SARS-CoV-2 Seroconversion in Humans: A Detailed Protocol for a Serological Assay, Antigen Production, and Test Setup

Daniel Stadlbauer,[1,6] Fatima Amanat,[1,2,6] Veronika Chromikova,[1] Kaijun Jiang,[1] Shirin Strohmeier,[1,3] Guha Asthagiri Arunkumar,[1,2] Jessica Tan,[1,2] Disha Bhavsar,[1] Christina Capuano,[1] Ericka Kirkpatrick,[1,2] Philip Meade,[1,2] Ruhi Nichalle Brito,[1] Catherine Teo,[1] Meagan McMahon,[1] Viviana Simon,[1,4,5] and Florian Krammer[1,7]

[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, New York
[2]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, New York
[3]Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
[4]Global Health Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York
[5]Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, New York
[6]These authors contributed equally to this work
[7]Corresponding author: *florian.krammer@mssm.edu*

In late 2019, cases of atypical pneumonia were detected in China. The etiological agent was quickly identified as a betacoronavirus (named SARS-CoV-2), which has since caused a pandemic. Several methods allowing for the specific detection of viral nucleic acids have been established, but these only allow detection of the virus during a short period of time, generally during acute infection. Serological assays are urgently needed to conduct serosurveys, to understand the antibody responses mounted in response to the virus, and to identify individuals who are potentially immune to re-infection. Here we describe a detailed protocol for expression of antigens derived from the spike protein of SARS-CoV-2 that can serve as a substrate for immunological assays, as well as a two-stage serological enzyme-linked immunosorbent assay (ELISA). These assays can be used for research studies and for testing in clinical laboratories.
© 2020 The Authors.

Basic Protocol 1: Mammalian cell transfection and protein purification
Basic Protocol 2: A two-stage ELISA for high-throughput screening of human serum samples for antibodies binding to the spike protein of SARS-CoV-2

Keywords: COVID19 • COVID-19 • ELISA • protein expression • SARS-CoV-2 • serological assay

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols in Microbiology e100, Volume 57
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmc.100
© 2020 The Authors. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

How to cite this article:
Stadlbauer, D., Amanat, F., Chromikova, V., Jiang, K., Strohmeier, S., Arunkumar, G. A., Tan, J., Bhavsar, D., Capuano, C., Kirkpatrick, E., Meade, P., Brito, R. N., Teo, C., McMahon, M., Simon, V., & Krammer, F. (2020). SARS-CoV-2 seroconversion in humans: A detailed protocol for a serological assay, antigen production, and test setup. *Current Protocols in Microbiology*, *57,* e100. doi: 10.1002/cpmc.100

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COronaVIrus Disease 2019 (COVID19; often written COVID-19), emerged in late 2019 in Wuhan, China (Wu et al., 2020; Zhu et al., 2020). Rapid, global spread of the virus is presently 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 (Chu et al., 2020; Corman et al., 2020). However, the development of serological assays is lagging 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. 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 re-infection, at least in the small number of animals used in the study, does not occur (Bao et al., 2020) 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 years (Callow, Parry, Sergeant, & Tyrrell, 1990). Therefore, individuals who have mounted an immune response to SARS-CoV-2 are likely immune, which means that they are unlikely to transmit the virus to others. As an example, healthcare workers who are immune could potentially care for COVID19 patients with minimal risk to themselves, their colleagues, and other patients. In addition, the use of convalescent plasma may serve as a valuable treatment option for patients with severe COVID19, especially in the absence of other options. A serological assay is critical for identifying potential plasma donors.

The surface glycoprotein of the virus, termed the spike (S) protein, mediates attachment of the virus to human cells via its receptor-binding domain (RBD; Wrapp et al., 2020) and mediates fusion of viral and cellular membranes. Antibodies binding to the spike protein, and especially to the RBD domain, can neutralize SARS-CoV-2. Therefore, we used different recombinant spike protein preparations as the antigens for our ELISA. We reported in our earlier work that individuals not exposed to SARS-CoV-2 are completely naïve to the spike protein, and their serum samples show little or no reactivity in an ELISA (Amanat et al., 2020). It is, therefore, easy to distinguish between exposed/immune and naïve individuals.

In this report, we provide detailed protocols for expressing the required antigen(s) (Basic Protocol 1) as well as setting up the ELISA that we have developed (Basic Protocol 2). An overview of these protocols is shown in Figure 1. We believe that these protocols will be useful not only for research laboratories around the globe, but also for testing in diagnostic/clinical laboratories. The described protocol setup works well for us, but it can easily be modified, adapted to local needs, and improved by the research

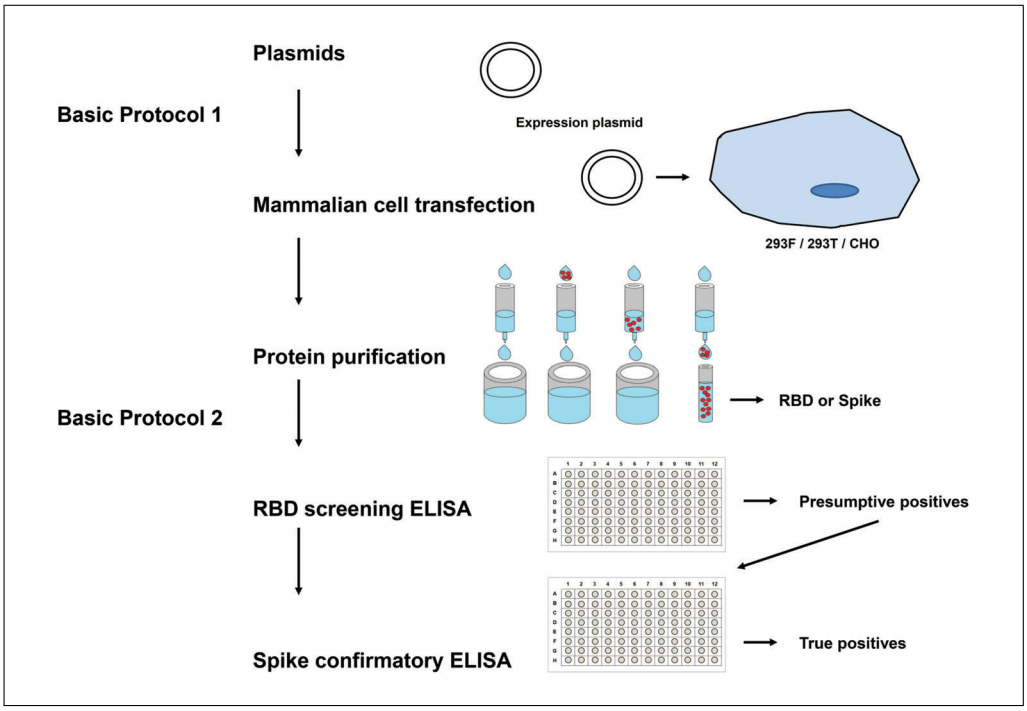

Figure 1 Graphical protocol overview.

community in the future. Not every aspect of these protocols has been optimized in detail, and we provide notes and comments whenever further optimizations and testing are recommended. Mammalian expression plasmids for the generation of the recombinant proteins are available from the corresponding author and from BEI Resources.

MAMMALIAN CELL TRANSFECTION AND PROTEIN PURIFICATION

BASIC PROTOCOL 1

This protocol can be used for both expression vectors: the one expressing secreted RBD as well as the one expressing a soluble, trimeric version of the SARS-CoV-2 spike protein. Expression levels of the RBD are very high in our hands (>20 mg/L culture), while expression levels for the full-length spike are lower (approximately 4 to 5 mg/L). Therefore, we use the recombinant RBD for initial screening ELISAs and the full-length spike for confirmatory ELISAs (as described in Basic Protocol 2). The expression vector constructs were described previously (Amanat et al., 2020). In brief, the sequences used for both proteins are based on the genomic sequence of the first isolate, Wuhan-Hu-1, which was released on January 10, 2020 (GenBank: MN908947.3). Sequences were codon-optimized for mammalian cell expression. The full-length spike protein sequence was modified to remove the polybasic cleavage site, which is recognized by furin, and to add a pair of stabilizing mutations (Figure 2). These two modifications were included to enhance the stability of the protein based on published literature (Amanat et al., 2020). The plasmids are grown in *E. coli* at 37°C (or 30°C) at 225 rpm in Luria-Bertani (LB) broth with ampicillin (LB-amp) in shaker flasks overnight. High-quality plasmid DNA can be obtained using commercially available maxiprep kits (ideally with an endotoxin-removal step). Importantly, other cell lines (293T, CHO, etc.), other media, transfection reagents, and more sophisticated protein purification methods might be used as alternatives if available.

Definitions

RBD = receptor-binding domain of SARS-CoV-2 (NR-52306)
PBS = phosphate-buffered saline
RT = room temperature (18° to 25°C)
MEM = Minimum Essential Medium

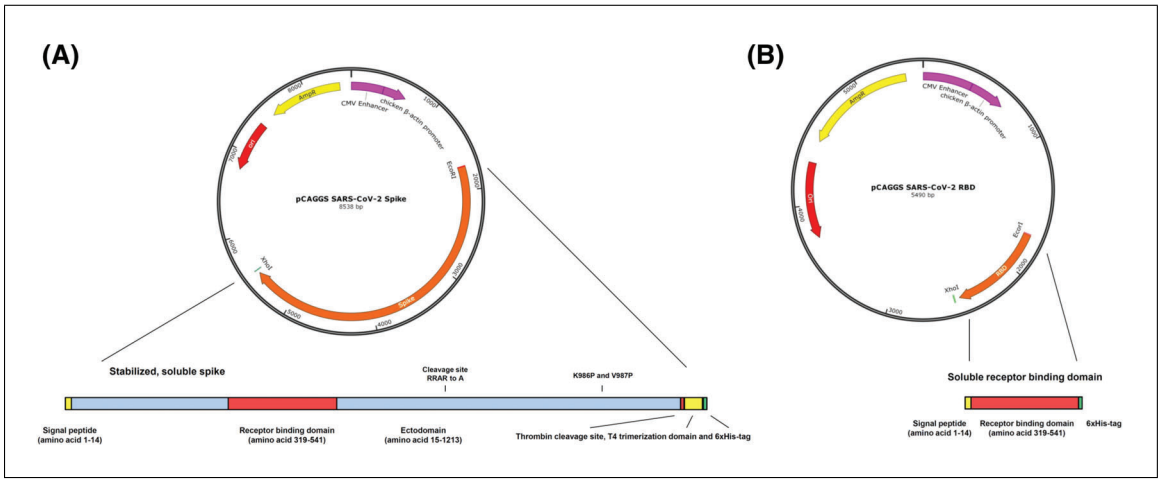

Figure 2 Vector map showing the pCAGGS expression vectors. (**A**) Shows the plasmid map of pCAGGS containing the sequence of the stabilized, soluble spike. The schematic below indicates the signal peptide, receptor binding domain, ectodomain with stabilizing mutations, thrombin cleavage site, T4 trimerization domain, and hexahistidine-tag. (**B**) illustrates the pCAGGS vector encoding for the soluble receptor binding domain. The signal peptide, receptor binding domain, and hexahistidine-tag are indicated.

DNA = deoxyribonucleic acid
Ni-NTA = nickel-nitrilotriacetic acid

Materials

Expi293F cells (Gibco #A14527)
Expi293 Expression Medium (Gibco #A1435102)
Opti-MEM™ I Reduced Serum Medium (Gibco #31985088)
ExpiFectamine™ 293 Transfection Kit (Gibco #A14524)
pCAGGS containing the SARS Coronavirus 2, Wuhan-Hu-1 spike glycoprotein gene RBD with C-terminal hexa-histidine tag (available at BEI Resources #NR-52309, *https://www.beiresources.org/Catalog/BEIPlasmid Vectors/NR-52309.aspx*)
pCAGGS containing the SARS Coronavirus 2, Wuhan-Hu-1 ectodomain spike glycoprotein gene with C-terminal hexa-histidine tag (will be made available at BEI Resources as #NR-52394 and can be requested from the corresponding author)
Ni-NTA agarose resin (Qiagen #30230 or equivalent)
1× PBS (Gibco #10010-023 or equivalent)
Wash buffer (see recipe)
Elution buffer (see recipe)
SDS-PAGE gels (Bio-Rad #4561094 or equivalent)

Class II biological safety cabinet
Cell counting slides (Invitrogen #C10312 or equivalent) or Countess II cell counter (or equivalent)
CO_2 incubator with built-in orbital shaker (Eppendorf New Brunswick S41i or equivalent)
Disposable polycarbonate Erlenmeyer flasks (Corning #431147)
Polypropylene sterile conical tubes:
15-ml (Fisher Denville Scientific #C1018P, or equivalent)
50-ml (Fisher Denville Scientific #C1060P, or equivalent)
Refrigerated centrifuge (Eppendorf 5810R or equivalent)
Stericup Quick Release-GP Sterile Vacuum Filtration System (MilliporeSigma #S2GPU05RE or equivalent)
Benchtop shaker (Benchmark #BT3000 or equivalent)
5-ml polypropylene columns (Qiagen #34964 or equivalent)

Amicon™ Ultra Centrifugal Filter Units, 10 kDa (MilliporeSigma #UFC901024 or equivalent)
Amicon™ Ultra Centrifugal Filter Units, 50 kDa (MilliporeSigma #UFC905024 or equivalent)
Sterile, serological pipettes
5-ml (Falcon #356543 or equivalent)
10-ml (Falcon #357551 or equivalent)
25-ml (Falcon #357535 or equivalent)
50-ml (Falcon #356550 or equivalent)
Micropipette tips
20-µl barrier tips (Denville Scientific #P1121 or equivalent)
200-µl barrier tips (Denville Scientific #P1122 or equivalent)
200-µl tips (USA Scientific #1111-1700 or equivalent)
1000-µl barrier tips (Denville Scientific #P1126 or equivalent)
1.5-ml microcentrifuge tubes (Denville #C2170 or equivalent)
Pipet-Aid
Timer
Refrigerator at 4°C (±1°C)
Ultra-low freezer (−80°C)

Additional reagents and equipment for mammalian cell culture including counting viable cells (see Current Protocols article: Phelan & May, 2015), metal-chelate affinity chromatography (see Current Protocols article: Petty, 1996), protein assays (see Current Protocols article: Lovrien & Matulis, 2005), and SDS-polyacrylamide electrophoresis of proteins (SDS-PAGE; see Current Protocols article: Manns, 2011)

Mammalian cell transfection

1. HEK 293F cells are counted using an automated cell counter (or a regular counting chamber and trypan blue solution; see Current Protocols article: Phelan & May, 2015) and seeded at a density of 600,000 cells/ml in Expi293 Expression Medium.

   The viability of the cells must be greater than 90% at all times.

2. Cells are passaged every 3 to 4 days and incubated in an orbital shaking incubator at 37°C with shaking at 125 rpm and 8% CO_2. See Current Protocols article Phelan and May (2015) for basic cell culture techniques.

   A maximum cell density of 4−5 × 10^6 cells/ml is recommended, at which point cells should be immediately passaged.

3. 600 × 10^6 cells are suspended in 200 ml (3 × 10^6 cell/ml) of Expi293 expression medium in a 1-L Erlenmeyer flask.

   Transfections are performed according to manufacturer's instructions.

4. 12 ml of Opti-MEM is added to two 50-ml sterile polypropylene conical tubes: one tube receives 200 µg (1 µg/µl final dilution in the total volume of culture) of respective plasmid DNA (for RBD or full-length spike), while the other tube receives 640 µl of ExpiFectamine transfection reagent from the kit.

5. The contents of both 50-ml tubes are mixed together and incubated at RT for 10 min to prepare the transfection mixture, after which the transfection mixture is added dropwise to the cells slowly, over a course of a few seconds using a micropipette while swirling the cells in a circular pattern.

6. Cells are then returned to the shaking incubator.

7. At 16 hr post-transfection, 1.2 ml of Expifectamine 293 Transfection Enhancer 1 and 12.0 ml of Expifectamine 293 Transfection Enhancer 2 from the kit are

added to the culture, and subsequently the culture is returned to the shaking incubator.

8. At 3 days post-transfection, the cells are harvested and centrifuged 20 min at 4000 × *g*, 4°C.

9. The supernatant is filtered using a 0.22-μm Stericup filter; the cell pellet can be discarded.

   Alternatively, cells can be spun down at 200 × g for 10 min, supernatant can be collected, and the same cells can be resuspended in 200 ml of fresh Expi293 expression medium and returned to the shaking incubator for another 3 days. This makes it possible to collect more protein in the fresh supernatant (the cells continue to express the protein) and can be used to increase protein yield. The protein integrity needs to be verified in the same way as for the initial protein harvest. This alternate strategy works well with the RBD, but is less suitable for the full-length spike (we have detected protein degradation in that case).

10. Continue to process the supernatant and purify protein immediately.

    Alternatively, if the supernatant is stored, it must be kept at 4°C (and for no longer than overnight/16 hr) in order to prevent denaturation of the protein at room temperature.

Protein purification via gravity flow

These steps can be replaced by more advanced purification methodologies, for example, if an Äkta purifier is available. The methods described below work even in labs not geared toward protein purification. See Current Protocols article Petty (1996) for additional detail on metal-chelate affinity chromatography.

11. Prior to use, Ni-NTA resin (6 ml per 200 ml culture) is washed once with fresh PBS (transfer resin into a 50-ml tube and fill up with PBS), then spun 10 min at 2000 × *g*, 4°C.

12. Once the centrifugation is complete, the PBS is discarded and resin is resuspended with the cell culture supernatant and inverted two or three times.

13. The resin is then incubated with the supernatant for 2 hr on a shaker (65 rpm) at RT.

14. Two clean 5-ml polypropylene columns are loaded with the supernatant-resin mixture and then washed with one column volume of wash buffer four times.

15. Columns are then eluted using the elution buffer.

16. Four fractions are collected from each column by incubating the resin in the column with 3 ml of elution buffer for each fraction. Incubate resin with elution buffer for 5 min after each addition of elution buffer.

17. Eluate is collected directly in a 50-ml polypropylene conical tube placed on ice.

    The total volume of eluate should be 24 ml from the two columns. More columns can be used to speed up the purification time, depending on the volume of the culture.

18. Eluate is spun through 10-kDa Amicon Ultra Centrifugal Filter Units (for RBD) or 50-kDa Amicon Ultra Centrifugal Filter Units (for full-length spike) at 4000 × *g* for 30 min (or longer if eluate takes longer to pass through the membrane) at 4°C or until only 200 to 300 μl remain in the unit.

    Amicon Filter Units should be equilibrated with PBS before use.

19. PBS is added twice to the Amicon Ultra Centrifugal Filter Unit from step 18 and the unit is spun at 4000 × *g* for 30 min at 4°C or until only 200 to 300 μl remain in the unit.

    This step exchanges the buffer to PBS.

20. Finally, the protein is collected from the Amicon Ultra Centrifugal Filter Unit, its concentration is measured (e.g., using the Bradford protein assay or similar methods; see Current Protocols article; Lovrien & Matulis, 2005), and a denaturing SDS-PAGE (4% to 20% gradient; see Current Protocols article: Manns, 2011) is run to check the integrity of the purified protein.

    The size of the expected bands is about 30 kDa for the RBD and about 190 kDa for the full-length spike (N-linked glycans increase the molecular weight of the protein). Monoclonal antibodies like CR3022 can also be used for quality control in an ELISA.

21. After the elution step, protein should always be kept on ice or stored at 4°C.

    For storage longer than 24 hr, protein should be frozen at −80°C to avoid degradation. A concentration of 2 mg/ml and an aliquot size of 50 to 200 μl is recommended.

A TWO-STAGE ELISA FOR HIGH-THROUGHPUT SCREENING OF HUMAN SERUM SAMPLES FOR ANTIBODIES BINDING TO THE SPIKE PROTEIN OF SARS-CoV-2

BASIC PROTOCOL 2

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

We developed this as a two-stage ELISA in which the first stage ('a' steps below) includes relatively high-throughput screening of samples in a single serum dilution against the RBD (which expresses very well and therefore can be produced in greater quantities). This is followed by a second stage ('b' steps below) in which positive samples from the first stage undergo a confirmatory ELISA against the full-length spike protein (which is harder to express; therefore there is usually less available). For the second stage, a dilution curve is performed. Typically, if only one operator is available, screening ELISAs can be run in the morning (760 samples/10 plates per run) and confirmatory ELISAs can be run in the afternoon (140 samples/10 plates per run). Of note, we describe the assay here as it is set up in our laboratory. We use a plate washer and a plate reader, but no automated system. The protocol can be adapted to use with an automated liquid handler. In addition, one of the difficulties in setting up the assay is the availability of appropriate negative and positive controls. Negative controls are easier to come by, and can be serum pools taken before 2020. Positive controls can be convalescent samples from COVID19 patients or monoclonal antibodies (mAbs) like CR3022 (ter Meulen et al., 2006; Tian et al., 2020). If no human sera or mAbs are available, mouse mAbs, mouse sera against SARS-CoV-2, other animal sera against SARS-CoV-2, or anti−His tag antibodies (the proteins are His-tagged) can be used. However, in this case, a different secondary antibody for the species from which the primary antibody is derived is needed for the positive control. Also, we recommend generating large batches of positive controls, which can be used for many runs. The positive control should be selected to result in a strong signal (recommend OD_{490} of about 2.0), and should be clearly distinguishable from the negative controls. ELISAs can be run with either serum or plasma.

CAUTION: Before starting to work with COVID19 samples, please consult with your local biosafety officer regarding which precautions, personal protective equipment and protective measures are required.

NOTE:

Definitions

ELISA = enzyme-linked immunosorbent assay
PBS = phosphate-buffered saline

RT = room temperature (18° to 25°C)
HRP = horseradish peroxidase
HCl = hydrochloric acid
OPD = *O*-phenylenediamine dihydrochloride

NOTE: RBD or full-length spike might be used for both ELISA stages if only one antigen is available. In addition, only the "a" steps (not recommended) or only the "b" steps might be performed, if fewer resources are available.

Materials

Recombinant RBD protein (Basic Protocol 1)
Recombinant full-length spike protein (Basic Protocol 1)
1× PBS [Gibco #10010-023 or equivalent, or prepare from 10× PBS (Corning™ 46013CM or equivalent)], sterile
Serum or plasma samples to be tested
PBS-T (see recipe)
Milk powder (AmericanBio #AB10109-01000 or equivalent)
Anti−human IgG (Fab-specific)−horseradish peroxidase (HRP) labeled secondary antibody produced in goat (Sigma #A0293)
SIGMA*FAST*™ OPD (Sigma-Aldrich #P9187 or equivalent)
Water for injection (WFI) for cell culture (Gibco #A1287301 or equivalent)
3 M hydrochloric acid (Fisher Scientific #S25856 or equivalent)

ELISA plates: flat-bottom Immuno nonsterile 96-well plates 4 HBX (Thermo Scientific #3855 or equivalent)
Multichannel pipettor(s) capable of pipetting 50 to 250 µl
Sterile reservoirs (Fisher Scientific #07-200-127 or equivalent)
Water bath with temperature control
Aquamax 2000 Plate Washer (Molecular Devices #AQUAMAX 2000 or equivalent)
Timer
Class II biological safety cabinet
1.5-ml microcentrifuge tubes (Denville #C2170 or equivalent)
Flat-bottom cell culture plates (Corning #3599 or equivalent)
Kimberly-Clark Kimwipes (Kimberly-Clark Professional #34721 or equivalent)
Biotek SynergyH1 microplate reader or equivalent
Polypropylene sterile conical tubes:
  15-ml (Denville Scientific #C1018P or equivalent)
  50-ml (Fisher Denville Scientific #C1060P or equivalent)
Sterile, serological pipettes:
  5-ml (Falcon #356543 or equivalent)
  10-ml (Falcon #357551 or equivalent)
  25-ml (Falcon #357535 or equivalent)
  50-ml (Falcon #356550 or equivalent)
Micropipette tips:
  20-µl barrier tips (Denville Scientific #P1121 or equivalent)
  200-µl barrier tips (Denville Scientific #P1122 or equivalent)
  200-µl tips (USA Scientific #1111-1700 or equivalent)
  1000-µl barrier tips (Denville Scientific #P1126 or equivalent)
Pipet-Aid
Micropipettors
Ultra-low freezer (−80°C)
Refrigerator at 4°C (+/−1°C)

RBD screening ELISA

1a. Coating ELISA plates (day 1).

i. 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 vials before diluting in 1× PBS.

Prepare approximately 5 ml for each plate to be coated.

ii. Coat plates with 50 µl of diluted protein per well using a multichannel pipettor and a reservoir. Lightly tap plates against surface to ensure protein is evenly coating the bottom of every well.

iii. Incubate at 4°C overnight.

Always keep a plate cover on top of coated plates during all steps of the protocol! Plates can likely be stored at 4°C for up to 1 week, but this needs to be validated locally to ascertain that it does not change the results.

2a. Heat inactivation of samples (day 1, this is a general safety precaution for work with human serum).

CAUTION: *We have not tested if this procedure inactivates SARS-CoV-2; please consult with your local biosafety officer to discuss proper safety precautions.*

i. Set the water bath to 56°C. Once temperature is reached, place the serum/plasma samples in the water bath and immediately start the timer for 1 hr.

ii. Remove samples when the timer goes off. Do not leave samples at 56°C for longer than 1 hr.

Store at 4°C overnight or until use.

3a. Blocking ELISA plates (day 2).

i. Calculate to prepare at least 30 ml of blocking solution per plate.

Blocking solution consists of PBS-T + 3% (w/v) milk powder.

ii. Using an automated plate washer, wash coated ELISA plates three times with PBS-T.

This step (and wherever a plate washer is mentioned below) can also be performed by washing plates with a multichannel pipettor manually if no plate washer is available.

iii. Add 200 µl blocking solution to all wells of the plates, starting the timer for 1 hr (do not exceed 4 hr) after completing the first plate. Place plates in a 20°C (RT) incubator until step 6a.

4a. Pre-diluting samples (day 2).

i. In a biological safety cabinet, set up sterile 1.5-ml microcentrifuge tubes to pre-dilute serum samples at a 1:5 ratio.

ii. Add 40 µl of sterile 1× PBS to all tubes.

iii. Gently vortex each serum sample to mix and add 10 µl to the microcentrifuge tubes, vortexing once more. Do this for all remaining samples including the positive and negative controls.

The volume of serum not needed in these 'a' steps will be stored and used for the 'b' steps, below.

5a. Setting up dilution plates (day 2).

i. Calculate and prepare at least 30 ml of PBS-T + 1% (w/v) milk powder.

ii. Prepare one dilution plate (separate flat-bottomed cell culture plate) per antigen-coated plate prepared.

iii. Add 180 µl of PBS-T containing 1% milk to all wells of the dilution plate (including blank wells).

iv. Leaving columns 1 and 12 as blanks, add 20 µl of pre-diluted sample (or control) to the designated wells.

This results in a final serum dilution of 1:50.

	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
E	Blank											Blank
F	Blank											Blank
G	Blank											Blank
H	Blank		...	...	...	Sample 73	Sample 74	Sample 75	Sample 76	(+) Ctrl.	(-) Ctrl.	Blank

Figure 3 RBD screening ELISA reference plate layout. The layout in which samples should be prepared in a 96-well cell culture plate (dilution plate) is shown. Wells designated for positive (+) and negative (−) controls are indicated.

v. Continue until all samples and controls have been added to the designated wells. See reference plate layout in Figure 3.

6a. Transferring serum dilution (day 2).

i. After the blocking incubation in step 3a, substep iii, remove ELISA plates from the RT incubator and throw off the blocking solution. Tap the plates dry on a Kimwipe or other absorbent material.

ii. Using a multichannel pipettor, pipette up and down four to six times in the wells of the first row of the dilution plate to mix.

iii. Transfer 100 µl from each well of the first row of the dilution plate to the corresponding wells in the ELISA plate. Change tips and continue to transfer the second row of the dilution plate to the ELISA plate in the same manner.

iv. Start the timer for 2 hr as soon as the contents of all of the rows have been transferred to the first ELISA plate.

v. Place plates in a 20°C (RT) incubator.

Do not exceed 4 hr of incubation at RT before proceeding to step 7a.

7a. Incubating with secondary antibody (day 2).

i. After 2 hr incubation at RT, wash the ELISA plates from step 6a three times with PBS-T using an automated plate washer.

ii. 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.

iii. Add 50 µl of the secondary antibody solution to all wells of the plate using a multichannel pipettor.

Be sure to avoid touching the walls of the wells with the pipette tips, to avoid carry-over and high background signals.

iv. Start the timer for 1 hr (stay in a range of 50 to 65 min) as soon as the secondary antibody has been added to the first plate.
v. Place plates in a 20°C (RT) incubator.

8a. Developing and reading plates (day 2).
i. After 1 hr, wash plates three times with PBS-T using an automated plate washer.
ii. Prepare SigmaFast OPD solution and calculate amount needed.

One set of tablets (1 gold + 1 silver tablet) dissolved in 20 ml water for injection (WFI) can be used for two 96-well plates.

iii. Fully dissolve one gold tablet in 20 ml WFI. Do not add silver tablet to solution until ready to start adding to the plates. The OPD solution needs to be prepared immediately before use.
iv. Add 100 µl OPD solution to all wells of the plate. Begin the timer for 10 min as soon as OPD has been added to the first row of the first plate.

Do not exceed 10 min of developing before stopping the reaction.

v. To stop the reaction after exactly 10 min, add 50 µl of 3 M HCl to all wells.

Ideally, read plates immediately after adding HCl.

vi. Read ELISA plates in a plate reader at an absorbance of 490 nm (immediately after adding HCl) and record data.

Samples that exceed a certain OD_{490} cutoff value (proposed cutoff: $OD_{490} = 0.15$ to 0.2, or mean of negative controls plus 3 times the standard deviation of the negative controls) are assigned as presumptive positives and will be tested in the confirmatory ELISA using full-length spike protein ('b' steps, below).

The OD_{490} cutoff has to be experimentally determined and depends on assay background and noise.

Spike confirmatory ELISA

1b. Coating ELISA plates (day 1).
i. 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 µg/ml. Once thawed, mix by gently vortexing vials before diluting in 1× PBS.

Prepare approximately 5 ml for each plate to be coated.

ii. Coat plates with 50 µl of diluted protein per well using a multichannel pipettor and a reservoir. Lightly tap plates against surface to ensure protein is evenly coating the bottom of every well.
iii. Incubate at 4°C overnight.

Always keep a plate cover on top of coated plates during all steps of the protocol! Plates can likely be stored in 4°C for up to 1 week but this needs to be validated locally to ascertain that it does not change the results.

2b. Blocking ELISA plates (day 2).
i. Calculate to prepare at least 30 ml of blocking solution per plate.

Blocking solution consists of PBS-T + 3% (w/v) milk powder.

ii. Using an automated plate washer, wash coated ELISA plates three times with PBS-T.
iii. Add 200 µl blocking solution to all wells of the plates, starting the timer for 1 hr (do not exceed 4 hr) after completing the first plate.
iv. Place plates in a 20°C (RT) incubator until step 4b.

3b. Pre-diluting samples (day 2).
Retrieve 1:5 pre-diluted samples from step 4a, above, to be tested and confirmed (samples that are above certain threshold in RBD screening ELISA based on a set OD_{490} value; see annotations after step 8a, substep vi).

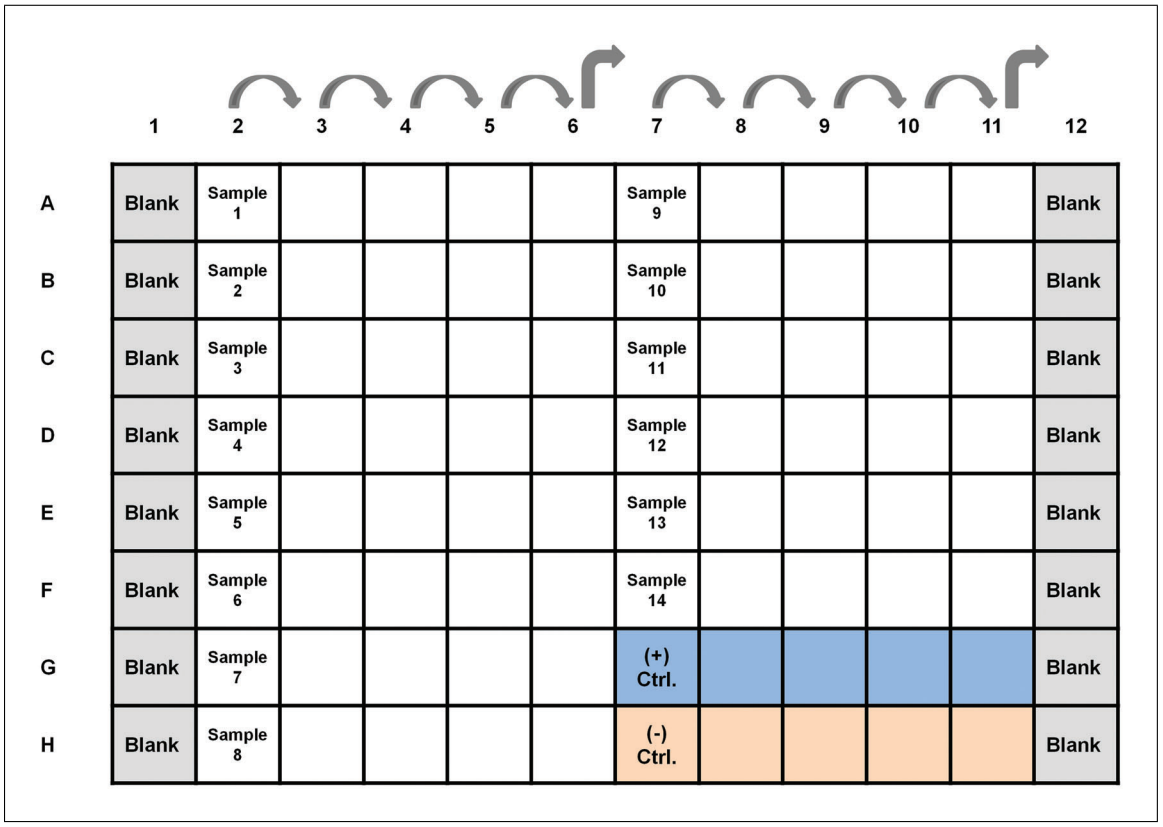

Figure 4 Confirmatory spike ELISA reference plate layout. The sample layout on the ELISA plate is shown, including the serial dilution steps that need to be performed. Wells designated for positive (+) and negative (−) controls are indicated.

4b. Performing serial dilutions (day 2).

i. Calculate and prepare at least 20 ml of PBS-T + 1% (w/v) milk powder per plate.

ii. After the blocking incubation in step 2b, substep iii, remove plates from the RT incubator and throw off the blocking solution. Tap the plates dry on a Kimwipe or other absorbent material.

iii. Using a multichannel pipettor, add 120 µl of PBS-T containing 1% milk to all wells of each plate.

iv. Leaving columns 1 and 12 as blanks, add an extra 51 µl of PBS-T containing 1% milk to wells only in columns 2 and 7.

Wells of column 2 and 7 will be the sample wells.

v. 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.

vi. In column 7, add samples to wells A through F.

vii. Transfer positive and negative controls into wells G and H respectively. See reference plate layout in Figure 4.

viii. With the multichannel pipettor, pipette up and down four to six 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 four to six times to mix. Repeat this until column 6; discard 60 µl before column 7.

ix. Taking fresh tips, mix column 7 by pipetting up and down four to six times. Repeat the same process of transferring, mixing, and discarding tips from columns 7 to 11. Once column 11 is reached, discard 60 µl.

x. Start the timer for 2 hr once the first ELISA plate has been serially diluted.

Do not exceed 4 hr of incubation at RT before proceeding to step 5b.

xi. Place plates in a 20°C (RT) incubator.

5b. Incubating with secondary antibody (day 2).

i. After 2 hr of incubation at RT, wash the plates from step 4b with PBS-T using the automated plate washer.

ii. 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.

iii. Add 50 µl of the secondary antibody solution to all wells of the plate using a multichannel pipettor.

Be sure to avoid touching the tips of the pipette to the walls of the well.

iv. Start the timer for 1 hr (stay in a range of 50 to 65 min) as soon as the secondary antibody has been added to the first plate.

v. Place plates in a 20°C (RT) incubator.

6b. Developing and reading plates (day 2).

i. After 1 hr, wash plates three times with PBS-T using an automated plate washer.

ii. Prepare SigmaFast OPD solution and calculate amount needed.

One set of tablets (1 gold + 1 silver tablet) dissolved in 20 ml water for injection (WFI) can be used for two plates.

iii. Fully dissolve one gold tablet in 20 ml WFI. Do not add silver tablet to solution until ready to start adding to the plates. The OPD solution needs to be prepared immediately before use.

iv. Add 100 µl to all wells of the plate. Begin timer for 10 min as soon as OPD has been added to the first row of the first plate.

Do not exceed 10 min of developing before stopping the reaction.

v. To stop the reaction after exactly 10 min, add 50 µl of 3 M HCl to all wells.

vi. Read ELISA plates in plate reader at an absorbance of 490 nm 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.

REAGENTS AND SOLUTIONS

Elution buffer (4 L)

31.74 g $NaH_2PO_4.H_2O$
70.16 g NaCl
64.0 g imidazole (Sigma-Aldrich # I5513 or equivalent; final concentration is 235 mM)
4 L distilled water
Store at room temperature up to 4 months

Use distilled water filtered using a 0.22-µm Stericup vacuum filtration system.

Phosphate-buffered saline with 0.1% Tween 20 (PBS-T; 50 L)

45 L distilled water
5 L 10× PBS (Corning™ #46013CM or equivalent))
50 ml Tween 20 (Fisher Bioreagents #BP337-500 or equivalent)
Store at room temperature for to 4 months

Wash buffer (4 L)

31.74 g $NaH_2PO_4.H_2O$
70.16 g NaCl
5.44 g imidazole (Sigma-Aldrich # I5513 or equivalent; final concentration is 20 mM)

4 L distilled water
Store at room temperature up to 4 months

Use distilled water filtered using a 0.22-µm Stericup vacuum filtration system.

COMMENTARY

Background Information

The protein expression and purification methods (Basic Protocol 1) described in this article are based on well-established techniques. The expression plasmids and protein sequences have been optimized to increase protein stability and yield (Amanat et al., 2020). Plasmids can be requested from the Krammer laboratory or can be found on BEI Resources. The ELISA protocol (Basic Protocol 2) has been designed to allow for high-throughput screening of many samples per day, followed by a confirmatory step to verify presumptive positive results. The ELISA assay itself is based on well-established protocols and has been optimized for the use of SARS-CoV-2 antigens.

Critical Parameters and Troubleshooting

The most common problem for the transfection (Basic Protocol 1) is low cell viability before performing the transfection. The cells need to be 90% to 95% viable. The absence of antibiotics/antifungals requires good sterile technique to prevent contamination. Sterile plasmid preparations are also recommended, and it is important to add the enhancer to the shaking flasks 16 hr post-transfection.

For the protein purification, we recommend always using fresh Ni-NTA resin to prevent cross-contamination with other proteins. Harvested supernatant should be ideally processed immediately to ensure protein integrity. To make filtering of the supernatant easier, an additional centrifugation step (after pelleting the cells) is recommended to pellet residual cells and other particles. When performing buffer exchange using Amicon Ultra Centrifugal Filter Units, make sure to use the right-size cut-off (use smaller cut-off for RBD). It is recommended that purified protein be diluted to a concentration of about 2 mg/ml. Storage at higher concentrations may result in aggregation of protein.

For the ELISA (Basic Protocol 2), performing all of the washing steps and adhering to the incubation times are important to achieve low background reactivity. Most critical are the incubation times for the secondary antibody and the substrate (OPD and HCl for stopping the reaction). In addition, touching wells with tips when transferring secondary antibody and substrate can result in higher background and possibly false positive wells, and needs to be avoided. In preparing the OPD, it is also important to dissolve the gold tablet fully and only add the silver tablet right before the substrate is added to the ELISA plate.

Understanding Results

We expect expression levels of the RBD to be above 20 mg per L of culture cells and expression of the full-length spike protein to be approximately 4 mg per L of 293Fs, using a gravity-flow protein-purification strategy. When running the SDS-PAGE to confirm protein integrity, clear single bands are expected for the RBD and full-length spike at around 25 to 30 kDa and ∼190 kDa, respectively. Additionally, ELISAs with positive and negative controls (e.g., monoclonal antibodies) are performed to confirm correct protein folding. We expect a good binding profile for the positive control and low-to-no background reactivity for the negative control.

Time Considerations

Basic Protocols 1 and 2 can be completed in about 6 days. Basic Protocol 1 takes about 4 days. Growing up a cryostock of 293F cells, bringing them to passage 4 (recommended before transfection), and obtaining a sufficient cell number would take another few days; this is not taken into account in the protocol. Basic Protocol 2 takes at least 2 days (antigen coating on day 1 and running the ELISA on day 2). The screening ELISA could be performed in the morning and the confirmatory ELISA in the afternoon, or the assays can be done on consecutive days.

Acknowledgements

We thank Dr. Raffael Nachbagauer (Icahn School for Medicine at Mount Sinai) and Dr. Aubree Gordon (University of Michigan) for critical reading and constructive comments. Development of this protocol was partially supported by the NIAID Centers of Excellence for Influenza Research and Surveillance (CEIRS) contract HHSN272201400008C.

Philanthropic donations in support of our work are much appreciated, since the reagents

are shared free of charge with the scientific community. Please contact Vanesa Saric (*vanesa.saric@mountsinai.org*) for further information.

Literature Cited

Amanat, F., Nguyen, T., Chromikova, V., Strohmeier, S., Stadlbauer, D., Javier, A., … Krammer, F. (2020). A serological assay to detect SARS-CoV-2 seroconversion in humans. *medRxiv*, 2020.2003.2017.20037713. doi: 10.1101/2020.03.17.20037713.

Bao, L., Deng, W., Gao, H., Xiao, C., Liu, J., Xue, J., … Qin, C. (2020). Reinfection could not occur in SARS-CoV-2 infected rhesus macaques. *bioRxiv*, 2020.2003.2013.990226. doi:10.1101/2020.03.13.990226.

Callow, K. A., Parry, H. F., Sergeant, M., & Tyrrell, D. A. (1990). The time course of the immune response to experimental coronavirus infection of man. *Epidemiology and Infection*, *105*(2), 435−446. doi: 10.1017/s0950268800048019.

Chu, D. K. W., Pan, Y., Cheng, S. M. S., Hui, K. P. Y., Krishnan, P., Liu, Y., … Poon, L. L. M. (2020). Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clinical Chemistry*, *66*(4), 549−555. doi: 10.1093/clinchem/hvaa029.

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K. W., … Drosten, C. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance*, *25*(3). doi: 10.2807/1560-7917.ES.2020.25.3.2000045.

Lovrien, R., & Matulis, M. (2005). Assays for total protein. *Current Protocols in Microbiology*, *00*, A.3A.1–A.3A.14. doi: 10.1002/9780471729259.mca03as00.

Manns, J. M. (2011). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins. *Current Protocols in Microbiology*, *22*, A.3M.1–A.3M.13. doi: 10.1002/9780471729259.mca03ms22.

Phelan, K., & May, K. M. (2015). Basic techniques in mammalian cell culture. *Current Protocols in Cell Biology*, *66*, 1.1.1–1.1.22. doi: 10.1002/0471143030.cb0101s66.

ter Meulen, J., van den Brink, E. N., Poon, L. L., Marissen, W. E., Leung, C. S., Cox, F., … Goudsmit, J. (2006). Human monoclonal antibody combination against SARS coronavirus: synergy and coverage of escape mutants. *PLoS Medicine*, *3*(7), e237. doi: 10.1371/journal.pmed.0030237.

Tian, X., Li, C., Huang, A., Xia, S., Lu, S., Shi, Z., … Ying, T. (2020). Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. *Emerging Microbes & Infections*, *9*(1), 382–385. doi: 10.1080/22221751.2020.1729069.

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., … McLellan, J. S. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science*, *367*(6483), 1260–1263. doi: 10.1126/science.abb2507.

Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., … Zhang, Y. Z. (2020). A new coronavirus associated with human respiratory disease in China. *Nature*. *579*(7798), 265−269. doi: 10.1038/s41586-020-2008-3.

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., … Team, C. N. C. I. a. R. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *New England Journal of Medicine*, *382*(8), 727–733. doi: 10.1056/NEJMoa2001017.