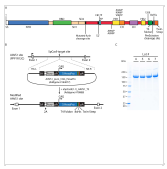

May 13, 2021

Artisanal Production of Prefusion-Stabilized SARS-CoV-2 Spikes

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Coronavirus Method Development Community Doyon Lab

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ABSTRACT

The two-part protocol presented here describes the establishment of a stable pool of 293-F cells expressing the HexaPro variant of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) spike (S) protein and its production and purification from the culture medium. CRISPR-Cas9-driven targeted integration of the expression cassette at the *AAVS1* safe harborlocus streamlines cell line production. Engineered 293-F cells grown as suspension cultures in animal origin-free, chemically defined, protein-free medium allow purification to be performed directly from the culture medium with minimal cell processing steps. Yields of native and purified Spikes typically average 30-50 mg per liter of culture medium following tandem affinity purification using nickel-coupled and Strep-Tactin resins. Purified proteins bind recombinant ACE2 and mAb CR3022 in ELISA-based assays. Establishing an engineered cell line from cryopreserved 293-F cells takes approximately 4 weeks, and subsequent production and purification take between 9 and 14 days. As shown for the D614G mutation, this system can be readily adapted to study Spike variants.

ATTACHMENTS

Artisanal Production of Prefusion-Stabilized SARS-CoV-2 Spikes_manuscript.pdf

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KEYWORDS

SARS-CoV2, spike protein, 293-F, stable cell line, genomic safe harbor, CRISPR-Cas9, AAVS1 targetting

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MATERIALS TEXT

Equipment

Note: All vessels used for cell culture should be sterilized if necessary. All glassware used for protein purification should be rinsed before use with ultrapure water.

A
0,45 µm sterile filters
1.5 × 10 cm glass chromatography column (Bio-Rad #7375011)
15 and 50 ml sterile polypropylene conical tubes
250 ml Erlenmeyer culture flasks with vented cap
2,8 L Fernbach baffled culture flask with vented cap
2.5 × 20 cm glass chromatography column (Bio-Rad #7374252)
37°C tissue culture incubator with shaking capacity up to 120 RPM and adhesive mats
37°C water bath
2.5 × 20 cm glass chromatography column (Bio-Rad #7374252)
37°C tissue culture incubator with shaking capacity up to 120 RPM and adhesive mats
37°C water bath
50 mL Erlenmeyer flask with phenolic screw cap
500 ml Nalgene PPCO Centrifuge Bottles
12-wells cell culture plates
Beckman Avanti series centrifuge with JLA-10.500 fixed angle rotor
Benchtop centrifuge
Benchtop rocking platform
Hemocytometer or automated cell counter
Magnetic stirrer
Stain-free enabled imager (optional)

List of reagents

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A	B	C
REAGENTS	**Supplier**	**Catalog number**
For cell culture and protein production		
293-F Cells	Gibco	11625019
AAVS1_puro_CAG_HexaPro	Addgene	164077
eSpCas9(1.1)_No_FLAG_AAVS1_T2	Addgene	79888
FreeStyle 293 Expression Medium	Gibco	12338018
Lipofectamine 3000	Invitrogen	L3000015
Opti-MEM I Reduced Serum Medium	Gibco	31985062
Puromycin Dihydrochloride	Gibco	A1113803
Trypan Blue Solution, 0.4%	Gibco	15250061
For protein purification		
10x Buffer BXT	IBA Lifesciences	2-1042-025
10x Buffer W	IBA Lifesciences	2-1003-100
2,2,2-Trichloroethanol (optional)	Sigma	T54801
4–20% Mini-PROTEAN TGX Stain-Free Gels (or hand-cast acrylamide gels)	Bio-Rad	4568095
Imidazole	Fisher BioReagents	BP30550
Magnesium chloride, anhydrous	Sigma	M8266
Ni Sepharose 6 Fast Flow	Cytiva	17531801
Potassium chloride	Sigma	P9541
Potassium phosphate monobasic	Sigma	P5379
QC Colloidal Coomassie Stain	Bio-Rad	1610803
Sodium chloride	Sigma	S7653
Sodium hydroxide solution, 1N	Fisher	SS266-1
Sodium phosphate dibasic	Sigma	S0876
Sodium phosphate monobasic	Sigma	S0751
Strep-Tactin HRP	IBA Lifesciences	2-1502-001
Strep-Tactin XT 4Flow high-capacity resin	IBA Lifesciences	2-5030-010

REAGENTS SETUP
PBS 10X (1 L):

Add the powders to 600 ml of ultrapure water in a 1 L glass bottle while stirring, adjust pH to 7,4 using NaOH and fill to 1 L with ultrapure water. Dilute to 1X with ultrapure water before use.

NaCl	80g
KCl	2g
Na2HPO4	14,4g
KH2PO4	2,4g

His wash buffer (1 L):
Add the powders to 600 ml of ultrapure water in a 1 L glass bottle while stirring, adjust pH to 8,0 using NaOH and fill to 1 L with ultrapure water.

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NaH2PO4 (50 mM)	6 g
NaCl (300 mM)	17,54 g
Imidazole (20 mM)	1,36 g

His elution buffer (1 L):
Add the powders to 600 ml of ultrapure water in a 1 L glass bottle while stirring, adjust pH to 8,0 using NaOH and fill to 1 L with ultrapure water.

NaH2PO4 (50 mM)	6 g
NaCl (300 mM)	17,54 g
Imidazole (250 mM)	17 g

Buffer W and QXT 1X (1 L):
These buffers are provided as 10X stocks. Dilute to 1X with ultrapure water before use.

Puromycin diluted solution (1 ml):
The puromycin used here is provided at a stock concentration of 10mg/ml. We recommend diluting this stock with sterile PBS to a concentration of 0,5mg/ml. The stock solution should be kept at -20°C for long term storage. We recommend keeping the diluted solution at 4°C up to a year. To make 1ml of diluted puromycin:

Puromycin stock	50µl
Sterile PBS	950µl

Thawing and general handling of 293-F cells before transfection (Timing: 11 days) 1w 4d

1 Before thawing the cells, prewarm 30 ml of FreeStyle 293 Expression Medium in a 250 ml tissue culture shaker flask at 37°C, preferably in a humidified atmosphere with 8% CO2. Also, prewarm a 9 ml aliquot of culture medium at 37°C in a 50 ml sterile polypropylene conical tube.

Note: While we used commercial 293-F cells from Gibco, it is likely that this protocol can be adapted for other 293-derived cell lines adapted for suspension and serum-free growth. We recommend culturing 293-F cells in medium without antibiotic, as we have seen reduced growth fitness when using Pen-Strep. Cells should be tested for the absence of mycoplasma contamination before use.

2 Rapidly thaw the cells in a 37°C water bath, disinfect with 70% ethanol and resuspend in the 9 ml medium aliquot. Centrifuge the cells at 340g for 5 minutes and discard the supernatant. Resuspend the cell pellet in the 30 ml aliquot of prewarmed medium.

3 Assess post-thaw cell count and viability using trypan blue and a hemocytometer or an automated cell counter. Incubate the cells in a 37°C incubator in a humidified atmosphere with 8% CO2 and orbital shaking at 120 RPM.

4 Monitor cell growth and viability everyday, until cells reach a confluency of about 2-3 x 10^6 cells/ml. At this point, cells should be split at 0,5 x 10^6 cells/ml. 4d

5 We recommend passaging the cells at least twice after thawing to ensure proper fitness before transfection. We also recommend transfecting cells at most at 10 passages post-thaw to create a stable cell line, hence the importance of creating a stock of low-passage frozen cells after the initial thaw. 1w

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Lipofectamine transfection of 293-F cells (Timing: 3 days) 3d 0h 10m

6 Prepare the two plasmid vectors ordered from Addgene (eSpCas9(1.1)_No_FLAG_AAVS1_T2 and AAVS1_puro_CAG_HexaPro) as high-quality DNA preps. We recommend transfecting a control vector such as a mammalian GFP expression plasmid (750 ng) to monitor transfection efficiency and cell death during the subsequent puromycin selection step.

Note: Lipofection conditions, such as the ratios of Lipofectamine 3000 and P3000 reagents, may benefit from further optimisation. However, we found that the conditions presented here were acceptable for generating stable cell lines using 293-F cells. The transfection conditions used here can be scaled up or down.

7 Count and plate 2 x 10^5 cells in a volume of 2ml per well in a 12-wells cell culture plate for each transfection condition.

8 Using Opti-MEM medium prewarmed at 37°C, prepare the following mixes in two tubes:

Tube A	
Reagent	**Amount**
Lipofectamine 3000	3 µl
Opti-MEM medium	Complete to 50 µl

A	B
Tube B	
Reagent	**Amount**
eSpCas9(1.1)_No_FLAG_AAVS1_T2	250 ng
AAVS1_puro_CAG_HexaPro	500 ng
P3000 reagent	2 µl
Opti-MEM medium	Complete to 50 µl

9 Transfer the content of tube **A** to tube **B** and mix thoroughly by pipetting. Incubate this mix 10 minutes at room temperature. 10m

10 Add the content of tube **B** to the corresponding well of the 12-wells plate.

11 Incubate the cells in a 37°C incubator in a humidified atmosphere with 8% CO_2 without shaking for 72h. 3d

Puromycin selection of the transfected 293-F cells (Timing: 8-11 days) 1w 4d

12 Prewarm 8 ml FreeStyle Expression Medium in a sterile 50 ml Erlenmeyer flask. Transfer the transfected cells to the flask. Cells may clump and attach to the bottom of the wells, in which case gentle up and down pipetting is generally sufficient to resuspend them. Incubate the cells overnight in a 37°C incubator in a humidified atmosphere with 8% CO2 and orbital shaking at 125 RPM. We recommend lightly unscrewing the cap from the flasks during incubation, as narrow flask caps such as these are usually not vented. 1d

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13 The next day, add 10 µl of the diluted puromycin solution to the medium so that the final concentration is 0,5 µg/ml in a 10 ml total volume.

14 Monitor cell death and growth every 2-3 days until a confluency of 2-2,5 x 10^6 cells/ml, generally 7-10 days after adding the antibiotic. At this point, the puromycin selection is generally complete. If cells reach confluency too early, an additional 1:2 to 1:3 split may be performed. 1w 3d

15 Once the pool of puromycin-selected cells reaches confluency, split the cells in culture medium without puromycin. At this point, we highly recommend taking an aliquot of medium from a confluent culture to confirm the secretion of the spike protein by western blot with a Strep-Tactin HRP conjugate.

Production and harvest from a stable spike-expressing cell line (Timing: 7-12 days) 1w 5d

16 To start a production, split the cells in a 2,8L Fernbach baffled culture flask to obtain 0,5 x 10^6 cells/ml in a total volume of 500ml fresh 293 FreeStyle Expression medium.

> Note: Volumes of medium and reagents are provided for protein production from 500ml of culture medium and may be scaled up or down accordingly. As with other chromatography columns, ensure that the volume of liquid inside the column is always sufficient to keep the resin submerged.

17 Incubate the cells 7-12 days in a 37°C incubator in a humidified atmosphere with 8% CO2 and orbital shaking at 55 RPM. **Figure 2A** illustrates spike accumulation in the culture medium at several time points. 1w 5d

18 Once the desired time point has been reached, harvest the cultures, and divide equally between two 500 ml PPCO Nalgene bottles. Centrifuge at 1850g for 20 minutes. Transfer the supernatant to two new Nalgene bottles and repeat centrifugation. 20m

19 Harvest the supernatant from both bottles after the second centrifugation step and pass through a 0,45 µm sterile filter. Use filtered supernatant for subsequent purification.

Histidine tag purification of the spike protein from filtered culture supernatant (Timing: 1 day) 1d

20 Transfer the filtered supernatant to a clean 1 L container and add 30 ml of Ni Sepharose 6 Fast Flow resin pre-rinsed with PBS 1X. Incubate overnight on a rocking platform at room temperature. **Optional:** save an aliquot of the initial filtered supernatant, referred to here as “Hisinput”. 12h

21 Transfer the supernatant mixed with the resin to a clean 2,5 cm x 20 cm chromatography column and wait for the beads to settle down. Next, collect the flowthrough. **Optional:** save an aliquot of this fraction, referred to here as “His flowthrough”.

22 Add 150 ml of 1X PBS to wash the column and collect the flowthrough. **Optional:** save an aliquot of this fraction, referred to here as “His wash 1”.

23 Add 150 ml of His wash buffer and collect the flowthrough. **Optional:** save an aliquot of this fraction, referred to here as “His wash 2”.

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24 Add 150 ml of His elution buffer and collect the following fractions:

E1: 20ml (E1 His)

E2: 40ml (E2 His)

E3: 30ml (E3 His)

E4: 30ml (E4 His)

E5: 30 ml (E5 His)

25 Wash the column with 240 ml PBS and store at 4°C.

26 Assess protein recovery and purification efficiency by loading the different fractions onto an 8% polyacrylamide gel and performing polyacrylamide gel electrophoresis (PAGE). Total proteins can be visualized by staining the gel with colloidal Coomassie Blue. A typical gel showing the fractions obtained at the different steps of purification is shown in **Figure 2.** Total proteins can also be rapidly visualized by employing Stain-Free gels and a stain-free enabled imager. A less expensive alternative to commercial Stain-Free gels can be achieved by adding 0,5% (v/v) 2,2,2-Trichloroethanol (TCE) to a handcast polyacrylamide gel and visualizing total proteins on a stain-free enabled imager or a UV gel imager[26]. In our experience, the second elution (E2 His) contains most of the purified protein and is selected for subsequent Twin-Strep tag purification.

Twin-Strep tag purification of the spike protein from a histidine purified sample (Timing: 1 day) 1d

27 Add 10ml of Strep-Tactin XT 4Flow high-capacity resin to the selected His eluate fraction(s) in a clean 50-100 ml container. Incubate overnight on a rocking platform at room temperature. 12h

28 Transfer the His eluate mixed with the resin on a 1,5 cm x 10 cm chromatography column and wait for the beads to settle down. Collect the flowthrough. **Optional:** save an aliquot of this fraction, referred to here as “Strep-Tactin flowthrough”.

29 Wash the column with 5 x 10 ml of 1X buffer W and collect the flowthrough. **Optional:** save an aliquot of each of these five fractions, referred to here as “Strep-Tactin wash 1-5”.

30 Elute with 30 ml of 1X buffer BXT and collect the following fractions:

E1: 5 ml (E1 ST)

E2: 20 ml (E2 ST)

E3: 5 ml (E3 ST)

31 Pass 150 ml of 3M MgCl2 to regenerate the column, and re-equilibrate using 80 ml of 1X buffer W. Store the column at 4°C.

32 Assess protein recovery and purification efficiency by loading the different fractions onto an acrylamide gel as described in **step 26**. Protein yield may be assessed by methods such as Bradford assay and absorbance at 280nm using a UV spectrophotometer. We found that both resins performed well when reused to purify up to 3-4 batches. The

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resulting purified HexaPro spike protein can be used in downstream applications. We observed that the protein seemed to remain stable at 4°C on the short term but advise flash-freezing followed by -80°C keeping for long term storage.

Anticipated results

33 By following this protocol, we consistently obtain yields of 30-50 mg of purified protein per liter of culture supernatant as determined by Bradford assay and A280 absorbance. Most of the purified protein is eluted in the second (E2 ST) and third (E3 ST) fractions of the final elution step, at concentrations of up to 2 mg/ml. **Figure 1C** shows the consistency in purity obtained from different production lots using the protocol described here. **Figure 3** shows that the purified protein binds both to recombinant ACE2 and mAb CR3022 and that this protocol can be adapted to produce a D614G HexaPro spike variant. The vectors used in this protocol are available from Addgene (#79888 and #164077).

Figures

34

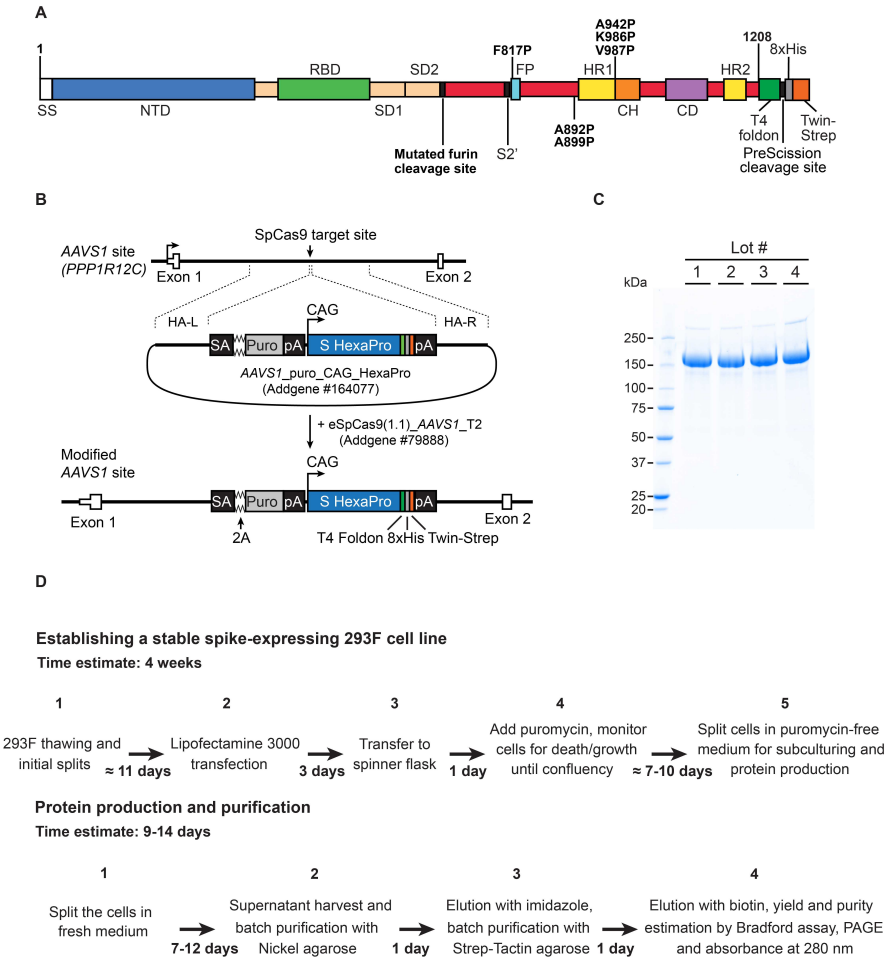

Figure 1 | Production and purification of the HexaPro variant of the SARS-CoV2 spike protein from *AAVS1* targeted 293-F cells. **A)** Representation of the HexaPro variant of the spike protein, adapted from *Wrapp et al.* SS, signal sequence; NTD, N-terminal domain; RBD, receptor-binding domain; SD1/SD2, subdomains 1 and 2; S2', protease cleavage site; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2. The six stabilizing proline substitutions are indicated. **B)** Schematic of the *AAVS1* safe harbor locus before and after CRISPR-driven targeting of the HexaPro expression construct. SA, splice acceptor site; 2A, 2A self-cleaving peptide sequence; Puro, puromycin resistance gene; pA, polyadenylation sequence; CAG, CMV early enhancer/chicken β-actin promoter; T4 Foldon, T4 fibritin trimerization motif; 8xHis, octa-histidine affinity tag; Twin-Strep, Twin-Strep affinity tag. The Addgene numbers of the vectors used in this protocol are shown. **C)** Batch-to-batch consistency in purity between different lots of protein produced and purified using this protocol. Purified proteins (10µg per sample) were loaded on a 4–20% acrylamide gel for PAGE followed by colloidal Coomassie staining. **D)** Illustration of the general workflow of this protocol. The first part of the procedure is the establishment of a stable pool of 293-F cells expressing the HexaPro spike protein. The second part is the production and purification of this protein from the culture medium.

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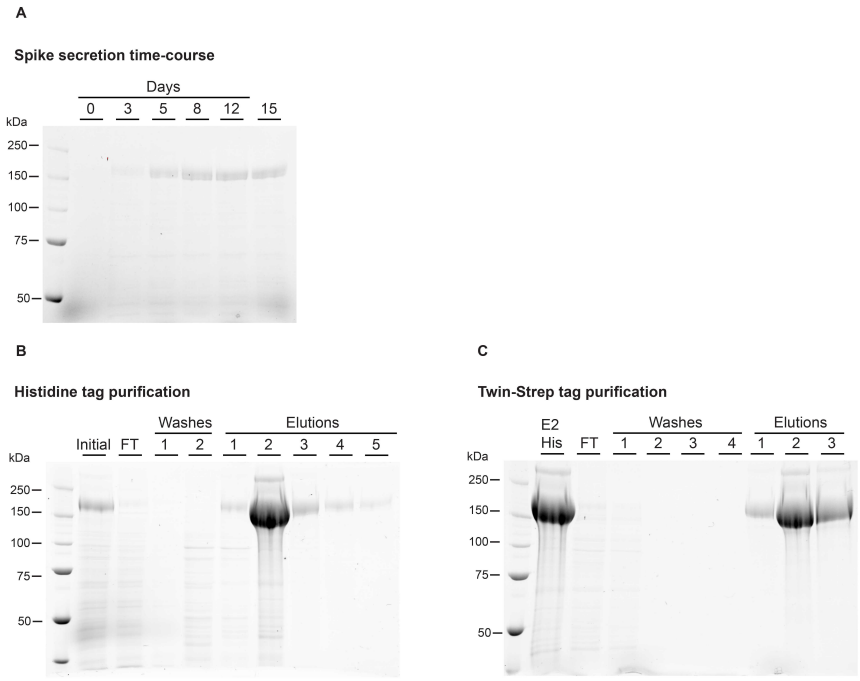

Figure 2 | Spike secretion in the culture medium and acrylamide gel visualization of typical purification fractions. **A)** Aliquots of culture medium from a culture of spike-expressing 293-F cells were taken at the indicated time points. 20µl per sample of culture medium mixed with Laemmli buffer were loaded on a handcast acrylamide gel containing 0,5% (v/v) 2,2,2-Trichloroethanol (TCE) for PAGE. The gels were visualized with a stain free-enabled imager (ChemiDoc MP, Bio-Rad). **B)** The different fractions from 8xHis tag purification with Ni Sepharose 6 Fast Flow that are described in the protocol were loaded on a gel and imaged as in **A)**. **C)** Same as in **B)** for Twin-Strep tag purification with Strep-Tactin XT 4Flow high-capacity resin. FT: flowthrough, E2 His: second eluted fraction from the histidine tag purification.

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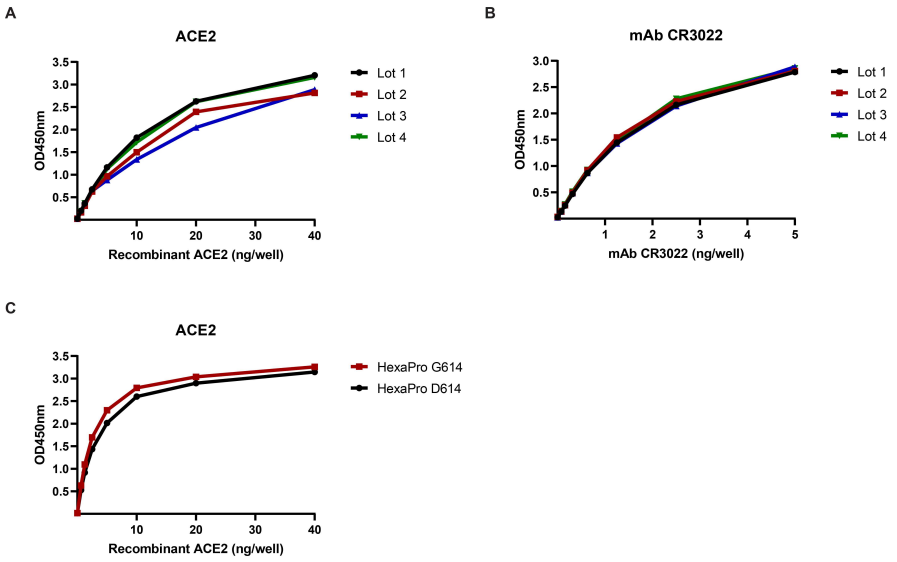

Figure 3 | Functional binding of the purified spike in ELISA assays. ELISA assays were conducted with four different lots of recombinant spike produced using this protocol against either **A)** recombinant ACE2-Fc or **B)** the mAb CR3022 antibody. **C)** Same as in **A)** but with protein from lot 4 (HexaPro D614) and protein harboring the D614G mutation (HexaPro G614). Each data point is the average of two technical duplicates. ELISA assays were performed using a modified version of the protocol developed by *Anamat et al.*. Briefly, wells from a 96-wells plates were coated overnight at 4°C with 100µl per well of 2µg/ml of purified spike diluted in PBS. Washes were performed between each subsequent step with 200µl of PBS 1X with 0,1% (vol/vol) Tween-20 (PBST). Blocking was performed by incubating 1h at room temperature with 200µl per well of 2% (wt/vol) bovine serum albumin (BSA, Sigma) prepared in PBST. Next, 100µl per well of blocking solution (PBST with 2% (wt/vol) BSA) containing either recombinant ACE2-Fc (a gift from Manuel Carruso) or mAb CR3022 were added to each well before incubation 1h at room temperature. After blocking, 100µl of a 1:60,000 (vol/vol) dilution of goat anti-human IgG-horseradish peroxidase (HRP) conjugated secondary antibody (AP112, Emd Millipore) prepared in the blocking buffer was added per well, followed by incubation 1h at room temperature. Revelation was performed by adding 100µl of Pierce TMB (3,3',5,5' tetramethylbenzidine) substrate (Thermo Scientific) per well. The plates were incubated 20 min at room temperature in the dark before the reaction was stopped by adding 100µl of 2M sulfuric acid per well. Optical density at 450nm was measured using a Tecan Spark 10M microplate reader.

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