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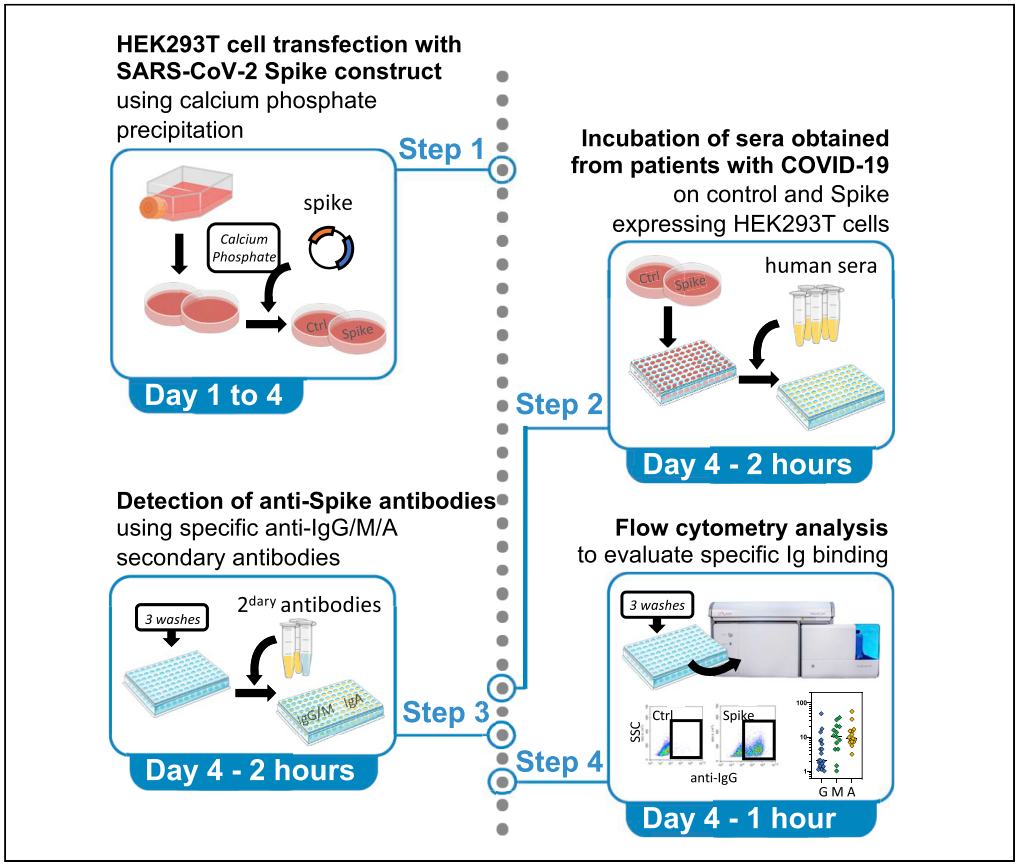
A cell-based system combined with flow cytometry to evaluate antibody responses against SARS-CoV-2 transmembrane proteins in patients with COVID-19



This protocol describes a flow cytometry approach to evaluate antibody responses against SARS-CoV-2 transmembrane proteins in COVID-19-positive patient sera samples without the need of specific laboratory facilities for viral infection. We developed a human-cell-based system using spike-expressing HEK293T cells that mimics membrane insertion and N-glycosylation of viral integral membrane proteins in host cells. This assay represents a powerful tool to test antibody responses against SARS-CoV-2 variants and vaccine effectiveness.

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Highlights

A human-cell-based assay was developed to access antibody responses against SARS-CoV-2

The assay recapitulates spike membrane insertion and posttranslational modifications

The assay represents a powerful tool to test spike variants and vaccine effectiveness

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A cell-based system combined with flow cytometry to evaluate antibody responses against SARS-CoV-2 transmembrane proteins in patients with COVID-19

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SUMMARY

This protocol describes a flow cytometry approach to evaluate antibody responses against SARS-CoV-2 transmembrane proteins in COVID-19-positive patient sera samples without the need of specific laboratory facilities for viral infection. We developed a human-cell-based system using spike-expressing HEK293T cells that mimics membrane insertion and N-glycosylation of viral integral membrane proteins in host cells. This assay represents a powerful tool to test antibody responses against SARS-CoV-2 variants and vaccine effectiveness.

For complete details on the use and execution of this protocol, please refer to Martin et al. (2021).

BEFORE YOU BEGIN

The following part describes specific steps to obtain HEK293T cells (derived from human embryonic kidney cells expressing the large T antigen from SV40) expressing SARS-CoV-2 Spike protein. This cell-based system has also been applied to M (membrane) and E (envelope) SARS-CoV-2 proteins as well as to Spike D614 and G614 variants. This protocol could be adapted to any other cell types, such as epithelial Hela cells, although transfection method used for those cells should be adapted.

Preparation of the SARS-CoV-2 spike construct

Timing: 3 days

The SARS-CoV-2 Spike construct obtained from the Krogan's laboratory (see key resources table) was amplified using stable competent *E. coli* bacteria (NEB) and purified using NucleoBond Xtra Maxi kit from Macherey-Nagel as described below.

1. Transform high efficiency stable competent (NEB) *E. coli* bacteria with the SARS-CoV-2 Spike construct
   a. Mix gently 50 µL of bacteria suspension with 1 µg of plasmid in a 1.5 mL Eppendorf tube on ice;
   b. Incubate 30 min on ice
   c. Incubate bacteria 30 s at 42°C

d. Put back directly bacteria on ice;
e. Incubate 5 min on ice;
f. Add 650 µL of pre-warmed SOC medium on transformed bacteria;
g. Incubate one hour at 37°C under agitation;
h. Centrifuge the bacteria suspension at 1,200 g for 30 s;
i. Remove 600 µL of supernatant
j. Resuspend the bacteria pellet in the remaining 100 µL supernatant
k. Plate 100 µL of the bacteria suspension on agar plates containing ampicillin at 100 µg/mL
l. Incubate 16 h at 37°C in an incubator

2. Grow transformed bacteria
   a. Collect one colony in 5 mL LB medium containing ampicillin at 100 mg/mL (LBA)
   b. Incubate 4 h at 37°C under agitation at 250 rpm
   c. Transfer 5 mL bacteria in 250 mL LBA
   d. Incubate 16 h at 37°C under agitation at 250 rpm
3. Purify the SARS-CoV-2 Spike plasmid using NucleoBond Xtra Maxi kit from Macherey-Nagel
   a. Collect 250 mL of bacterial culture in a 250 mL tube
   b. Pellet the bacteria at 4,500 g for 30 min at 4°C
   c. Discard the supernatant
   d. Resuspend the bacteria in 12 mL of resuspension buffer (RES)
   e. Add 12 mL of lysis buffer (LYS)
   f. Mix gently by inverting the 250 mL tube (do not vortex)
   g. Incubate 5 min at 20°C
   h. Prepare the NucleoBond column by adding 25 mL of equilibration buffer (EQU) to the column filter as described in the manufacturer's protocol (https://www.mn-net.com/media/pdf/ed/82/0f/Instruction-NucleoBond-Xtra.pdf)
   i. Add 12 mL of neutralization buffer (NEU) to the bacteria suspension and immediately mix the lysates gently by inverting the 250 mL tube (do not vortex) until the blue suspension turns colorless completely
   j. Invert the tube 3 times before applying the lysate to the equilibrated NucleoBond column as described in the manufacturer's protocol (https://www.mn-net.com/media/pdf/ed/82/0f/Instruction-NucleoBond-Xtra.pdf)
   k. Allow the column to empty by gravity flow
   l. Wash the NucleoBond filter and column with 15 mL of EQU buffer
   m. Allow the column to empty by gravity flow
   n. Discard the NucleoBond filter
   o. Wash the NucleoBond column with 25 mL of wash buffer (WASH)
   p. Allow the column to empty by gravity flow
   q. Place a 50 mL Falcon tube to collect the plasmid DNA elution under the column
   r. Elute the plasmid DNA by applying 15 mL of pre-warmed (at 50°C) elution buffer (ELU) on the column
   s. Add 10.5 mL of isopropanol (pre-warmed at 20°C) to precipitate the eluted plasmid DNA
   t. Centrifuge at 4,500 g for 45 min at 4°C
   u. Carefully discard the supernatant
   v. Add 1 mL of ethanol 70% to the DNA pellet and transfer it to a 1.5 mL Eppendorf tube
   w. Centrifuge at 15 000 g for 5 min at 20°C
   x. Remove the supernatant
   y. Add 1 mL of ethanol 70% to the DNA pellet
   z. Centrifuge at 15 000 g for 5 min at 20°C
   aa. Remove completely the ethanol with a pipette tip
   bb. Dry the DNA pellet at 20°C
   cc. Add 250 µL of Tris EDTA (TE) buffer
   dd. Resuspend carefully the DNA and store at −20°C

ee. Determine the plasmid DNA concentration and quality using an UV-visible spectrophotometer (i.e., a DeNovix DS-11 nanodrop spectrophotometer). The plasmid concentration was between 2–4 μg/μL (between 1 to 2 mg in total) with an A260/A280 ratio between 1.8 to 2.

⚠ CRITICAL: Before bacteria transformation, (1) prepare ampicillin-containing LB agar plates and media, (2) set the water bath at 42°C, and (3) pre-warm the SOC medium at 20°C.

⚠ CRITICAL: Before the SARS-CoV-2 Spike plasmid purification, pre-warm the elution buffer (ELU) at 50°C.

Note: For step 3a, if needed, split the 250 mL bacterial culture into five 50 mL tubes for centrifugation; and then pool the bacteria pellets when resuspending in 12 mL of resuspension buffer (RES) (step 3d).

HE293T cell culture and transfection with the SARS-CoV-2 spike plasmid

HEK293T cells used in this protocol were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) and transfected using calcium phosphate precipitation. The composition of the transfection solutions i.e., HBS solution and calcium chloride solutions is described in the following tables. This protocol could be adapted for other cell lines including epithelial or lung cell lines. To estimate the number of cells needed, take into account that each serum would be tested on non-transfected (one well) and SARS-CoV-2 Spike-expressing HEK293T cells (two wells for IgG/M and IgA detection). Generally, between 2 to 3 million of HEK293T cells were obtained from one 10 cm Petri dish that were sufficient to test between 4 to 6 sera.

Preparation of HBS 2× solution

Reagent	Final concentration	Amount
NaCl	274 mM	8 g
$Na_2HPO_4 - 7H_2O$	1.5 mM	0.2 g
Hepes	55 mM	6.6 g
H_2O	n/a	500 mL
Total	**n/a**	**500 mL**

Adjusted at pH 7.05, filtered at 0.22 μm and stored at −20°C (10 mL-aliquots).

Preparation of calcium chloride solution (Timing: 4 days)

Reagent	Final concentration	Amount
$CaCl_2$	2 M	11,1 g
H_2O	n/a	50 mL
Total	**n/a**	**50 mL**

Filtered at 0.22 μm and keep at 20°C.

4. Grow HEK293T cells in DMEM 4.5 g/L glucose medium containing 10% FBS

HEK293T cells were grown in 15 mL of DMEM 4.5 g/L glucose medium containing 10% FBS in 75 cm^2 tissue culture flasks

5. Plate HEK293T cells in 10 cm Petri dish
   a. Wash gently HEK293T cells monolayer amplified in 75 cm^2 tissue culture flasks with 5 mL of PBS
   b. Detach HEK293T cells from the plate using 4 mL of PBS and 1 mL of 0.05% trypsin
   c. Mix gently
   d. Incubate 1 min at 20°C
   e. Flush vigorously with a 5 mL pipette the cell layer to facilitate the cell detachment

f. Collect detached cells in a 50 mL Falcon tubes containing 1 mL FBS
g. Determine the cell number
h. Plate 1 million of HEK293T cells in 10 mL DMEM 10 %FBS medium in 10 cm Petri dish
i. Incubate 16 h at 37°C in an incubator 5% CO2 in a humid atmosphere

6. Prepare the transfection solution
   a. Pre-warm HBS 2× solution at 20°C
   b. Mix 430 μL H2O with 70 μL of $CaCl_2$ solution vigorously with a pipette tip
   c. Add 10 μg of plasmid and immediately mix vigorously with a pipette tip
   d. Add 500 μL of HBS 2× solution and immediately mix vigorously with a pipette tip
   e. Incubate exactly 10 min at 20°C
   f. For the control condition, prepare the transfection solution without plasmid
7. Transfect the HEK293T cells with SARS-CoV-2 Spike plasmid
   a. Replace the culture medium of the HEK293T cells by 9 mL of fresh DMEM 10% FBS medium
   b. Resuspend the transfection reagent
   c. Add drop by drop 1 mL of transfection solution on HEK293T cells seeded in 10 cm Petri dish
   d. For the control condition, add drop by drop transfection solution without plasmid on the control HEK293T plate
   e. Incubate for 48 h at 37°C in an incubator 5% CO2 in a humid atmosphere

⚠ CRITICAL: Before plating HEK293T cells in 10 cm Petri dish, ensure that HEK293T cells were at a confluence of about 70%.

⚠ CRITICAL: Before transfection, pre-warm the HBS 2× solution.

Monitoring of the viral spike protein expression on transfected HEK293T cells using an anti-Spike antibody

Timing: 2 h

8. Collect the non-transfected and SARS-CoV-2 Spike-expressing HEK293T cells
   a. Wash HEK293T cells monolayer with 5 mL of PBS
   b. Detach HEK293T cells from the plate using 4 mL of PBS and 1 mL of 0.05% trypsin
   c. Mix gently
   d. Incubate 1 min at 20°C
   e. Flush vigorously with a 5 mL pipette the cell layer to facilitate the cell detachment
   f. Collect detached cells in a 15 mL tube containing 1 mL FBS
   g. Centrifuge the cell suspension at 550 g for 7 min
   h. Discard the supernatant
   i. Resuspend the cell pellet in 5 mL of staining buffer
   j. Determine the cell number
9. Stain non-transfected and SARS-CoV-2 Spike-expressing HEK293T cells with the anti-Spike antibody
   a. Distribute 2.5 × 10^5 cells per well in a 96 well U-bottom plate
   b. Centrifuge the 96 well U-bottom plate at 550 g for 7 min
   c. Remove the supernatant by flicking the 96 well U-bottom plate
   d. Resuspend the cell pellets with 50 μL of diluted anti-Spike antibody solution (1 in 250)
   e. Incubate 30 min at 4°C
   f. Add 150 μL of staining buffer
   g. Centrifuge the cell suspension at 550 g for 7 min
   h. Remove the supernatant by flicking the 96 well U-bottom plate
   i. Resuspend the cell pellets with 200 μL of staining buffer
   j. Repeat twice step 2.g to step 2.i

10. Stain non-transfected and SARS-CoV-2 Spike-expressing HEK293T cells with the secondary antibody
    a. Resuspend the cell pellets with 100 µL of diluted AF488-coupled anti-rabbit antibody (1 in 500)
    b. Incubate 30 min at 4°C in the dark
    c. Add 150 µL of staining buffer
    d. Centrifuge the cell suspension at 550 g for 7 min
    e. Remove the supernatant by flicking the 96 well U-bottom plate
    f. Resuspend the cell pellets with 200 µL of staining buffer
    g. Repeat twice step 2.d to step 2.f
    h. Resuspend the cell pellets with 200 µL of staining buffer and 5 µL of 7AAD used as a live dead cell dye
    i. Proceed to the flow cytometry analysis
11. Analyze the cell suspension by flow cytometry
    a. Set up the flow cytometer for detection of the single live cell population of interest (7-AAD negative) labeled with an AF488 fluorochrome by preparing the gating approach (i.e., FSC/Per-CP dot plot -> FSC-H/FSC-A dot plot (to exclude the doublets) -> FSC/SSC dot plot (to check the cell population) -> AF488/SSC dot plot or AF488 histogram) described as indicated in the 'materials and equipment' part (Figure 1).
    b. Acquire 10 000 events in the gate of interest
    c. Use the non-transfected HEK293T cells as a negative control
    d. Determine the expression level of SARS-CoV-2 Spike protein by calculating the ratio of the geo-mean of fluorescence intensity (gMFI of AF488) as followed: (gMFI observed with SARS-CoV-2 Spike-expressing HEK293T cells) / gMFI observed with non-transfected HEK293T cells)

⚠ CRITICAL: Before monitoring the HEK293T cells, (1) prepare the staining buffer (see the materials and equipment part) and (2) set up the flow cytometer (see step 4 and the materials and equipment part).

Note: To strengthen the conclusion of the staining, dead cells could be excluded from the analysis using 7AAD (see protocol step 9.g); and more events could be acquired. If needed, Spike expression could be also monitored using intracellular staining of SARS-CoV-2 Spike-expressing HEK293T cells with an anti-Tag antibody. Indeed, as SARS-CoV-2 Spike construct used in this protocol is tagged with two Strep-Tag II motifs, fluorescent-conjugated streptavidin or strepTactin could be used for detection of Spike. More details in Martin et al. (2021).

Sera collection and storage

Timing: 90 min

12. Collect blood from healthy volunteers or patients with COVID-19 in dry tubes
    a. Collect blood in a 6 mL BD Vacutainer (CAT) tube (Ref: 368815)
    b. Fill tube completely
    c. Thoroughly mix the blood by inverting the tube five times
    d. Allow blood to clot for 60 min at 20°C (tube standing upright)
13. Prepare the sera for freezing
    a. Centrifuge the dry tube containing the clotted blood at 2,000 g for 11 min at 20°C
    b. Transfer 500 µL of serum per cryotube in 2D barcoded matrix tubes (Thermo Fisher Scientific, Ref: 3743-WP1D)
14. Preserve the sera at −80°C

⚠ CRITICAL: To avoid useless freezing and thawing cycles, sera were re-aliquoted in small volumes (i.e., 100 µL)

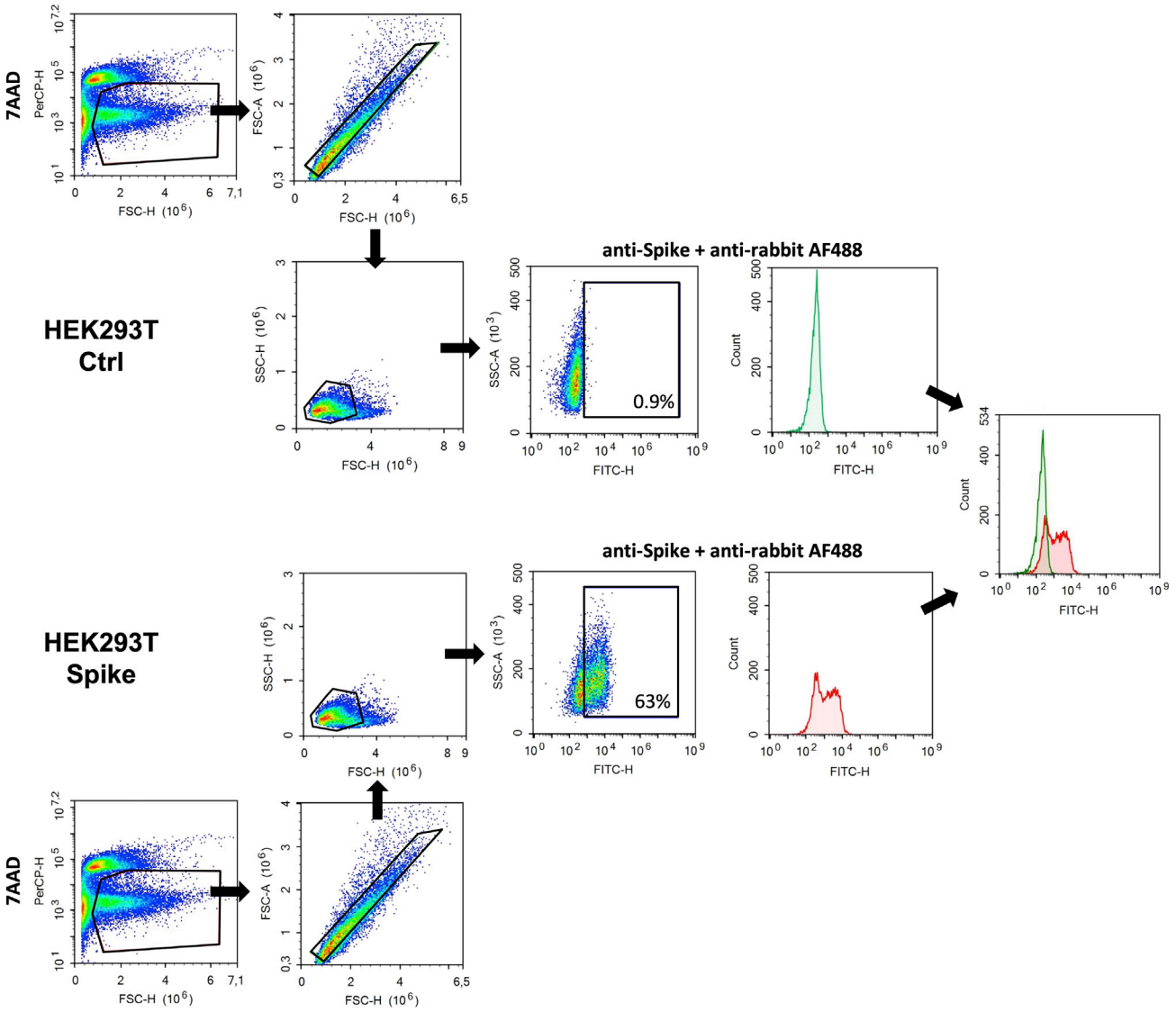

Figure 1. Setting of the flow cytometer for monitoring of the viral Spike protein expression on transfected HEK293T cells

The viable cell population of interest (negative for 7AAD staining) was first gated using a FSC/PerCP dot plot followed by a FSC-H/FSC-A dot plot (to exclude doublets from the analysis) followed by a FSC/SSC dot plot. Spike expression was then detected using anti-Spike antibody with a AF488/SSC dot-plot. The percentage of positive cells was calculated from a final gate adjusted with non-transfected HEK293T cells used as negative controls. Spike expression could also be represented using a AF488 histogram (non-transfected HEK293T cells: green histograms; Spike-expressing HEK293T cells: red histograms). Spike expression level is given a ratio of (geometric mean (gMFI) AF488 observed with Spike-expressing HEK293T cells) / (gMFI AF488 observed with non-transfected HEK293T cells).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Donkey polyclonal anti-rabbit IgG (H+L) Alexa Fluor 488 (Dilution: 1 in 500)	Jackson ImmunoResearch, Ely, UK	711-545-152 (RRID: AB_2313584)
Donkey polyclonal F(ab')2 anti-human IgG (H+L) Alexa Fluor 488 (Dilution: 1 in 500)	Jackson ImmunoResearch, Ely, UK	709-546-149 (RRID: AB_2340569)

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Donkey polyclonal F(ab')2 anti-human IgM Fc5µ Alexa Fluor 647 (Dilution: 1 in 1000)	Jackson ImmunoResearch, Ely, UK	709-606-073 (RRID: AB_2340579)
Goat polyclonal F(ab')2 anti-human IgA FITC (Dilution: 1 in 500)	Thermo Fisher Scientific, Illkirch, France	A24459 (RRID: AB_2535928)
Rabbit monoclonal anti-SARS-CoV-2 Spike S1 (Dilution: 1 in 250)	Sino Biologicals, Wayne, PA, USA	40150-R007 (RRID: AB_2827979)
Bacterial and virus strains		
High efficiency stable competent *E. coli* bacteria	New England Biolabs	C3040
Biological samples		
Plasmid pTwist EF1alpha nCoV-2019 S (D614 variant) 2×Strep	Krogan laboratory UCSF San Francisco, CA, USA	Gordon et al., 2020 PMID: 32353859
Serum samples – SEROCoV collection	Rennes Biobank, Rennes BRIF: BB-0033-00056	DC-2019-3585
Donkey serum	Sigma-Aldrich St Quentin-Fallavier, France	S30-100ML
Chemicals, peptides, and recombinant proteins		
7AAD	BD Biosciences, Le pont de Claix, France	559925 (RRID: AB_2869266)
Critical commercial assays		
NucleoBond Xtra Maxi kit	MACHEREY-NAGEL, Hoerdt, France	740416.50
Experimental models: Cell lines		
HEK293T cell line	ATCC	CRL-3216
Software and algorithms		
Novocyte Express	ACEA (Agilent), Les Ulis, France	n/a
Prism 7.0 software	GraphPad Software	www.graphpad.com/scientific-software/prism/
Other		
Flow cytometer Novocyte 3000	ACEA (Agilent), Les Ulis, France	n/a

MATERIALS AND EQUIPMENT

This protocol requires a specific staining buffer that contained bovine and donkey sera to limit the unspecific binding of secondary antibodies generated in donkey. This staining buffer was therefore used to wash cells and to dilute sera and secondary antibodies. Staining buffer recipe and antibody dilutions are provided in the following tables:

Recipe for the staining buffer		
Reagent	Final concentration	Amount
Fetal bovine serum (FBS)	2%	1 mL
Donkey serum (DS)	5%	10 mL
PBS 1×	n/a	39 mL
Total	**n/a**	**50 mL**
Stored at 4°C for one month.		

Preparation of secondary antibodies anti-human IgG and IgM		
Reagent	Final concentration	Amount
Antibodies anti-human IgG AF488 + anti-human IgM AF647	3 µg/mL 1.5 µg/mL	(for 45 tests) 10 µL 5 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Staining buffer	n/a	5 mL
Total	**n/a**	**5 mL**

To be prepared the day of the experiment and kept on ice and in the dark.

Preparation of secondary antibodies anti-human IgA		
Reagent	Final concentration	Amount
Antibodies anti-human IgA FITC	3 μg/mL	(for 45 tests) 10 μL
Staining buffer	n/a	5 mL
Total	**n/a**	**5 mL**

To be prepared the day of the experiment and kept on ice and in the dark.

The flow cytometer Novocyte 3000 (ACEA) used for this protocol is equipped of an automated sampling using 96-well plates and 3 lasers including blue and red lasers that allowed the detection of fluorochromes including fluorescein isothiocyanate (FITC), Alexa Fluor 488 (AF488) and 647 (AF647), brilliant violet 650 (BV650) and for 7-amino-actinomycin (7AAD). The setting of the flow cytometer is detailed in Figures 1, 2, and 3.

Alternatives: Any other flow cytometers and fluorochromes could be used as long as suitable laser channels and detectors were appropriate for the corresponding fluorochromes.

STEP-BY-STEP METHOD DETAILS

Preparation of non-transfected and SARS-CoV-2 spike expressing HEK293T cells

Timing: 30 min

This step is dedicated to cell preparation. HEK293T cells were previously transfected with SARS-CoV-2 Spike construct (described in the 'before you begin' part). To estimate the number of cells needed, take into account that each serum would be tested on non-transfected (one well) and SARS-CoV-2 Spike-expressing HEK293T cells (two wells for IgG/M and IgA detection). Generally, between 2 to 3 million of HEK293T cells obtained from one 10 cm Petri dish were sufficient to test between 4 to 6 sera. Do not forget to acquire non-transfected and Spike-expressing HEK293T cells with secondary antibodies alone. The staining buffer limits the non-specific binding of antibodies.

1. Collect the non-transfected and SARS-CoV-2 Spike-expressing HEK293T cells
   a. Wash HEK293T cells monolayer with 5 mL of PBS
   b. Detach HEK293T cells from the plates using 4 mL of PBS and 1 mL of 0.05% trypsin
   c. Mix gently
   d. Incubate 1 min at 20°C
   e. Flush vigorously with a 5 mL pipette the cell layer to facilitate the cell detachment
   f. Collect detached cells in a 15 mL tube containing 1 mL FBS
   g. Centrifuge the cell suspension for 7 min at 550 g
   h. Discard the supernatant
   i. Resuspend the cell pellet in 5 mL of staining buffer
2. Determine the cell number
3. Distribute cells in a 96 well U-bottom plate taking into account that each serum would be tested on non-transfected and SARS-CoV-2 Spike-expressing HEK293T cells
   a. Distribute 2.5×10^5 cells per well

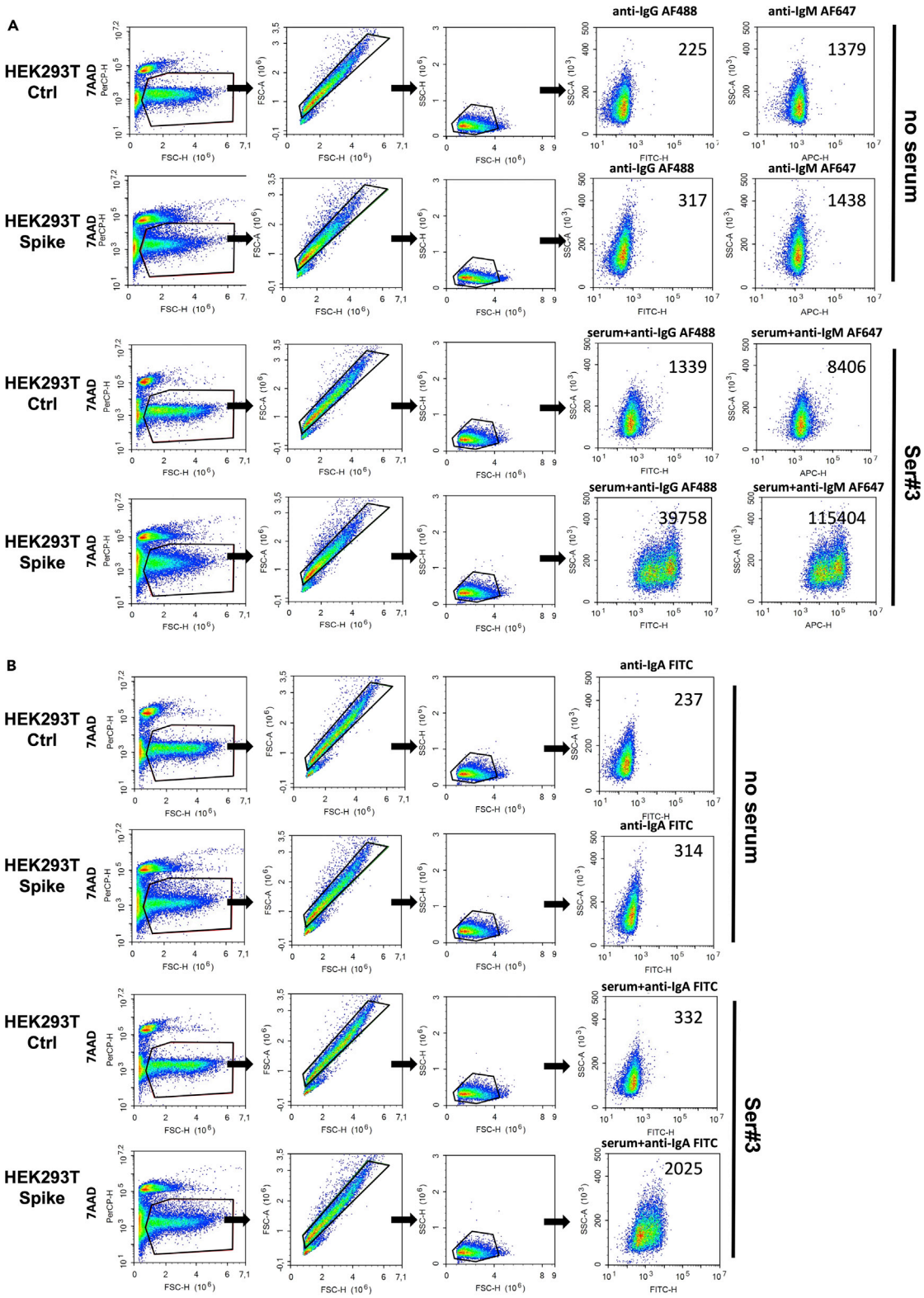
A
HEK293T Ctrl
HEK293T Spike
7AAD PerCP-H
FSC-H (10^6)
FSC-A (10^6)
SSC-H (10^6)
SSC-A (10^3)
anti-IgG AF488
anti-IgM AF647
FITC-H
APC-H
225
1379
317
1438
serum+anti-IgG AF488
serum+anti-IgM AF647
1339
8406
39758
115404
no serum
Ser#3
B
anti-IgA FITC
237
314
serum+anti-IgA FITC
332
2025

Figure 2. Setting of the flow cytometer for detecting anti-Spike IgG, M, and A from sera of patients with COVID-19

(A and B) The viable cell population of interest (negative for 7AAD staining) was first gated using a FSC/PerCP dot plot followed by a FSC-H/FSC-A dot plot (to exclude doublets from the analysis). Anti-spike antibodies were then detected using anti-human IgG AF488, IgM AF647 antibodies (A) and anti-IgA FITC (B) with a AF488/SSC, an AF647/SSC (A) and a FITC/SSC (B) dot-plot respectively. The gMFI of AF488, AF647 (A) and FITC (B) were further used to determine the level of anti-Spike IgG, IgM and IgA antibodies (see the 'quantification and statistical analysis' part).

b. Centrifuge the cell suspension for 7 min at 550 g
c. Remove the supernatant by flicking the 96 well U-bottom plate

⚠ CRITICAL: Spike expression should be verified before testing the human sera as described in the 'before you begin' part.

Incubation of non-transfected and SARS-CoV-2 spike expressing HEK293T cells with patients-derived sera

Timing: 1 h

This step describes the cell staining with human sera derived from healthy volunteers and patients with COVID-19. Serum 1 in 50 dilution is efficient for testing sera from asymptomatic, mild and severe patients with COVID-19. Negative (i.e., sera prior COVID-19 pandemic) and positive (i.e., controls provided by SeroBio France as a validation tool for diagnostic laboratories) sera controls should be used to validate the assay (see Martin et al., 2021).

4. Dilute healthy volunteers- and patients-derived sera
   a. Defrost the sera on ice
   b. Dilute sera at 1 in 50 with the staining buffer (10 µL of serum in 490 µL of the staining buffer)
5. Incubate the diluted serum with non-transfected and SARS-CoV-2 Spike expressing HEK293T cells
   a. Add 50 µL of diluted serum per well
   b. Resuspend the cells in diluted serum by pipetting up and down three times
   c. Incubate 30 min at 4°C
6. Wash the cells with the staining buffer
   a. Add 150 µL of staining buffer per well
   b. Centrifuge the cell suspension at 550 g for 7 min
   c. Remove the supernatant by flicking the 96 well U-bottom plate
   d. Resuspend the cell pellets with 200 µL of staining buffer
   e. Repeat twice step 6.b to step 6.d
   f. Remove the supernatant by flicking the 96 well U-bottom plate

Staining of non-transfected and SARS-CoV-2 spike expressing HEK293T cells with fluorescent anti-human immunoglobulins antibodies

Timing: 90 min

This step describes the cell staining for detecting the binding of different human anti-Spike immunoglobulins IgG, M and A (using AF488-, AF647- and FITC-coupled secondary antibodies respectively) on non-transfected and Spike-expressing HEK293T cells pre-incubated sera of patients with COVID-19. In this protocol, IgG and IgM antibodies were detected at the same time using AF488- and AF647-coupled secondary antibodies respectively; and IgA antibodies were detected separately using and FITC-conjugated secondary antibodies. Alternative methods could be adapted to detect all Ig subtypes at the same time (for instance using anti-IgA antibodies coupled to other fluorochromes). Do not forget to acquire non-transfected and Spike-expressing HEK293T cells with secondary antibodies alone.

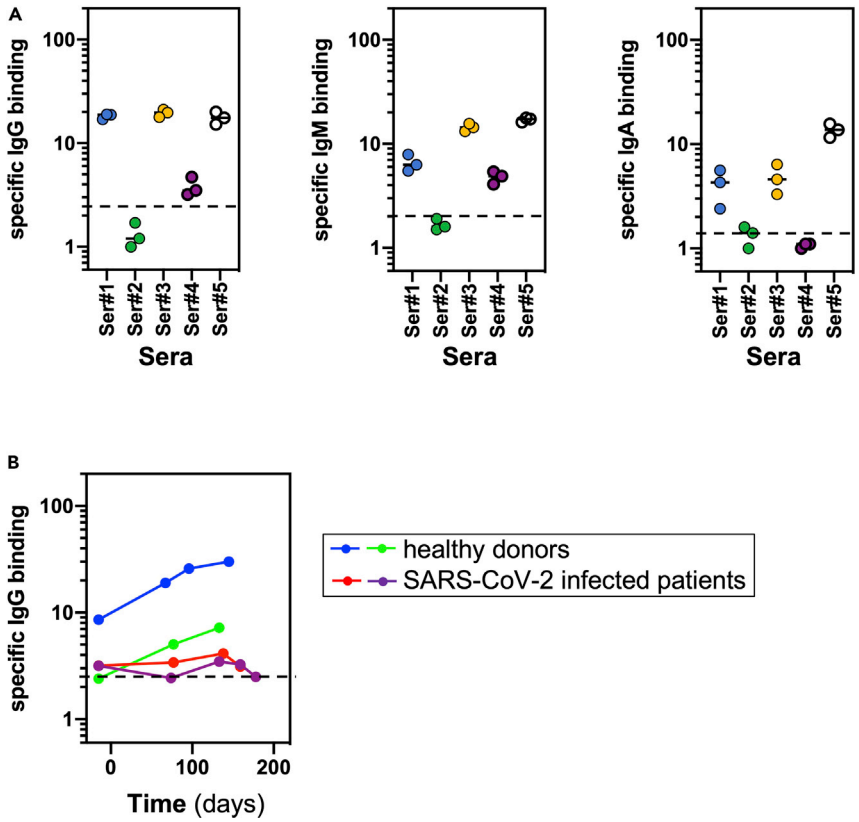

Figure 3. Representation of results outcome for detecting anti-Spike IgG, M, and A from sera of patients with COVID-19

Sera from SARS-CoV-2-infected patients were tested for their positivity against viral Spike proteins using the SARS-CoV-2 serological assay described in this protocol.

(A) Anti-Spike antibodies were detected using fluorescent-coupled anti-human IgG, IgM and anti-IgA antibodies. The means of fluorescence intensities were further used to determine the specific level of anti-Spike IgG, IgM and IgA antibodies (see the 'quantification and statistical analysis' part). Negative thresholds (dotted lines) were obtained with the basal levels of Ig binding from negative control sera.

(B) Specific level of anti-SARS-CoV-2 Spike IgG was followed over time after analysis of sera obtained from healthy donors (red and purple lines) and SARS-CoV-2-infected patients (blue and green lines). Negative thresholds (dotted lines) were obtained with the basal levels of Ig binding from negative control sera. For more details, refer to Martin et al. (2021).

7. Prepare the secondary antibodies dilutions as indicated in the 'materials and equipment' part
8. Incubate the secondary antibodies with non-transfected and SARS-CoV-2 Spike expressing HEK293T cells
   a. Add 100 µL of diluted secondary antibodies per well
   b. Resuspend the cells by pipetting up and down three times
   c. Incubate 30 min at 4°C in the dark
9. Wash the cells with the staining buffer
   a. Add 100 µL of staining buffer per well
   b. Centrifuge the cell suspension at 550 g for 7 min
   c. Remove the supernatant by flicking the 96 well U-bottom plate
   d. Resuspend the cell pellets with 200 µL of staining buffer
   e. Repeat twice step 10.b to step 10.d
   f. Remove the supernatant by flicking the 96 well U-bottom plate
   g. Resuspend the cell pellets with 200 µL of staining buffer and 5 µL of 7AAD

Flow cytometry analysis

Timing: 1 h

The step describes the analysis of Ig binding on non-transfected and Spike-expressing HEK293T cells. Additional information is provided in 'materials and equipment' and 'quantification and statistical analysis' parts.

10. Set up the flow cytometry for detection of the single cell population of interest :
    a. labeled with a AF488, AF647 fluorochromes and 7AAD for IgG and M detection by preparing the gating approach (i.e., FSC/PerCP dot plot -> FSC-H/FSC-A dot plot -> AF488/SSC and AF647/SSC dot-plots as indicated in the 'materials and equipment' part (Figure 2A)
    b. labeled with a FITC fluorochrome and 7AAD for IgA detection by preparing the gating approach (i.e., FSC/PerCP dot plot -> FSC-H/FSC-A dot plot -> FITC/SSC dot-plot as indicated in the 'materials and equipment' part (Figure 2B)
11. Acquire 10 000 events in the gate of interest
12. Use the non-transfected HEK293T cells as a negative control
13. Determine the mean of fluorescence intensity observed for AF488, AF647 and FITC fluorochromes (corresponding to IgG, IgM and IgA binding respectively) for each condition including non-transfected and Spike-expressing HEK293T cells (Figures 2A and 2B)
14. Determine the corresponding Ig binding levels observed with non-transfected HEK293T cells for each serum studied as followed: ratio of the geomean of fluorescence intensity (gMFI) = (gMFI observed with HEK293T cells incubated with serum and fluorescent anti-Ig antibodies) / (gMFI observed with HEK293T cells incubated only with fluorescent anti-Ig antibodies). See the 'materials and equipment' part
15. Determine the corresponding Ig binding levels observed with Spike-expressing HEK293T cells for each serum studied as described in step 14. See the 'materials and equipment' part
16. Determine the specific Ig binding as followed: specific Ig binding = (ratio observed with Spike-expressing HEK293T cells) / (ratio observed with non-transfected HEK293T cells). See the 'materials and equipment' part
17. The specific Ig binding determined for each serum was the mean of specific Ig binding obtained in three independent experiments.

Note: To strengthen the conclusion of the staining, more events could be acquired.

EXPECTED OUTCOMES

A representative analysis is provided in Figures 2A and 2B using sera from 5 different patients with COVID-19.

QUANTIFICATION AND STATISTICAL ANALYSIS

The following tables show examples of analyses for IgG, IgM and IgA obtained with 5 different patients with COVID-19 (Tables 1 and 2). The final specific Ig binding determined for each serum was the mean of specific Ig binding obtained in three independent experiments (Table 3). A final visualization of the data for these patients is proposed in Figure 3A. A follow-up of anti-SARS-CoV-2 antibodies could be obtained using this protocol (Figure 3B). Please refer to Martin et al. (2021) for an overview of the whole dataset.

LIMITATIONS

This protocol could be applied to any viral proteins that are expressed at the cell surface. In our recent work, [Martin et al., iScience 2021] we did observe anti-SARS-CoV-2 Spike and Membrane antibodies but failed to detect anti-SARS-CoV-2 Envelope antibodies due to the fact that this viral protein was probably not expressed at the cell surface and retained intracellularly. Considering the

Table 1. Examples of raw data obtained for anti-Spike IgG/M detection from patients with COVID-19

Specimen	Sample_Staining	gMFI AF488	gMFI AF647	IgG binding	Specific IgG binding	IgM binding	Specific IgM binding
no serum	Ctrl_IgG/M	225	1379				
Ser#1	Ctrl_IgG/M	853	4363	3.8		3.2	
Ser#2	Ctrl_IgG/M	1067	6138	4.7		4.5	
Ser#3	Ctrl_IgG/M	1339	8406	6.0		6.1	
Ser#4	Ctrl_IgG/M	1336	6044	5.9		4.4	
Ser#5	Ctrl_IgG/M	1009	7616	4.5		5.5	
no serum	Spike_IgG/M	317	1438				
Ser#1	Spike_IgG/M	20398	24808	64.3	17.0	17.3	5.5
Ser#2	Spike_IgG/M	2629	9556	8.3	1.7	6.6	1.5
Ser#3	Spike_IgG/M	39758	115404	125.4	21.1	80.3	13.2
Ser#4	Spike_IgG/M	6110	34055	19.3	3.2	23.7	5.4
Ser#5	Spike_IgG/M	24960	129008	78.7	17.6	89.7	16.2

The IgG binding is given by the ratio of (gMFI AF488 Ctrl with serum / gMFI AF488 Ctrl without serum); for instance for Ser#1: IgG binding = 853 / 225 = 3.8. The IgM binding is given by the ratio of (gMFI AF647 Ctrl with serum / gMFI AF647 Ctrl without serum); for instance for Ser#1: IgM binding = 4363 / 1379 = 3.2. The specific IgG binding is given by the ratio of (IgG binding obtained with Spike-expressing HEK293T cells / IgG binding obtained with non-transfected (Ctrl) HEK293T cells); for instance for Ser#1: specific IgG binding = 64.3 / 3.8 = 17.0. The specific IgM binding is given by the ratio of (IgM binding obtained with Spike-expressing HEK293T cells / IgM binding obtained with non-transfected (Ctrl) HEK293T cells); for instance for Ser#1: specific IgM binding = 17.3 / 3.2 = 17.0.

many Spike variants emerging, this protocol could be adapted using plasmids containing specific sequences of Spike variants.

TROUBLESHOOTING

We observed increased IgG binding on non-transfected HEK293T cells using sera of patients under immunotherapy with humanized antibodies, probably due to non-specific binding of these antibodies to HEK293T cells (step 14). This problem was solved by normalizing IgG binding as described in the 'quantification and statistical analysis' part.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tony Avril (t.avril@rennes.unicancer.fr).

Table 2. Examples of raw data obtained for anti-Spike IgA detection from patients with COVID-19

Specimen	Sample_Staining	gMFI FITC	IgA binding	Specific IgA binding
no serum	Ctrl_IgA	237		
Ser#1	Ctrl_IgA	284	1.2	
Ser#2	Ctrl_IgA	287	1.2	
Ser#3	Ctrl_IgA	332	1.4	
Ser#4	Ctrl_IgA	243	1.0	
Ser#5	Ctrl_IgA	576	2.4	
no serum	Spike_IgA	314		
Ser#1	Spike_IgA	898	2.9	2.4
Ser#2	Spike_IgA	390	1.2	1.0
Ser#3	Spike_IgA	2025	6.4	4.6
Ser#4	Spike_IgA	321	1.0	1.0
Ser#5	Spike_IgA	10426	33.2	13.7

The IgA binding is given by the ratio of (gMFI FITC Ctrl with serum / gMFI FITC Ctrl without serum); for instance for Ser#1: IgA binding = 284 / 237 = 1.2. The specific IgA binding is given by the ratio of (IgA binding obtained with Spike-expressing HEK293T cells / IgA binding obtained with non-transfected (Ctrl) HEK293T cells); for instance for Ser#1: specific IgG binding = 2.9 / 1.2 = 2.4.

Table 3. Individual data obtained for IgG, IgM, and IgA detection from patients with COVID-19

Specimen	Specific IgG binding	Specific IgM binding	Specific IgA binding
Ser#1	17.0	5.5	2.4
	18.8	7.9	5.6
	19.0	6.3	4.3
Mean ± *SD*	18.3 ± *1.1*	6.6 ± *1.2*	4.1 ± *1.6*
Ser#2	1.7	1.5	1.0
	1.2	1.9	1.4
	1.0	1.6	1.6
Mean ± *SD*	1.3 ± *0.4*	1.7 ± *0.2*	1.4 ± *0.3*
Ser#3	21.1	13.2	4.6
	19.7	14.4	3.3
	17.8	15.6	6.4
Mean ± *SD*	19.6 ± *1.7*	14.4 ± *1.2*	4.8 ± *1.6*
Ser#4	3.2	5.4	1.0
	4.7	4.9	1.1
	3.5	4.1	1.1
Mean ± *SD*	3.8 ± *0.8*	4.8 ± *0.7*	1.1 ± *0.1*
Ser#5	17.6	16.2	13.7
	15.2	17.7	11.6
	20.0	17.3	15.6
Mean ± *SD*	17.6 ± *2.4*	17.1 ± *0.8*	13.6 ± *2.0*

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request. This paper does not report the original code.

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AUTHOR CONTRIBUTIONS

Methodology, S.M., A.N., G.J., and T.A.; investigation, S.M., A.N., G.J., and T.A.; formal analysis, S.M., A.N., G.J., and T.A.; resources, F.G.; conceptualization, M.L.G., E.C., and T.A.; writing – editing, M.L.G.; supervision, E.C. and T.A.; project administration, E.C. and T.A.; funding acquisition, E.C.; writing – review & editing, E.C. and T.A.; writing – original draft, T.A.

DECLARATION OF INTERESTS

E.C. is a founder of Cell Stress Discoveries Ltd (https://cellstressdiscoveries.com) and Thabor Therapeutics (https://www.thabor-tx.com/).

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