

1 **A detailed protocol for a serological assay to detect SARS-CoV-2**
2 **seroconversion in humans: antigen production and test setup**

3 *Daniel Stadlbauer^{1#}, Fatima Amanat^{1,2#}, Veronika Chromikova¹, Kaijun Jiang¹, Shirin*
4 *Strohmeier^{1,3}, Guha Asthagiri Arunkumar^{1,2}, Jessica Tan^{1,2}, Disha Bhavsar¹, Christina*
5 *Capuano¹, Ericka Kirkpatrick^{1,2}, Philip Meade^{1,2}, Nichalle Britto¹, Catherine Teo¹, Meagan*
6 *McMahon¹, Viviana Simon^{1,4,5}, Florian Krammer^{1*}*

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

10 *²Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York,*
11 *NY, USA*

12 *³Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna,*
13 *Austria*

14 *⁴Global Health Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, NY,*
15 *USA*

16 *⁵Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount*
17 *Sinai, New York, NY, USA*

18
19 *To whom correspondence should be addressed: florian.krammer@mssm.edu

20 #contributed equally

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29 **Abstract**

30 In late 2019, cases of atypical pneumonia were detected in China. The etiological agent was
31 quickly identified as betacoronavirus (named SARS-CoV-2) which has since caused a pandemic.
32 Several methods allowing for the specific detection of viral nucleic acids have been established
33 but only allow detection of the virus during a short period of time, generally during acute infection.
34 Serological assays are urgently needed to conduct serosurveys, to understand the antibody
35 responses mounted in response to the virus and last but not least for identifying individuals who
36 are potentially immune re-infection. Here we describe a detailed protocol for expression of
37 antigens derived from the spike protein of SARS-CoV-2 that can serve as substrate for
38 immunological assays as well as a two-step serological enzyme-linked immunosorbent assay
39 (ELISA). These assays can be used for research studies as well as for testing in clinical
40 laboratories.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes **CO**rona**VI**rus **D**isease 2019 (COVID19), emerged in late 2019 in Wuhan, China^{1,2}. The virus rapidly spread globally causing a pandemic. Currently, no drugs or antivirals are available and countermeasures are limited to non-pharmaceutical interventions (NPIs). Nucleic acid-based tests for detection of the virus during acute disease are in use worldwide^{3,4}.

However, the development of serological assays has been lagging behind due to lack of suitable reagents. Serological assays are needed to perform serosurveys aimed at determining the real infection rate and infection fatality rate in a given population. Furthermore, they are useful to characterize the immune response to the virus in a detailed qualitative and quantitative manner. In addition, serological assays are also of immediate practical use. They can be used to identify individuals who were infected (including severe, mild and asymptomatic cases) and who are now potentially immune. A recent study in non-human primates showed that reinfection, at least in the small number of animals used in this study, does not occur⁵ once antibody responses have been mounted. Infection with coronaviruses circulating in human populations such as HKU, NL63 etc. also leads to immunity that protects from re-infection for months to years⁶. Therefore, individuals

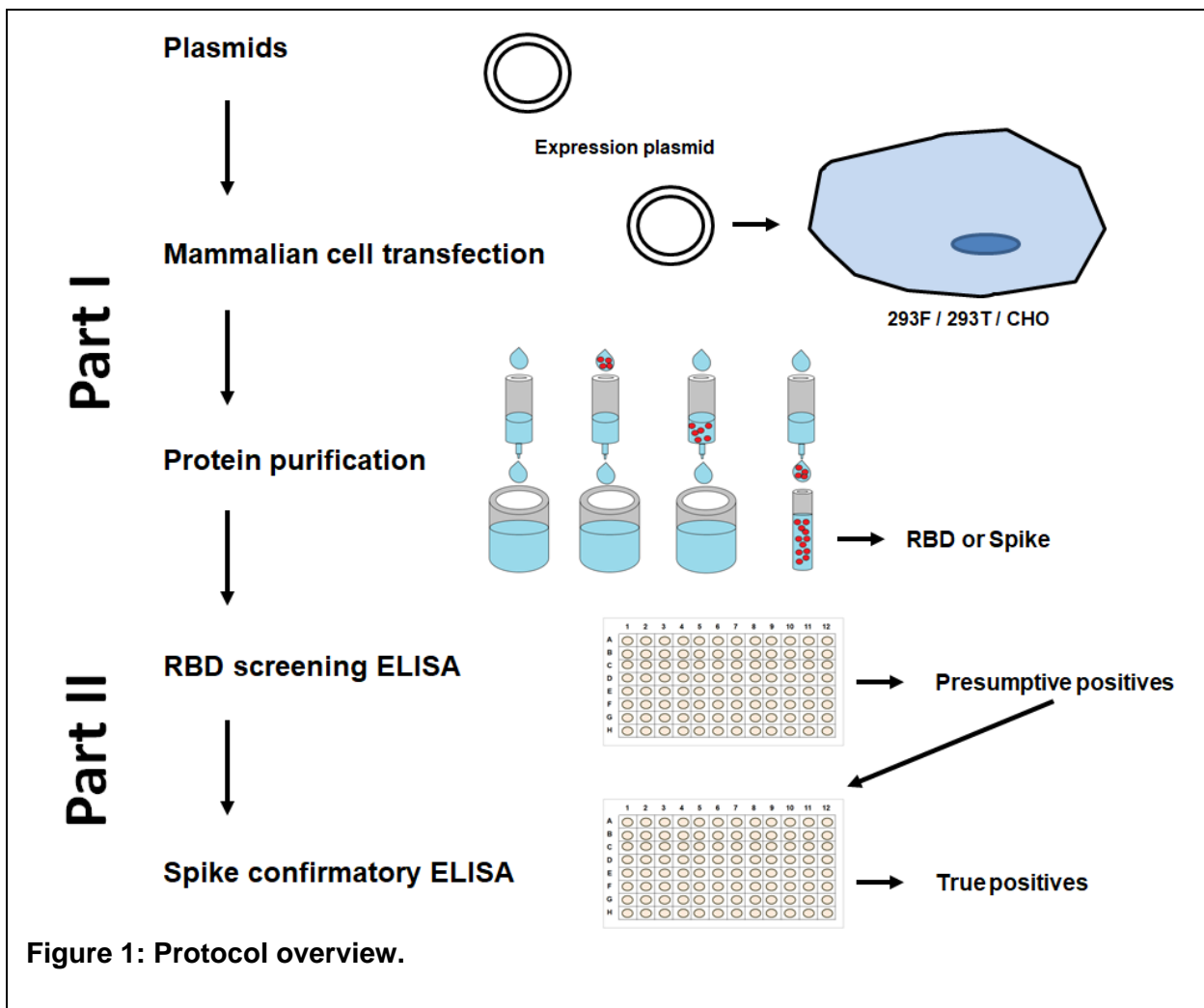


Figure 1: Protocol overview.

66 who have mounted an immune response to SARS-CoV-2 are likely immune, which means that
67 they are unlikely to transmit the virus to others. As an example, health care workers who are
68 immune can take care of COVID19 patients with minimal risk to themselves, their colleagues and
69 other patients. In addition, the use of convalescent serum may serve as valuable treatment option
70 for patients with severe COVID19, especially in the absence of other options. A serological assay
71 is critical for identifying potential blood donors.

72 The surface glycoprotein of the virus, termed spike (S) protein, mediates attachment of the virus
73 to human cells via its receptor binding domain (RBD)⁷ and mediates fusion of viral and cellular
74 membranes. Antibodies that bind to the spike protein, and especially to the RBD domain, can
75 neutralize coronaviruses. We used, therefore, different recombinant spike protein preparations as
76 antigen for our ELISA. We reported in our earlier work that individuals not exposed to SARS-
77 CoV-2 are completely naïve for the spike protein and show no reactivity in an ELISA⁸. It is,
78 therefore, easy to distinguish between exposed/immune and naïve people.

79 In this report, we provide detailed protocols for expressing the needed antigen(s) (**Part I**) as well
80 as for setting up the ELISA that we have developed (**Part II**) (**Figure 1**). We believe that this
81 protocol will be useful not only for research laboratories around the globe but also for testing in
82 diagnostic/clinical laboratories. The described protocol setup works well for us but it can easily be
83 modified, adapted to local needs and improved by the research community in the future.
84 Mammalian expression plasmids for the generation of the recombinant proteins are available from
85 the corresponding author.

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87 **Part I: Mammalian cell transfection and protein purification protocol**

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89 This protocol can be used for both expression vectors for the secreted RBD as well as a soluble
90 trimeric version of the SARS-CoV-2 spike protein. Expression levels of the RBD are very high in
91 our hands (>20 mg/l culture) while expression levels for the full length spike are lower
92 (approximately 1 mg/ml). Therefore, we use the recombinant RBD for initial screening ELISAs
93 and the full length spike for confirmatory ELISAs (as described in **Part II**). Preparation of plasmids
94 for mammalian cell expression is not described here. The plasmids all carry a betalactamase
95 (amp) resistance gene. They are grown in *E. coli* at 37°C (or 30°C) in shaker flasks over night.
96 High quality plasmid DNA can be obtained using commercially available maxiprep kits (ideally
97 with an endotoxin removal step). Importantly, other cell lines (293T, CHO etc.), other media,
98 transfection reagents and more sophisticated protein purification methods might be used as
99 alternatives. Of note, cells can also be transfected in regular flasks in regular incubators without
100 shaking.

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102 **MATERIALS**

- 103 ● Expi293 Expression Medium (Gibco #A1435102)
- 104 ● Opti-MEM™ I Reduced Serum Medium (Gibco #31985088)
- 105 ● ExpiFectamine™ 293 Transfection Kit (Gibco #A14524)
- 106 ● PBS (1X) (Gibco #10010-023 or equivalent)
- 107 ● Ni-NTA Agarose (Qiagen #30230 or equivalent)
- 108 ● SDS-PAGE gels (Bio-Rad #4561094 or equivalent)
- 109 ● SDS-PAGE cell and power supply
- 110 ● Sodium phosphate monobasic monohydrate NaH₂PO₄ · H₂O (Sigma Aldrich #S3522 or
111 equivalent)
- 112 ● Sodium Chloride NaCl (Sigma-Aldrich #S3014 or equivalent)
- 113 ● Imidazole (Sigma-Aldrich #I5513 or equivalent)

- 114 ● Disposable Polycarbonate Erlenmeyer Flasks (Corning #431147)
- 115 ● Trypan blue solution, 0.4 % (Gibco #15250-06 or equivalent)
- 116 ● Cell counting slides (Invitrogen #C10312 or equivalent)
- 117 ● 5mL Polypropylene columns (Qiagen #34964 or equivalent)
- 118 ● Amicon™ Ultra Centrifugal Filter Units 10 kDa (MilliporeSigma #UFC901024 or
- 119 equivalent)
- 120 ● Amicon™ Ultra Centrifugal Filter Units 50 kDa (MilliporeSigma #UFC905024 or
- 121 equivalent)
- 122 ● Polypropylene sterile conical tubes
 - 123 ○ 15 mL (Denville Scientific #C1018P or equivalent)
 - 124 ○ 50 mL (Fisher Denville Scientific #C1060P or equivalent)
- 125 ● Sterile, serological pipettes
 - 126 ○ 5mL (Falcon #356543 or equivalent)
 - 127 ○ 10mL (Falcon #357551 or equivalent)
 - 128 ○ 25 mL (Falcon #357535 or equivalent)
 - 129 ○ 50 mL (Falcon #356550 or equivalent)
- 130 ● Micropipette tips
 - 131 ○ 20 µL barrier tips (Denville Scientific #P1121 or equivalent)
 - 132 ○ 200 µL barrier tips (Denville Scientific #P1122 or equivalent)
 - 133 ○ 200 µL tips (USA Scientific #1111-1700 or equivalent)
 - 134 ○ 1000 µL barrier tips (Denville Scientific #P1126 or equivalent)
- 135 ● 1.5 mL Eppendorf tubes (Denville #C2170 or equivalent)
- 136 ● Stericup Quick Release-GP Sterile Vacuum Filtration System (MilliporeSigma
- 137 S2GPU05RE or equivalent)
- 138 ● Pipet-Aid
- 139 ● Micropipettes
- 140 ● Class II biological safety cabinet
- 141 ● Timer
- 142 ● Countess II cell counter or equivalent
- 143 ● CO₂ incubator with built in shaker (Eppendorf New Brunswick S41i or Equivalent)
- 144 ● Benchtop shaker (Benchmark #BT3000 or equivalent)
- 145 ● Cooling Centrifuge (Eppendorf 5810R or equivalent)
- 146 ● Refrigerator at 4°C (+/- 1°C)
- 147 ● Ultra-Low Freezer (-80°C)

148 DEFINITIONS

- 151 ● RBD = Receptor Binding Domain of SARS-CoV-2 (NR-52306)
- 152 ● PBS = Phosphate-Buffered Saline
- 153 ● RT = Room Temperature (18-25°C)
- 154 ● MEM = Minimum Essential Medium
- 155 ● DNA = Deoxyribonucleic Acid
- 156 ● Ni-NTA = Nickel-Nitrilotriacetic acid

157 PROCEDURE:

158 Transfection in mammalian cells:

161 HEK 293F cells are counted using an automated cell counter (or a regular counting chamber) and
162 seeded at a density of 600,000 cells/ml in Expi293 expression medium. The viability of the cells
163 must be greater than 90% at all times. Cells are passaged every 3-4 days and incubated in an
164 orbital shaking incubator at 37°C and 125 RPM with 8% CO₂. A maximum cell density of 4-5 x
165 10⁶ cells/ml is recommended and at this point, cells should be immediately passaged.

166 Transfections are performed according to manufacturer's instructions. 600 x 10⁶ cells are
167 suspended in 200 ml of Expi293 expression media in a 1 L shaker flask. Twelve ml of Opti-MEM
168 is added to two 50 ml falcon tubes: one tube receives 200 ug (1 ug/ul) of respective plasmid DNA
169 (for RBD or full-length spike) while the other tube receives 640 ul of ExpiFectamine transfection
170 reagent. The contents of both the 50 ml Falcon tubes are mixed together and incubated at RT for
171 10 minutes after which the transfection mixture is added dropwise to the cells. Cells are then
172 returned to the shaking incubator. Sixteen hours post transfection, 1.2 ml of Expifectamine 293
173 Transfection Enhancer 1 and 12.1 ml of Expifectamine 293 Transfection Enhancer 2 is added to
174 the culture and subsequently, the culture is returned to the shaking incubator.

175 Three days post-transfection, the cells are harvested and spun at 4,000g for 20 minutes at 4°C.
176 The supernatant is filtered using a 0.22 um stericup filter, the cell pellet can be discarded.
177 Alternately, cells can be spun at 200g for 10 minutes, supernatant can be collected, and the same
178 cells can be resuspended in 200 mls of fresh Expi293 expression medium and returned to the
179 shaking incubator for another 3 days. This alternate strategy works well with the RBD but is less
180 suitable for the full-length spike (we have detected protein degradation in that case).

181 Ideally is the supernatant containing the protein is further processed immediately. Alternatively, if
182 it is stored, it must be kept at 4°C (and for no longer than overnight/16h) in order to prevent
183 denaturation of the protein at room temperature.

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185 **Protein purification via gravity flow:**

186 *Note: This step can be substituted with more advanced purification methodology if e.g. an Aekta*
187 *purifier is available. The methods described below work, even in labs not geared towards protein*
188 *purification.*

189 Prior to use, Ni-NTA resin (6 ml per 200 ml culture) is washed with fresh PBS, then spun at 2000g
190 for 10 min in a centrifuge. Once the centrifugation is complete, PBS is discarded, and resin is
191 resuspended with the supernatant from cells and inverted about two or three times. The resin is
192 then incubated with the supernatant for 2 hours on a shaker at RT.

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194 Two clean polypropylene columns are loaded with the supernatant-resin mixture and then washed
195 with Wash Buffer four times. Columns are then eluted using the Elution Buffer. Which contains a
196 high concentration of imidazole Four fractions are collected from each column by incubating the
197 resin in the column with 3 ml of Elution Buffer for each fraction. Eluate is collected directly in a 50
198 ml falcon tube placed on ice. The total volume of eluate should be 24 ml from the two columns.
199 More columns can be used to speed up the purification time depending on the volume of the
200 culture.

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202 Eluate is spun through 10 kDa Amicon Centrifugal Filter Units (for RBD) or 50 kDa Amicon
203 Centrifugal Filter Units (for full-length spike) at 4000g for 30 minutes (or longer if eluate takes
204 longer to pass through the membrane) at 4°C until only 200-300 ul remain in the unit. The

205 Centrifugal Filter Unit is then washed with PBS twice by centrifugation at 4000g for 30 minutes at
206 4°C (washing means filling up with PBS and centrifugation until the volume in the unit is down to
207 200-300ul again). Finally, the protein is collected from the Amicon centrifugal unit, concentration
208 is measured (e.g. using Bradford reagent or similar methods), and a denaturing SDS-page is run
209 to check integrity of the purified protein.

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211 After the elution step, protein is always kept on ice. For storage longer than 24h it should be frozen
212 to -80°C to avoid degradation.

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214 **Wash buffer (4L):**

215 NaH₂PO₄·H₂O 31.74 g
216 NaCl 70.16 g
217 Imidazole 5.44 g (final concentration is 20 mM)
218 Distilled water* 4L

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221 **Elution buffer (4L):**

222 NaH₂PO₄·H₂O 31.74 g
223 NaCl 70.16 g
224 Imidazole 64.0 g (final concentration is 235 mM)
225 Distilled water* 4L

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227 *Use Distilled water filtered using a 0.22um stericup vacuum filtration system.

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230 **Part II: A two-step ELISA protocol for high throughput screening of human serum** 231 **samples for antibodies binding to the spike protein of SARS-CoV-2**

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233 The purpose of this part of the protocol is to describe the procedure for measuring human antibody
234 responses to the recombinant receptor binding domain (RBD) of the spike protein or full-length
235 spike protein of SARS-CoV-2 and to ensure the reproducibility and consistency of the obtained
236 results.

237 We developed this as a two-step ELISA in which the first step (**A**) includes relatively high
238 throughput screening of samples in a single serum dilution against the RBD (which expresses
239 very well and therefore there is typically more protein available). This is followed by a second step
240 (**B**) in which positive samples from the first step undergo a confirmatory ELISA against the full
241 length spike protein (which is harder to purify, therefore there is usually less available). For the
242 second step a dilution curve is performed. Typically, if only one operator is available, screening
243 ELISAs can be run in the morning (760 samples/10 plates per run) and confirmatory ELISAs can
244 be run in the afternoon (140 samples/10 plates per run). Of note, we describe the assay here as
245 set up in our laboratory. We use a plate washer and a plate reader but no automated system. The
246 protocol can be adapted to an automated liquid handler as well. In addition, one of the difficulties
247 to set up the assay is the availability of appropriate negative and positive controls. Negative
248 controls are easier to come by and can be serum pools of serum taken before 2020. Positive
249 controls can be convalescent samples from COVID19 patients or monoclonal antibodies (mAbs)
250 like CR3022^{9,10}. If no human sera or mAbs are available, mouse mAbs, mouse sera against
251 SARS-CoV-2, order animal sera against SARS-CoVo2 or anti-his tag antibodies (the proteins are
252 his-tagged) can be used. However, in this case a different secondary antibody for the species
253 from which the primary antibody is derived is needed for the positive control. Also, we recommend

254 generating large batches of positive controls, which can be used for many runs. The positive
255 control should be selected to exceed an OD₄₉₀ of the negative control plus 3 standard deviations
256 of the negative controls up to, at least, a 1:150 dilution. ELISAs can be run with both serum and
257 plasma.

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259 *Of note: RBD or full length spike might be used for both ELISA steps if only one antigen is*
260 *available. In addition, only step A (not recommended) or only step B might be performed if fewer*
261 *resources are available.*

262 ● MATERIALS

- 264 ○ Recombinant RBD protein
- 265 ○ Recombinant full-length spike protein
- 266 ○ Flat-Bottom Immuno Nonsterile 96-Well Plates 4 HBX (Thermo Scientific #3855,
267 or equivalent)
- 268 ○ Flat Bottom Cell Culture Plates (Corning #3599 or equivalent)
- 269 ○ Milk Powder (AmericanBio #AB10109-01000, or equivalent)
- 270 ○ PBS (1X) (Gibco #10010-023 or equivalent)
- 271 ○ Water For Injection (WFI) for Cell Culture (Gibco #A1287301 or equivalent)
- 272 ○ Tween 20 (Fisher Bioreagents #BP337-500, or equivalent)
- 273 ○ Phosphate Buffered Saline (10X) (Corning™ 46013CM or equivalent)
- 274 ○ Polypropylene sterile conical tubes
 - 275 ■ 15 mL (Denville Scientific #C1018P or equivalent)
 - 276 ■ 50 mL (Fisher Denville Scientific #C1060P or equivalent)
- 277 ○ Sterile, serological pipettes
 - 278 ■ 5mL (Falcon #356543 or equivalent)
 - 279 ■ 10mL (Falcon #357551 or equivalent)
 - 280 ■ 25 mL (Falcon #357535 or equivalent)
 - 281 ■ 50 mL (Falcon #356550 or equivalent)
- 282 ○ Micropipette tips
 - 283 ■ 20 µL barrier tips (Denville Scientific #P1121 or equivalent)
 - 284 ■ 200 µL barrier tips (Denville Scientific #P1122 or equivalent)
 - 285 ■ 200 µL tips (USA Scientific #1111-1700 or equivalent)
 - 286 ■ 1000 µL barrier tips (Denville Scientific #P1126 or equivalent)
- 287 ○ Sterile reservoirs (Fisher Scientific #07-200-127 or equivalent)
- 288 ○ Anti-Human IgG (Fab specific)-Peroxidase antibody produced in goat (Sigma
289 #A0293)
- 290 ○ Hydrochloric Acid 3.0M (Fisher Scientific #S25856, or equivalent)
- 291 ○ SIGMAFAST™ OPD (Sigma-Aldrich #P9187 or equivalent)
- 292 ○ Kimberly-Clark Kimwipes (Kimberly-Clark Professional #34721 or equivalent)
- 293 ○ Pipet-Aid
- 294 ○ Micropipettes
- 295 ○ Class II biological safety cabinet
- 296 ○ Ultra-Low Freezer (-80°C)
- 297 ○ Refrigerator at 4°C (+/- 1°C)
- 298 ○ Multichannel pipette(s) capable of pipetting 50-250 µL
- 299 ○ 1.5 mL Eppendorf tubes (Denville #C2170 or equivalent)
- 300 ○ Timer
- 301 ○ Aquamax 2000 Plate Washer (Molecular Devices #AQUAMAX 2000 or equivalent)
- 302 ○ Biotek SynergyH1 Microplate Reader or equivalent

303 ● DEFINITIONS

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- RBD = Receptor Binding Domain
- ELISA = Enzyme-Linked Immunosorbent Assay
- PBS = Phosphate-Buffered Saline
- PBS-T = Phosphate-Buffered Saline with 0.1% Tween 20
- RT = Room Temperature (18-25°C)
- HRP = Horseradish Peroxidase
- HCl = Hydrochloric Acid
- OPD = o-phenylenediamine dihydrochloride

● **A - RBD Screening ELISA**

1. Coating ELISA plates (day 1)
 - Thaw the required number of vials of antigen (SARS-CoV-2 **RBD** protein) to coat 96-well microtiter ELISA plates at a concentration of 2 µg/ml. Once thawed, mix by gently vortexing vial before diluting in 1X PBS.
 - Prepare approximately 5 mL for each plate to be coated.
 - Coat plates with 50 µl of diluted protein per well using a multichannel pipette and a reservoir. Lightly tap plates against surface to ensure protein is evenly coating the bottom of every well.
 - Incubate at 4°C overnight. Plates can be stored at 4°C for up to 1 week.
 - Always keep a cover plate on top of coated plates during all steps of the protocol!
2. Heat inactivation of samples (day 1, this is a safety precaution)
 - Set the water bath to 56°C. Once temperature is reached, place the serum/plasma samples in and start the timer for 1h immediately.
 - Remove samples when the timer goes off. Do not leave samples at 56°C for longer than 1h. Store at 4°C overnight or until use.
3. Block ELISA plate (day 2)
 - Calculate to prepare at least 30 ml of blocking solution per plate.
 - Blocking solution consists of PBS-T + 3% milk powder (weight/volume).
 - Using an automated plate washer, wash coated ELISA plates 3x with PBS-T.
 - Add 200 µl blocking solution to all wells of the plates, starting the timer for 1h (do not exceed 4h) after completing the first plate. Place plates in a 20°C (RT) incubator.
Note: This step (and wherever a plate washer is needed below) can also be performed by washing plates with a multichannel pipette by hand if no plate washer is available.
4. Pre-diluting samples (day 2)
 - In a biological safety cabinet, set up sterile Eppendorf tubes to pre-dilute serum samples 1:5.
 - Add 40 µl of sterile 1X PBS to all tubes.
 - Gently vortex serum sample to mix and add 10 µl to the Eppendorf tube, vortexing once more. Do this for all remaining samples including the positive and negative controls. *Volume not needed in this part A will be stored and used for part B.*
5. Dilution plate set-up (day 2)
 - Calculate and prepare at least 30 ml of PBS-T + 1% milk powder (weight/volume).

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- Prepare 1 dilution plate (separate flat bottomed cell culture plate) per antigen coated plate prepared.
- Add 180 µl of PBS-T containing 1% milk to all wells of the dilution plate (including blank wells)
- Leaving Columns 1 and 12 as blanks, add 20 µl of sample (or control) into the designated well. This results in a final serum dilution of 1:50.
- Continue until all samples and controls have been added to designated wells. See reference plate layout below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	(+) Ctrl.	(-) Ctrl.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Blank
B	Blank	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13			Blank
C	Blank											Blank
D	Blank											Blank
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H	Blank		Sample 73	Sample 74	Sample 75	Sample 76	(+) Ctrl.	(-) Ctrl.	Blank

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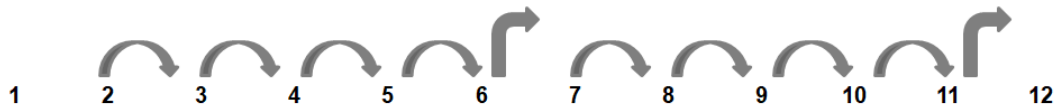
- Transfer serum dilution (day 2)
 - After blocking incubation, remove plates from the room temperature incubator and throw off the blocking solution. Tap the plates dry on a kimwipe.
 - Using a multichannel pipette, pipette up and down 4-6 times in the first row of dilution plate to mix.
 - Transfer 100 µl to the corresponding rows in the ELISA plate. Change tips and continue to transfer second row to the ELISA plate.
 - Start the timer for 2h as soon as all the rows have been transferred to the first ELISA plate. (Do not exceed 4h)
 - Place plates in a 20°C (RT) incubator.
- Secondary Antibody (day 2)
 - After 2h, wash the plates 3x with PBS-T using the automated plate washer.
 - Dilute anti-human IgG (Fab specific) HRP labeled secondary antibody 1:3000 in PBS-T containing 1% milk. Prepare at least 5 ml per plate.

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- Add 50 μ l to all wells of the plate using a multichannel pipette. Be sure to avoid touching the tips of the pipette to the walls of the well to avoid carry over and high background signals.
 - Start the timer for 1h (stay in a range of 50min to 65min) as soon as the secondary antibody has been added to the first plate. Place plates in a 20°C (RT) incubator.
8. Plate development and reading (day 2)
- After 1h, wash plates 3x with PBS-T using an automated plate washer.
 - Prepare SigmaFast OPD solution and calculate amount needed. One set of tablets (1 gold + 1 silver tablet) dissolved in 20 ml WFI can be used for 2 plates.
 - Fully dissolve one gold tablet in 20 mL WFI. Do not add silver tablet to solution until ready to start adding to the plates (needs to be prepared fresh right before use).
 - Add 100 μ l to all wells of the plate. Begin timer for 10 minutes as soon as OPD has been added to the first row on the first plate. Do not exceed 10 minutes of developing before stopping the reaction.
 - To stop the reaction after exactly 10 minutes, add 50 μ l of 3M HCl to all wells.
 - Read ELISA plates in plate reader at an absorbance of 490 nm (immediately after adding HCl) and record data.
 - Samples that exceed certain OD₄₉₀ cutoff value (proposed cutoff: OD₄₉₀ = 0.15-0.2 or mean of negative controls plus 3 times the standard deviation of the negative controls) are assigned presumptive positive and will be tested in confirmatory ELISA using full-length spike protein.
 - OD₄₉₀ cutoff has to be experimentally determined and depends on assay background and noise.
- **B - Spike confirmatory ELISA**
1. Coating ELISA plates (day 1)
- Thaw the required number of vials of antigen (SARS-CoV-2 **Spike** protein) to coat 96-well microtiter ELISA plates at a concentration of 2 μ g/ml. Once thawed, mix by gently vortexing vial before diluting in 1X PBS.
 - Prepare approximately 5 mL for each plate to be coated.
 - Coat plates with 50 μ l of diluted protein per well using a multichannel pipette and a reservoir. Lightly tap plates against surface to ensure protein is evenly coating the bottom of every well.
 - Incubate at 4°C overnight. Plates can likely be stored in 4°C for up to 1 week but this needs to be validate locally to ascertain that it does not change the results.
2. Block ELISA plate (day 2)
- Calculate to prepare at least 30 ml of blocking solution per plate.
 - Blocking solution consists of PBS-T + 3% milk powder (weight/volume).
 - Using an automated plate washer, wash coated ELISA plates 3x with PBS-T.
 - Add 200 μ l blocking solution to all wells of the plates, starting the timer for 1 h (do not exceed 4h) after completing the first plate. Place plates in a 20°C (RT) incubator.
3. Pre-diluting samples (day 2)
- Retrieve 1:5 pre-diluted samples from Part A to be tested and confirmed (samples that are above certain threshold in RBD screening ELISA based on a set OD₄₉₀ value – see end of **A**).

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4. Serial dilution (day 2)

- Calculate and prepare at least 20 ml of PBS-T + 1% milk powder (weight/volume) per plate.
- After blocking incubation, remove plates from the room temperature incubator and throw off the blocking solution. Tap the plates dry on a kimwipe.
- Using a multichannel pipette, add 120 µl of PBS-T containing 1% milk to all wells of the plate.
- Leaving Columns 1 and 12 as BLANKS, add an extra 51 µl only to Columns 2 and 7 (=sample wells).
- Add 9 µl of 1:5 pre-diluted sample (final dilution 1:100 on the plate) to the first well in Column 2 and continue to add samples to all 8 wells. In Column 7, add samples to wells 1 through 6. Transfer positive and negative control into wells 7 and 8, respectively. See reference plate layout below.
- With the multichannel pipette, pipette up and down 4-6 times in Column 2 to mix. Discard these tips. With new tips, transfer 60 µl (3-fold dilution) from Column 2 to Column 3 and pipette up and down once 4-6 times to mix. Repeat this until Column 6; discard 60 µl before Column 7.
- Taking fresh tips mix Column 7 by pipetting. Repeat the same process of transferring, mixing, and discarding tips from Columns 7-11. Once Column 11 is reached, discard 60 µl.
- Start timer for 2h (do not exceed 4h) once the first ELISA plate has been serially diluted.
- Place plates in a 20°C (RT) incubator.



A	Blank	Sample 1					Sample 9					Blank
B	Blank	Sample 2					Sample 10					Blank
C	Blank	Sample 3					Sample 11					Blank
D	Blank	Sample 4					Sample 12					Blank
E	Blank	Sample 5					Sample 13					Blank
F	Blank	Sample 6					Sample 14					Blank
G	Blank	Sample 7					(+) Ctrl.					Blank
H	Blank	Sample 8					(-) Ctrl.					Blank

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5. Secondary Antibody (day 2)
 - After 2h, wash the plates 3x with PBS-T using the automated plate washer.
 - Dilute anti-human IgG (Fab specific) HRP labeled secondary antibody 1:3000 in PBS-T containing 1% milk. Prepare at least 5 ml per plate.
 - Add 50 μ l to all wells of the plate using a multichannel pipette. Be sure to avoid touching the tips of the pipette to the walls of the well.
 - Start the timer for 1 h (50 – 65 min) as soon as the secondary antibody has been added to the first plate. Place plates in a 20°C (RT) incubator.

6. Plate development and reading (day 2)
 - After 1h, wash plates 3x with PBS-T using an automated plate washer.
 - Prepare SigmaFast OPD solution and calculate amount needed. One set of tablets (1 gold + 1 silver tablet) dissolved in 20 mL WFI can be used for 2 plates.
 - Fully dissolve one gold tablet in 20 mL WFI. Do not add silver tablet to solution until ready to start adding to the plates.
 - Add 100 μ l to all wells of the plate. Begin timer for 10 minutes as soon as OPD has been added to the first row of the first plate. Do not exceed 10 minutes of developing before stopping the reaction.
 - To stop the reaction after exactly 10 minutes, add 50 μ l of 3M HCl to all wells.
 - Read ELISA plates in plate reader at an absorbance of 490 nm (immediately after adding HCl) and record data.
 - True positive samples will have a signal higher than the negative control plus 3 standard deviations of the negative controls in at least two consecutive dilutions.

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