy would likely limit nosocomial spread of the virus. Of
177   course, the generated recombinant proteins are also excellent reagents for vaccine development and
178   can serve as baits for sorting B cells for monoclonal antibody generation. We are making the methods
179   and laboratory reagents widely available to the research community in order to support the global effort
180   to limit and mitigate spread of SARS-CoV-2.

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184   **Methods**

185   **Recombinant proteins**
186   The mammalian cell codon optimized nucleotide sequence coding for the spike protein of SARS-CoV-2
187   isolate (GenBank: MN908947.3) was synthesized commercially (GeneWiz). The receptor binding
188   domain (RBD, amino acid 319 to 541, RVQP….CVNF) along with the signal peptide (amino acid 1-14,
189   MFIF….TSGS) plus a hexahisitidine tag was cloned into mammalian expression vector pCAGGS as well as
190   in a modified pFastBacDual vectors for expression in baculovirus system. The soluble version of the spike
191   protein (amino acids 1-1213, MFIF….IKWP) including a C-terminal thrombin cleavage site, T4 foldon
192   trimerization domain and hexahistidine tag was also cloned into pCAGGS. The protein sequence was
193   modified to remove the polybasic cleavage site (RRAR to A) and two stabilizing mutations were
194   introduced as well (K986P and V987P, wild type numbering). Recombinant proteins were produced
195   using the well-established baculovirus expression system and this system has been published in great
196   detail in [16,24,25] including a video guide. Recombinant proteins were also produced in Expi293F cells
197   (ThermoFisher) by transfections of these cells with purified DNA using ExpiFectamine 293 Transfection
198   Kit (ThermoFisher). Supernatants from transfected cells were harvested on day 3 post-transfection by
199   centrifugation of the culture at 4000 g for 20 minutes. Supernatant was then incubated with 6 mls Ni-
200   NTA agarose (Qiagen) for 1-2 hours at room temperature. Next, gravity flow columns were used to
201   collect the Ni-NTA agarose and the protein was eluted. Each protein was concentrated in Amicon

202 centrifugal units (EMD Millipore) and re-suspended in phosphate buffered saline (PBS). Proteins were
203 analyzed on reducing SDS-PAGE. The DNA sequence for all constructs is available from the Krammer
204 laboratory. Several of the expression plasmids and proteins have also been submitted to BEI Resources
205 and can be requested from their web page for free (https://www.beiresources.org/).
206
207 **SDS-PAGE**
208 Recombinant proteins were analyzed via a standard SDS-PAGE gel to check protein integrity. One ug of
209 protein was mixed with 2X Laemmli buffer containing 5% beta-mercaptoethanol (BME) at a ratio of 1:1.
210 Samples were heated at 100 °Celsius for 15 minutes and then loaded onto a polyacrylamide gel (5% to
211 20% gradient; Bio-Rad). Gels were stained with SimplyBlue SafeStain (Invitrogen) for 1-2 hours and then
212 de-stained in distilled water overnight.
213
214
215 **Human samples**
216 Banked human samples were collected from study participants enrolled in ongoing IRB approved
217 longitudinal observational study protocols of the Mount Sinai Personalized Virology Initiative. Samples
218 were selected based on the date of collection (2019, early 2020) and whether participants had a
219 documented history of viral infection. Samples were collected in the Clinical Research Unit at the Icahn
220 School of Medicine at Mount Sinai after obtaining written consent and all participants agreed to sample
221 banking and future research use. Self-reported ethnicities of the individuals from which samples were
222 tested included Caucasian, Asian, African American and Hispanic. Samples included sera from a
223 participant with acute NL63 infection as determined by the Biofire Respiratory panel. We included
224 serum collected at day 3 post symptom onset as well as convalescent serum from the same person (day
225 30 post symptom onset). In addition, we tested convalescent sera from individuals with dengue,
226 chikungunya and hantavirus infections. These samples served as negative controls given that they were
227 collect prior to SARS-Cov-2 spread in the US. Six subjects were 20-29, 19 were 30-39, 13 were 40-49, 7
228 were 50-59 years old and six were 60 or older. For the mRBD ELISAs sera from additional nine t subjects
229 were tested (30-39: 2; 40-49: 4; 50-59: 2; 60+: 1).
230 De-identified samples from the University of Melbourne and University of Helsinki were used as positive
231 controls. For those, human experimental work was conducted according to the Declaration of Helsinki
232 Principles and according to the Australian National Health and Medical Research Council Code of
233 Practice. All participants provided written informed consent prior to the study. The studies were
234 approved by the Alfred Hospital (ID #280/14) and University of Melbourne (ID #1442952.1, 1955465.2)
235 Human Research Ethics Committees, and under research permit for project TYH2018322 of Helsinki
236 University Hospital Laboratory.
237
238 **ELISA**
239 The ELISA protocol was adapted from previously established protocols [26,27]. Ninety-six well plates
240 (Immulon 4 HBX; Thermo Scientific) were coated overnight at 4°Celsius with 50 ul per well of a 2 ug/ml
241 solution of each respective protein suspended in PBS (Gibco). The next morning, the coating solution
242 was removed and 100 ul per well of 3% non-fat milk prepared in PBS with 0.1% Tween 20 (TPBS) was
243 added to the plates at room temperature (RT) for 1 hour as blocking solution. Serum samples were
244 heated at 56°C for 1 hour before use to reduce risk from any potential residual virus in serum. Serial
245 dilutions of serum and antibody samples were prepared in 1% non-fat milk prepared in TPBS. The
246 blocking solution was removed and 100 ul of each serial dilution was added to the plates for 2 hours at
247 RT. Next, the plates were washed thrice with 250ul per well of 0.1% TPBS. Next, a 1:3000 dilution of goat
248 anti-human IgG-horseradish peroxidase (HRP) conjugated secondary antibody (ThermoFisher
249 Scientific) was prepared in 0.1% TPBS and 100 ul of this secondary antibody was added to each well for 1

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250 hour. Plates were again washed thrice with 0.1% TBS. Once completely dry, 100 ul of SigmaFast OPD (*o*-
251 phenylenediamine dihydrochloride; Sigma-Aldrich) solution was added to each well. This substrate was
252 left on the plates for 10 minutes and then the reaction was stopped by addition of 50 µL per well of 3 M
253 hydrochloric acid (HCl). The optical density at 490 nanometers was measured via a Synergy 4 (BioTek)
254 plate reader. The background value was set at and optical density 490nm of 0.11 and area under the
255 curve (AUC) was calculated. AUC values below 1 were assigned a value of 0.5 for graphing and
256 calculation purposes. Data was analyzed in Prism 7 (Graphpad).
257
258 To assess the distribution of the different antibody isotypes/subclasses in the samples that reacted well
259 in our standard ELISA, another ELISA was performed with different secondary antibodies [23]. These
260 antibodies include anti-human IgA (α-chain-specific) HRP antibody (Sigma A0295) (1:3,000), anti-human
261 IgM (µ-chain-specific) HRP antibody (Sigma A6907) (1:3,000), anti-human IgG1 Fc-HRP (Southern Biotech
262 9054-05) (1:3,000), anti-human IgG3hinge-HRP (Southern Biotech 9210-05) (1:3,000), and anti-human
263 IgG4 Fc-HRP (Southern Biotech 9200-05).
264

265 **Acknowledgements**

281

282 **Conflict of interest**

283 The authors declare no conflict of interest.

284

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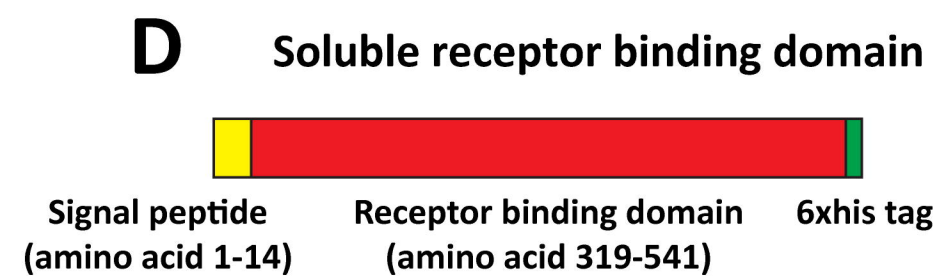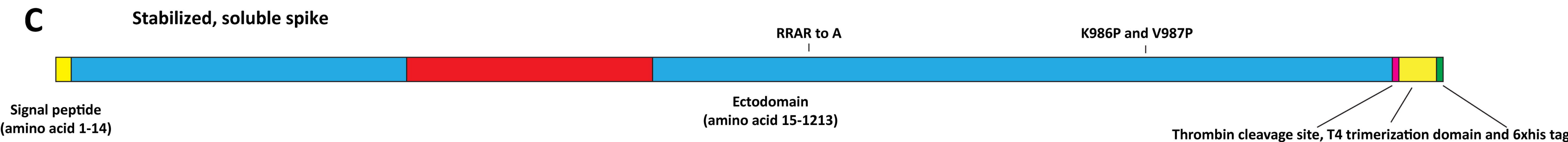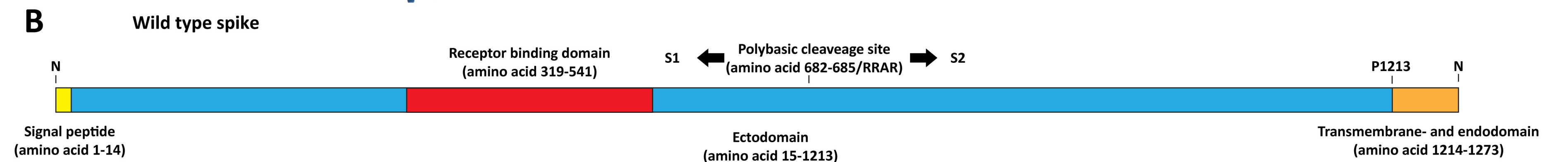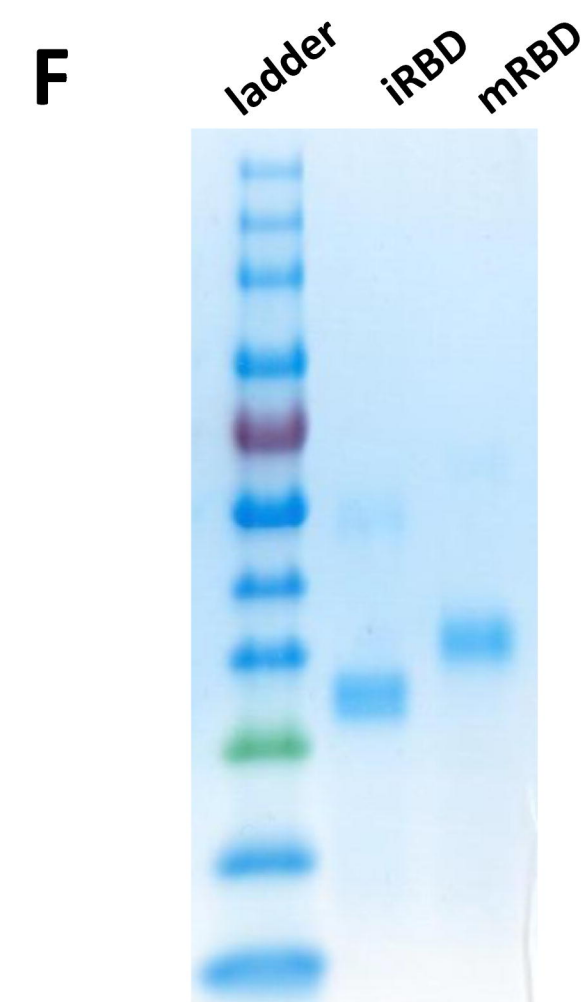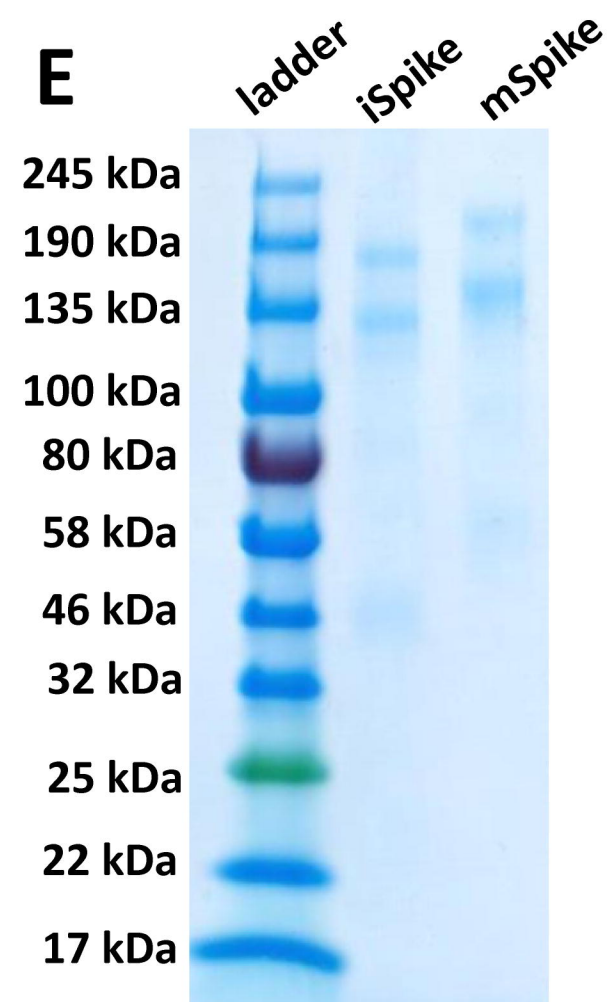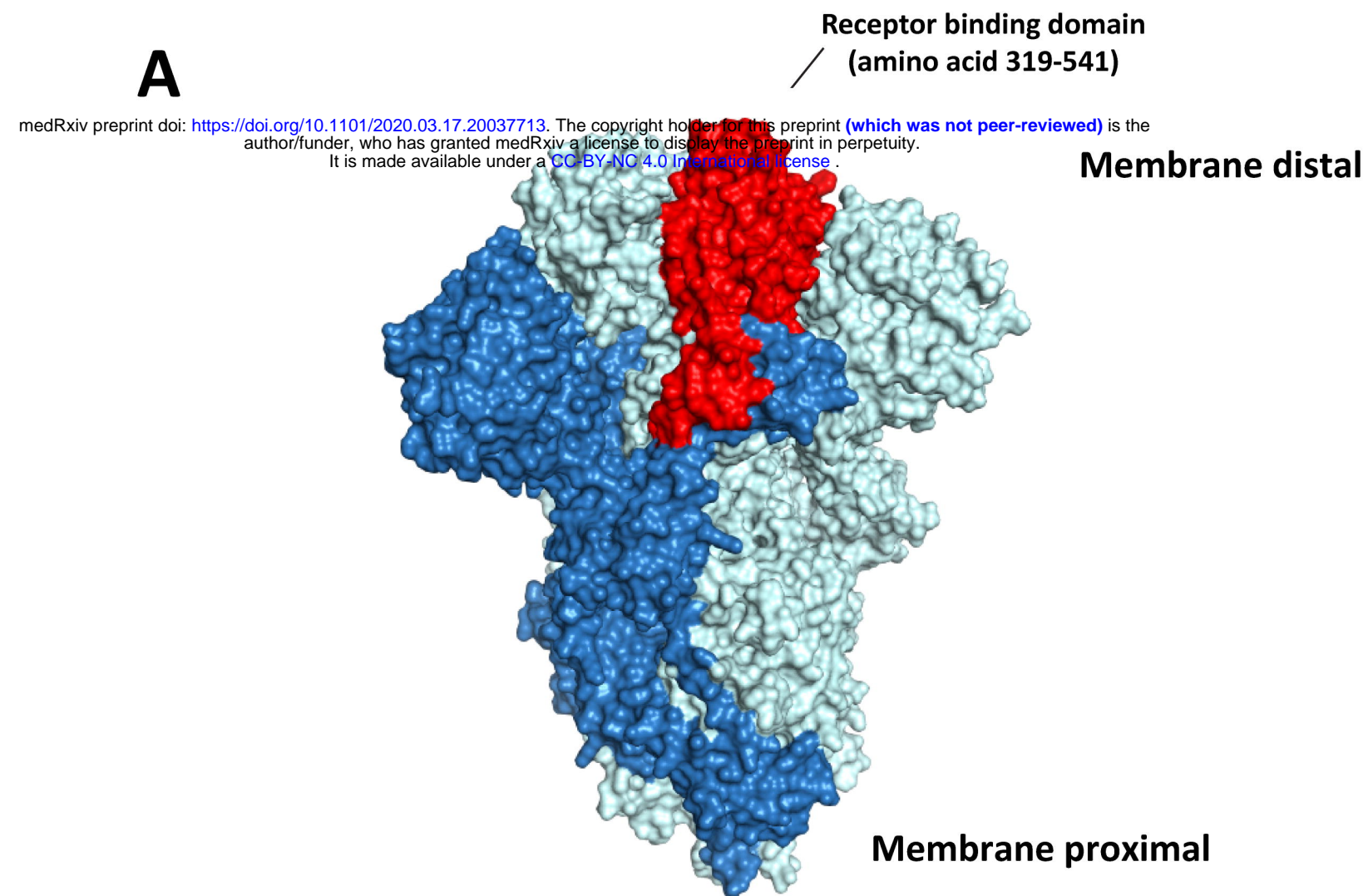
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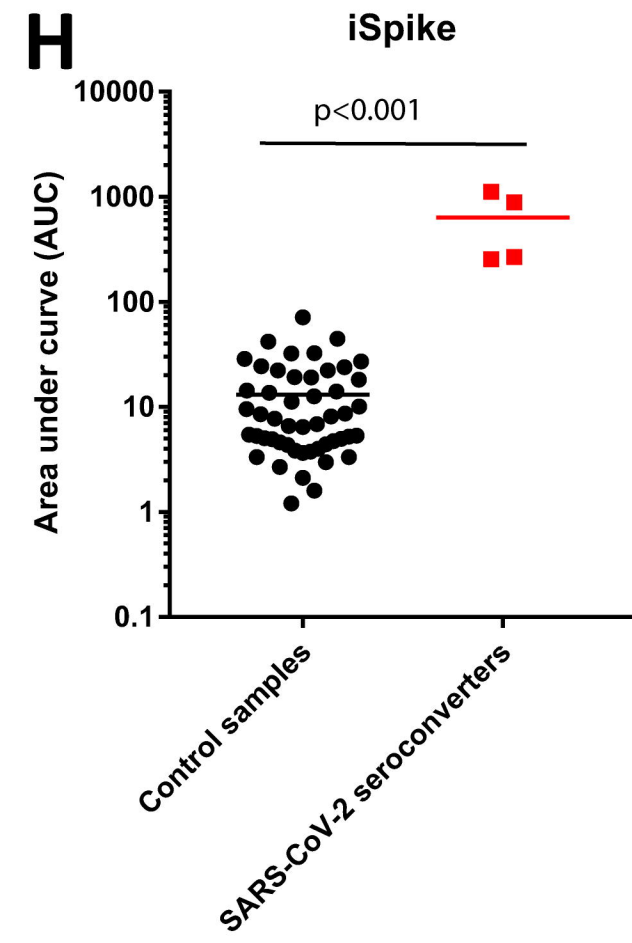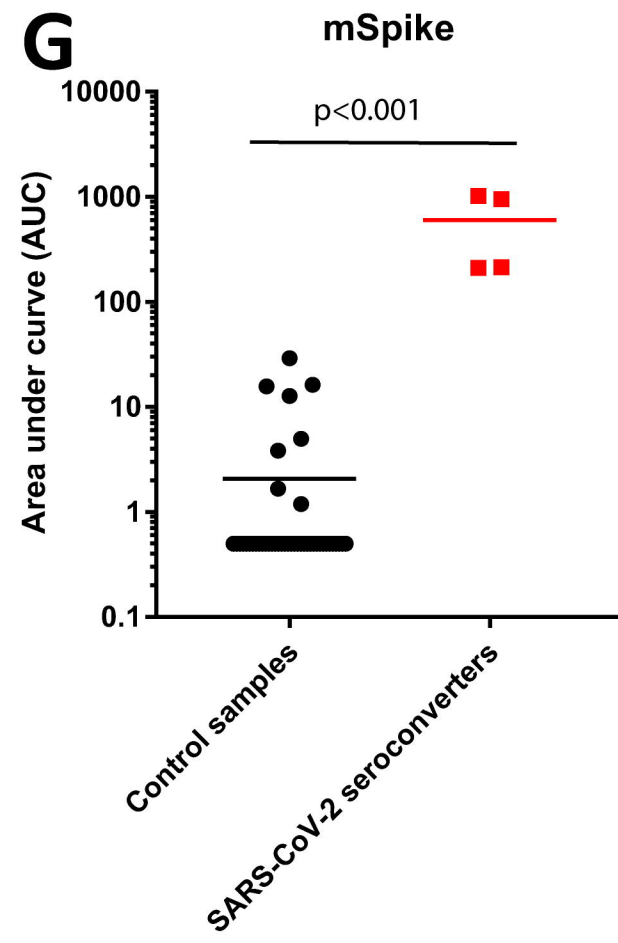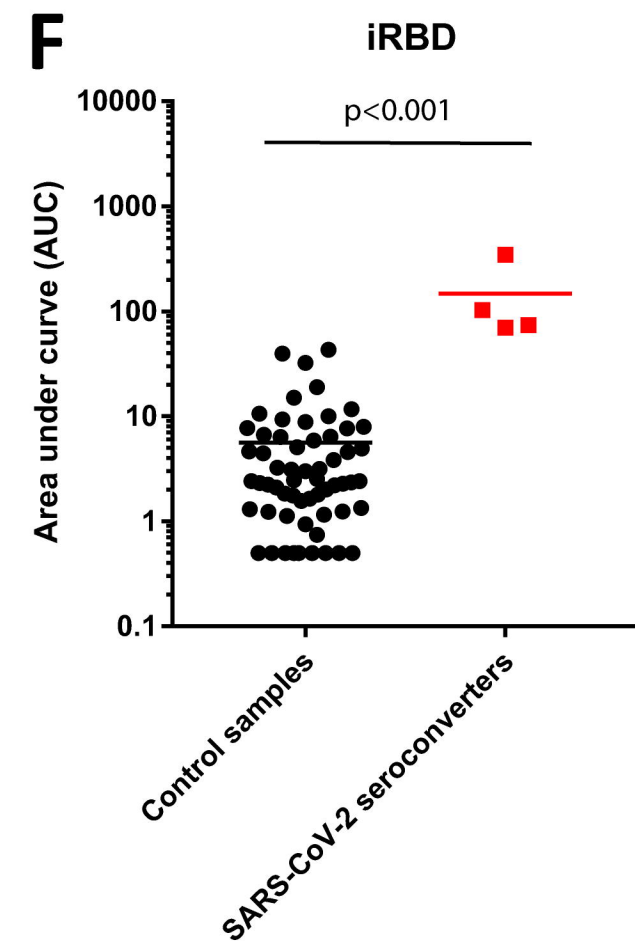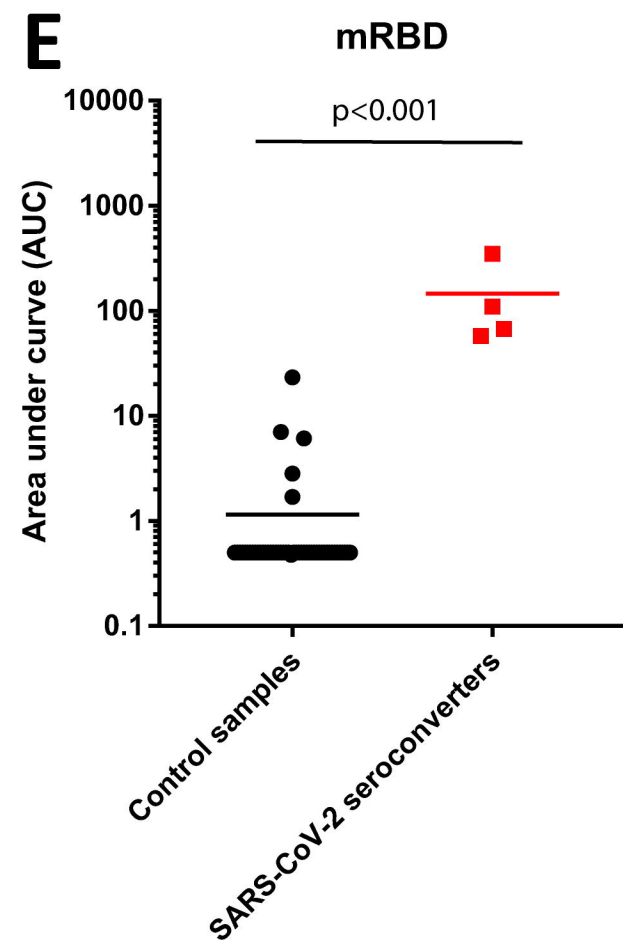345 2017;2:1415-24.

346

347 **Figure legends**

348

349 **Figure 1: Constructs for recombinant protein expression. A** Visualization of the trimeric spike protein of
350 SARS-CoV-2 based on PBD # 6VXX using Pymol.[8] One monomer is colored in dark blue while the
351 remaining two monomers are held in light blue. The receptor binding domain (RBD) of the dark blue
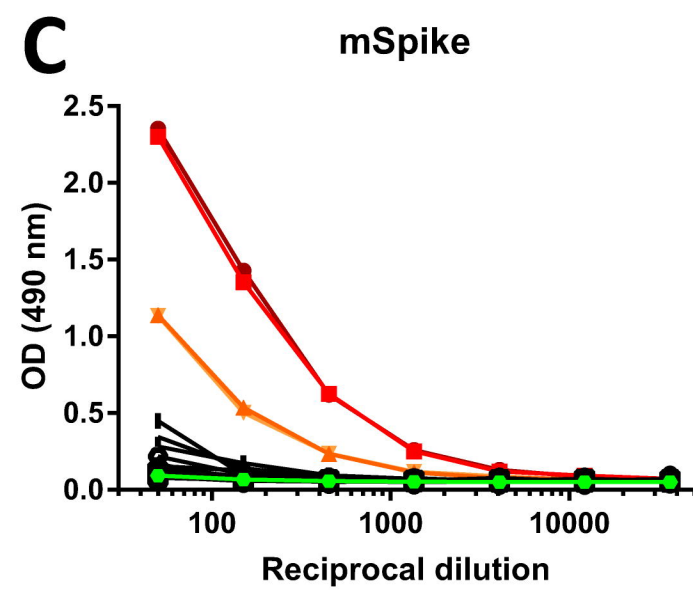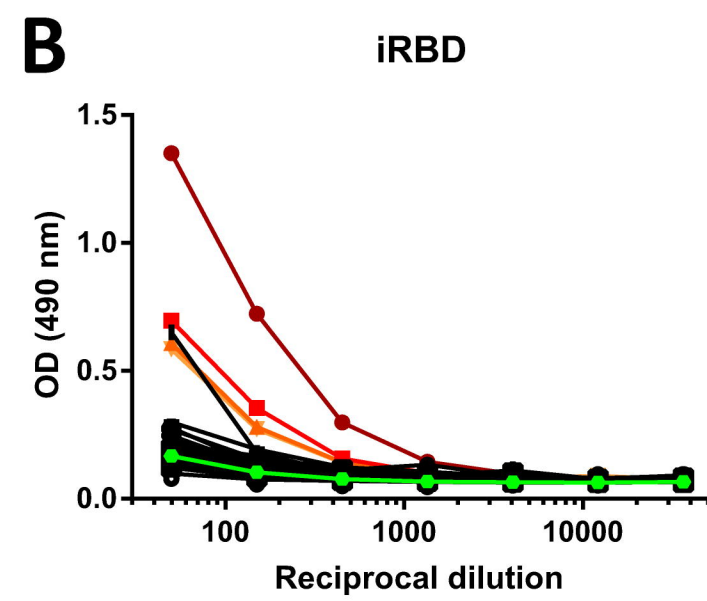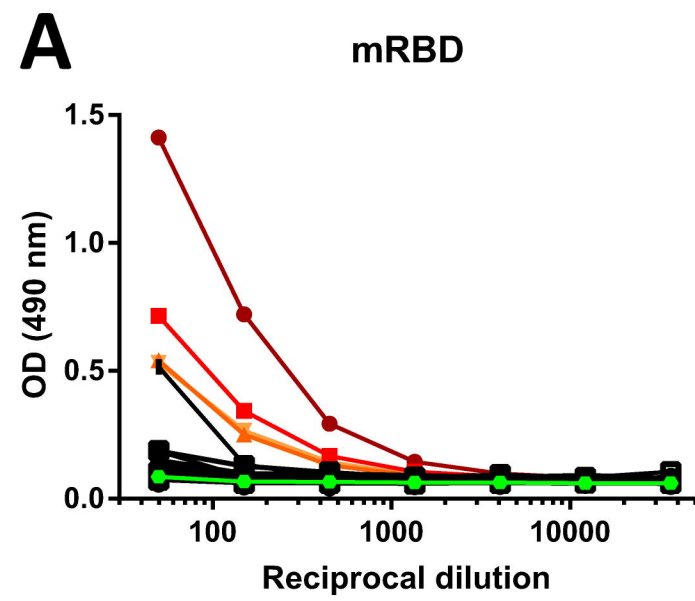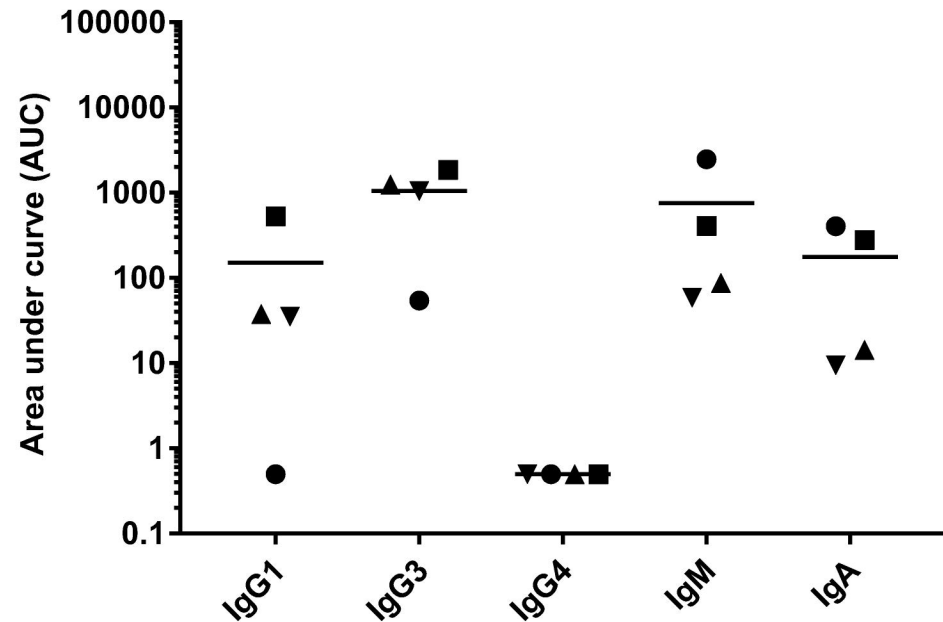352 trimer is highlighted in red. **B** Schematic of the wild type full length spike protein with signal peptide,
353 ectodomain, receptor binding domain, furin cleavage site, S1, S2, and transmembrane and endodomain
354 domain indicated. **C** Schematic of the soluble trimeric spike. The polybasic/furin cleavage site (RRAR)
355 was replaced by a single A. The transmembrane and endodomain were replaced by a furin cleavage site,
356 a T4 foldon tetramerization domain and a hexahistidine tag. Introduction of K986P and V987P has been
357 shown to stabilize the trimer in the pre-fusion conformation. **D** Schematic of the soluble receptor
358 binding domain construct. All constructs are to scale. **E** Reducing SDS PAGE of insect cell and mammalian
359 cell derived soluble trimerized spike protein (iSpike and rSpike). **F** Reducing SDS PAGE of insect cell
360 derived and mammalian cell derived recombinant receptor binding domain (iRBD and mRBD).

361 **Figure 2: Reactivity of control and SARS-CoV-2 convalescent sera to different spike antigens. A-D**
362 Reactivity to insect cell derived RBD (iRBD), mammalian cell derived RBD (mRBD), insect cell derived
363 soluble spike protein (iSpike) and mammalian cell derived soluble spike protein (sSpike). Sera from three
364 SARS-CoV-2 infected individuals were used and are shown in shades of red. Two samples are from the
365 same patient but from different time points (SARS-CoV-2 #3A and #3B). One sample, shown in green, is
366 a convalescent serum sample post NL63 infection. **E-F** shows data from the same experiment but
367 graphed as area under the curve (AUC) to get a better quantitative impression. The n for the control
368 samples is 50 except for the iRBD were it is 59. Statistics were performed using a student's t-test in
369 Graphpad Prism.

370 **Figure 3: Isotypes and subtypes of antibodies from SARS-CoV-2 convalescent sera to the soluble spike
371 protein.** Insect cell derived (**A**) and mammalian cell derived (**B**) spike protein was used to study
372 isotype/subclass distribution of antibodies. The different samples are indicated by different symbols.
373 Sera from three SARS-CoV-2 infected individuals were used and are shown in shades of red. Two
374 samples are from the same patient but from different time points (SARS-CoV-2 #3A and #3B).

A

Receptor binding domain
(amino acid 319-541)

Membrane distal

Membrane proximal

E

ladder   iSpike   mSpike

245 kDa
190 kDa
135 kDa
100 kDa
80 kDa
58 kDa
46 kDa
32 kDa
25 kDa
22 kDa
17 kDa

F

ladder   iRBD   mRBD

B   **Wild type spike**

N

Receptor binding domain
(amino acid 319-541)

S1   ← Polybasic cleaveage site →   S2
(amino acid 682-685/RRAR)

P1213   N

Signal peptide
(amino acid 1-14)

Ectodomain
(amino acid 15-1213)

Transmembrane- and endodomain
(amino acid 1214-1273)

C   **Stabilized, soluble spike**

RRAR to A

K986P and V987P

Signal peptide
(amino acid 1-14)

Ectodomain
(amino acid 15-1213)

Thrombin cleavage site, T4 trimerization domain and 6xhis tag

D   **Soluble receptor binding domain**

Signal peptide
(amino acid 1-14)

Receptor binding domain
(amino acid 319-541)

6xhis tag

A mSpike isotyping

B iSpike isotyping

- ■ SARS-CoV-2 #1
- ● SARS-CoV-2 #2
- ▲ SARS-CoV-2 #3A
- ▼ SARS-CoV-2 #3B