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Production of Trimeric SARS-CoV-2 Spike Protein by CHO Cells for Serological COVID-19 Testing

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ABSTRACT

We describe scalable and cost-efficient production of full length, His-tagged SARS-CoV-2 spike glycoprotein trimer by CHO cells that can be used to detect SARS-CoV-2 antibodies in patient sera at high specificity and sensitivity. Transient production of spike in both HEK and CHO cells mediated by PEI was increased significantly (up to 10.9-fold) by a reduction in culture temperature to 32ºC to permit extended duration cultures. Based on these data GS-CHO pools stably producing spike trimer under the control of a strong synthetic promoter were cultured in hypothermic conditions with combinations of bioactive small molecules to increase yield of purified spike product 4.9-fold to 53 mg/L. Purification of recombinant spike by Ni-chelate affinity chromatography initially yielded a variety of co-eluting protein impurities identified as host cell derived by mass spectrometry, which were separated from spike trimer using a modified imidazole gradient elution. Purified CHO spike trimer antigen was used in ELISA format to detect IgG antibodies against SARS-CoV-2 in sera from patient cohorts previously tested for viral infection by PCR, including those who had displayed COVID-19 symptoms. The antibody assay, validated to ISO 15189 Medical Laboratories standards, exhibited a specificity of 100% and sensitivity of 92.3%. Our data show that CHO cells are a suitable host for the production of larger quantities of recombinant SARS-CoV-2 trimer which can be used as antigen for mass serological testing.

Keywords: bioproduction; CHO cells; SARS-CoV-2; COVID-19; spike trimer; serological assay

Immune response represents the first line of defense against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection that has caused the coronavirus

disease 2019 (COVID-19) pandemic. The spike glycoprotein that protrudes from the surface of the virus is highly immunogenic with the receptor-binding domain (RBD) being the target of many neutralizing antibodies (Yuan et al., 2020). Utilizing a stabilized version of the full-length SARS-CoV-2 spike protein, a very robust and accurate serological enzyme-linked immunosorbent assay (ELISA) for antibodies in patient sera has recently been developed (Amanat et al., 2020) and approved for use by the US FDA. However, very low production titers (1–2 mg/L) of the spike trimer were reported using the human embryonic kidney (HEK) Expi293 Expression system (Esposito et al., 2020; see Supplementary Figure S1), therefore effectively limiting its widespread utilization as a preferred antigen in serological assays for COVID-19. The low production titer is not surprising considering that the SARS-CoV-2 spike is a large homotrimer (~670 kDa) with 22 *N*-linked glycosylation sites per monomer (Watanabe et al., 2020). In this work, using improved vector engineering and production process strategies we describe the development of a stable recombinant spike manufacturing platform utilizing Chinese hamster ovary (CHO) cells as a preferred production host. Transient expression was initially employed to fast-track production of recombinant protein to enable biophysical analyses and early clinical evaluation in serological assays, as well as to evaluate product manufacturability and refine production process and purification conditions.

We have previously shown that for difficult-to-express (DTE) proteins, transient production processes need to be tailored to negate the protein-specific negative effects of recombinant gene overexpression in host cells (e.g., unfolded protein response (UPR) induction, limited cell-specific productivity (qP); Johari et al., 2015). Using the plasmid construct from the Krammer Laboratory (see Materials and Methods for details), HEK Expi293F and CHO-S cells were transiently transfected

with the CAG-driven expression plasmid using PEI at an optimal gene dosage for spike production in both cases (data not shown). Further, we utilized a mild hypothermic condition, an effective process engineering intervention for production of DTE proteins (e.g., Estes et al., 2015; Johari et al., 2015) and to extend culture longevity (Figure 1A,B). As shown in Figure 1C, the qP of HEK cells increased 2.4-fold from 0.20 pg/cell/day to 0.48 pg/cell/day when the culture temperature was lowered from 37°C to 32°C. Additionally, the prolonged batch culture duration at 32°C enabled a 4.1-fold increase in titer, yielding 10.2 mg/L of purified spike. Greater enhancement was observed with CHO cells where mild hypothermia resulted in an 8.5-fold higher qP than that at 37°C, and a further increase in titer (10.9-fold, 5.4 mg/L) was obtained via increased cell accumulation. We anticipate that improved CHO systems (e.g., ExpiCHO-S cell line and ExpiCHO medium) as well as co-expression of genetic effectors and chemical chaperone addition (Cartwright et al., 2020; Johari et al., 2015) would significantly increase spike transient production in CHO cells. The CHO-derived spike exhibited a monomeric molecular mass of ~200 kDa by SDS-PAGE (Figure 1D) and a trimeric mass of ~670 kDa was measured using analytical size exclusion chromatography (Supplementary Figure S2). The material was further validated using peptide mapping in conjunction with mass spectrometry analysis (Supplementary Figure S3). Critically, the preliminary COVID-19 antibody serological test demonstrated its suitability for the ELISA (data not shown) thus permitting development of CHO stable production platform.

For DTE proteins, very low yielding transient expression systems can be an early indication of reduced stable production (Mason et al., 2012), where particular engineering strategies may be required to obtain stable cells with desirable production characteristics. To generate CHO cells stably expressing recombinant spike trimer, we

tested two in-house CHO synthetic promoters, namely 40RPU (~90% CMV activity) and 100RPU (~220% CMV activity) promoters (see Brown et al., 2017; Johari et al., 2019). Although the use of extremely strong promoters may be counterintuitive for DTE proteins, we reasoned that only those transfectants harboring a sub-UPR threshold productivity, and thus capable of proliferation would survive. Thus, if cell proliferation attenuation and apoptosis occur as ER functional capacity is exceeded, this is a condition that would directly deselect poorly performing stable transfectants. The promoters and spike gene were inserted into a vector construct encoding glutamine synthetase (driven by an SV40 promoter) and the electroporated CHO-S host cells were subjected to a single round of selection at 25 or 50 μM methionine sulfoximine (MSX), using suspension culture. After 19 days, recovered CHO cell populations were screened for the ability to produce spike in 3-day batch culture (Figure 2A). These data showed that transfectant pools derived from genetic constructs harboring the strong 100RPU promoter expressed recombinant spike whereas those using the weaker 40RPU promoter did not. More stringent selection conditions (50 μM MSX) yielded transfectant pools exhibiting lower productivity. Accordingly, CHO cell pools harboring the 100RPU promoter under 25 μM MSX were taken forward for the manufacturing process.

In order to rapidly produce recombinant spike, stable transfectant pools (rather than clonally derived populations) were employed. Based on CHO transient process data (Figure 1), we tested the hypothesis that an optimal 10-day fed-batch stable production process could be executed at 32°C and further enhanced by chemical chaperone additives (e.g., Johari et al., 2015). We compared this strategy to an alternative approach utilizing culture temperature shift to achieve maximal cell density (biphasic), as well as constant 37°C as control. These data are shown in

Figure 2B and C, whilst the screening data for eight small molecule chemical additives is shown in Supplementary Figure S4. Compared to the 37°C control culture, hypothermia resulted in a clear (33%) initial reduction in cell-specific proliferation rate over the first 5 days of culture (Figure 2B). However, the qP of the latter was 3.4-fold higher over control and addition of valproic acid (VPA) at Day 6 further enhanced qP 5.1-fold, yielding 51 mg/L of spike after purification by immobilized metal affinity chromatography (IMAC; Figure 2C). Similar enhancement was observed with betaine although there was no synergistic effect when the two molecules were utilized together. Reduction in culture temperature after 3 days culture improved the integral of viable cell density (IVCD) 1.4-fold and when combined with VPA addition at Day 4, 53 mg/L of spike was attained after IMAC purification. These data demonstrate that the optimal process engineering intervention for recombinant spike production identified for rapid transient gene expression was generally translatable to the stable production process. Further, as low-level, sub-UPR threshold expression is likely required to permit adequate cell growth (Supplementary Figure S1), we reasonably expect that application of mammalian inducible expression technology (e.g. cumate; Poulain et al., 2017) to switch on spike production using an intensified biphasic culture system would be particularly useful to maximize stable production.

To purify spike protein from culture supernatant, IMAC was initially performed using a step-elution of 250 mM imidazole according to Stadlbauer et al. (2020). Figure 3A shows SDS-PAGE of eluted proteins, and reveals the presence of protein impurities not derived from recombinant spike (Figure 3B), which were identified using tandem mass spectrometry as CHO host cell derived proteins (HCPs). Whilst all of the identified extracellular HCPs have previously been shown to be

present in CHO cell culture supernatant (Park et al., 2017), HSPG in particular has been reported to occur in CHO cells at relatively higher level than HEK cells (Goey et al., 2018; Lee et al., 2016), illustrating the need for an improved purification method especially at high IVCDs. In order to increase recombinant spike purity, a revised gradient elution profile up to 250 mM imidazole was implemented. As shown in Figure 3C, HCPs were eluted at a lower imidazole concentration than recombinant spike, permitting recovery of high purity (>95%) product for use in serological assays (Figure 3D).

COVID-19 antibody tests would help reveal the true scale of the pandemic in a population and the persistence of immunity, whether vaccines (many of which are based on the production of neutralizing antibodies against spike protein) designed to protect from infection are effective, as well as identify highly reactive human donors for convalescent plasma therapy. The CHO-spike anti-SARS-CoV-2 ELISA was developed based on the Krammer Laboratory's assay, and validated to ISO 15189 Medical Laboratories standards. Initially, we tested a panel of 234 negative samples taken pre-COVID-19 outbreak (June–August 2019) and 26 positive samples taken during the COVID-19 outbreak (≥15 days post-positive PCR test). ELISAs were performed by 1/20 dilution of the individual serum samples and the cut-off index of 1.4 was determined using the cut-off OD value (ROC curve with 100% specificity) and the negative control. In this particular evaluation, the assay had an overall specificity of 100% and sensitivity of 92.3% as illustrated in Figure 4A. To establish the reproducibility of the ELISA, positive samples were tested on 5 separate assays over 2 days at 3 different dilutions to determine the inter-assay variations. The data (Figure 4B) shows that the assay performed within the standard range for precision with inter-assay %CV of ≤5%. To be able to interpret serosurveys correctly, the

ELISA was evaluated for potential cross-reactivity from individuals with other medical conditions where zero positives were observed in all cases (Supplementary Table S1).

Overall, our work serves as an exemplar for a development process of characteristically difficult-to-express spike manufacturing platform utilizing CHO cells. This itself is a significant and useful finding, as many DTE recombinant proteins cannot be produced using this industry standard production vehicle — e.g., a recent study reported that for over 2,200 human genes encoding secreted proteins expressed in CHO cells, almost 50% did not yield target protein (Uhlen et al., 2018). On the other hand, the spike production in HEK cells was highly dependent on the very expensive Expi293 medium (we note that spike production using FreeStyle 293 medium resulted in an even lower titer (<40%); data not shown). Whilst it is potentially easier to produce a "monomer", the native trimeric structure of the spike protein on the surface of SARS-CoV-2 is rationally more desirable for optimal serological assay and research. Indeed, further modifications on trimeric spike's molecular architecture to improve its stability and production titers have recently been reported (Hsieh et al., 2020; Stuible et al., 2020; Xiong et al., 2020). Our data demonstrates how process and vector development could complement protein engineering efforts particularly where rapid product generation is required.

With one mg of spike providing serological assays for approximately 3,500 patient samples, the rapid, scalable transient platform was adequate for local population antibody tests and research studies. To enable large, constant clinical supply of spike, we showed that it was possible to generate CHO stable transfectants expressing the very complex glycoprotein, whilst high titers could be achieved via a

combination of process engineering approaches designed for both high qP and cell biomass accumulation. The refinement of the IMAC affinity purification process permitted greatly enhanced purity of the CHO spike product following an extended 10-day culture, ensuring suitability for use in serological immunity testing. The assay has been implemented at local hospitals with ~7,200 staff tested (as of 31 July 2020) which resulted in ~16% positive COVID-19 antibody detection, thus supporting the global effort to limit and mitigate the impact of SARS-CoV-2. Furthermore, it is highly likely that the cell and process engineering interventions designed for SARS-CoV-2 spike production is generically applicable to spike from different coronavirus strains.

MATERIALS AND METHODS

Cell Cultures and Chemical Chaperones

Expi293F cells were cultured in Expi293 Expression medium (ThermoFisher) in Erlenmeyer flasks maintained at 37°C, 125 rpm under 8% CO_2, 85% humidity. CHO-S clonal isolate cells (C1-80; Fernandez-Martell et al., 2018) were cultured in CD CHO medium (ThermoFisher) supplemented with 8 mM L-glutamine maintained at 37°C, 140 rpm under 5% CO_2, 85% humidity. Cells were seeded at 2×10^5 viable cells/mL and were sub-cultured every 3–4 days. Cell viability and VCD were measured using the Vi-CELL XR (Beckman Coulter). The IVCD was calculated as follows:

$$\mathrm{IVCD} = \left(\frac{\mathrm{VCD}_{t-1}+\mathrm{VCD}_t}{2} \times \Delta t\right) + \mathrm{IVCD}_{t-1} \qquad \text{(Eq. 1)}$$

where t is the time point (day). VPA, NaBu, DMSO, glycerol, betaine, TMAO and proline were obtained from Sigma while TUDCA was obtained from Merck.

Transient Production in HEK and CHO Cells

pCAGGS plasmids encoding the stabilized full-length SARS-CoV-2 spike protein trimer (polybasic furin cleavage site removed alongside K986P and V987P substitutions, P1213 addition of thrombin cleavage site, T4 trimerization domain and 6×His-tag; Amanat et al., 2020) or RBD were provided by the Krammer Laboratory (Icahn School of Medicine at Mount Sinai). The plasmids were amplified and purified using QIAGEN Plasmid *Plus* kit (Qiagen). For the optimized Expi293F transfection, cells were grown to 1.75×10^6 cells/mL, centrifuged and resuspended at a density of 3.5×10^6 cells/mL, followed by sequential addition of 0.85 µg of DNA and 2.55 µl of PEI MAX (each pre-diluted in 10 µL of 150 mM NaCl) per million cells. At 24 h post-transfection, the cells were diluted 2× by adding fresh medium, and where applicable culture was shifted to 32°C. For CHO transfection, cells were seeded one day before transfection and grown to 1.5×10^6 cells/mL. For every 1.5×10^6 cells, 1.3 µg of DNA and 4.55 µL of PEI MAX (each pre-diluted in 15 µL of 150 mM NaCl) were combined and incubated at RT for 2 min before being added into culture. Where applicable, culture was shifted to 32°C at 4 h post-transfection. For fed-batch production, 5% v/v CHO CD EfficientFeed B was added at Days 2, 4, 6 and 8.Generation of Stable CHO Pools and Fed-batch Production

A stable vector containing an SV40 promoter-driven GS gene was provided by AstraZeneca, UK. The spike gene was cloned by PCR, inserted into the vector downstream of 40RPU or 100RPU synthetic promoter (Brown et al., 2017) and the plasmid constructs were confirmed by DNA sequencing. 10×10^6 cells per cuvette were electroporated with 7 µg linearized DNA using Cell Line Nucleofector Kit V system (Lonza) and transferred to a TubeSpin containing 10 mL glutamine-free culture medium with the addition of 25 or 50 µM MSX after 48 h. The cells were left to recover under suspension conditions and recovered pools were cryopreserved when the cell viability reached >90%. For fed-batch production, 5% v/v CHO CD EfficientFeed B was added at Days 2, 4, 6 and 8.

Western Blotting

Proteins in culture supernatant were precipitated by TCA/DOC, resuspended in LDS loading buffer with BME and heated to 70°C. SDS-PAGE was performed using 4–

12% NuPAGE Bis-tris gels and resolved proteins were transferred to nitrocellulose membranes by iBlot system (ThermoFisher). Membranes were blocked in 5% milk/TBS-T before being incubated with HRP-conjugated anti-HisTag antibody (Bio-Rad) and visualized by enhanced chemiluminescence (ECL; ThermoFisher).

Recombinant Protein Purification and Quantification

Spike protein was harvested by centrifugation at 3,000×g for 20 min at 4°C and supernatant was filtered through a 0.22 μm filter. Protein was purified using the ÄKTA Pure system (Cytiva) and a 5-mL HisTrap HP column (Cytiva). The column was washed with 5 column volumes (CVs) of buffer B (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0), and equilibrated with 5 CVs of buffer A (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0). To reduce non-specific binding, the supernatant was adjusted to 20 mM imidazole using buffer B prior to sample loading. After sample loading, the column was washed in three steps using 5 CVs of buffer A, 5 CVs of 4.5% v/v buffer B, and 10 CVs of 9% v/v buffer B. Protein was eluted using 100% v/v buffer B. Eluted protein fractions were pooled and buffer exchanged into storage buffer (20 mM Tris, 200 mM NaCl, 10% v/v glycerol, pH 8.0) using a PD-10 desalting column (Cytiva). Protein was quantified using the Pierce Coomassie Plus (Bradford) Assay kit and bovine serum albumin for the calibration curve (ThermoFisher) and analyzed by reducing SDS-PAGE. An orthogonal quantification method was performed using an A280 measurement (NanoDrop One^{C}; ThermoFisher) with spike extinction coefficient of 428,255 M^{-1} cm^{-1} and M_W of 412.516 kDa. A complementary quantification of spike in culture supernatant was performed using CR3022 antibody ELISA (see below).

Protein Identification by Mass Spectrometry

All materials were supplied by ThermoFisher unless otherwise stated. Briefly, protein samples in 50 mM ammonium bicarbonate (ABC), 5 mM tris(2-carboxyethyl)phosphine-HCl were reduced by incubation at 37°C for 30 min. S-alkylation was performed by the addition of 1 µL 100 mM methyl methanethiosulfonate in isopropanol. For proteolytic digestion, 1.5 µL 0.2% ProteaseMax surfactant in 50 mM ABC and 2 µL 0.2g/L trypsin/endoproteinase Lys-C mixture (Promega) were added followed by incubation at 37°C for 16 h. Proteolysis was stopped and the surfactant hydrolyzed by the addition of 0.5% trifluoroacetic acid (TFA). The samples were desalted using HyperSep Hypercarb solid phase extraction tips and dried by vacuum centrifugation. For RPLC-MS, samples in 0.5% TFA, 3% acetonitrile (ACN) were injected. Peptides were separated using an RSLCnano system with a PepSwift PS-DVB monolithic column using a gradient from 97% solvent A (0.1% formic acid) to 35% solvent B (0.1% formic acid, 80% ACN). Mass spectra were acquired on a Q Exactive HF quadrupole-Orbitrap instrument, with automated data dependent switching between full-MS and tandem MS/MS scans. Proteins were identified by converting the MS data into Mascot Generic Format (MGF) files and analyzed against human and Chinese hamster reference proteome databases with the spike glycoprotein construct sequence inserted (www.uniprot.org) using Mascot Daemon v.2.5.1 with Mascot server v.2.5 (Matrix Science).

CR3022 Antibody ELISA for Spike Quantification

96-wells were coated overnight with 100 µL of anti-SARS-CoV spike CR3022 antibody (Absolute Antibody; 5 µg/mL in PBS) at 4°C. The coating solution was removed and washed twice (with 0.1% TBS-T). 100 µL of blocking solution (5% non-fat milk in 0.1% TBS-T) was added for 1 h and washed twice. 100 µl of sample

was added and incubated for 2 h at RT. A standard curve (Supplementary Figure S5) was generated from serially diluted, purified and quantified CHO spike (Figure 1D) using CD CHO medium as diluent. Plate was washed twice before incubation with 100 µl HRP-conjugated anti-HisTag antibody (Bio-Rad) diluted 1:500 with 5% milk/TBS-T for 2 h at RT. Plate was washed 3 times and 100 µl of SigmaFast OPD solution (Sigma) was added to each well. The reaction was allowed to proceed for 10 minutes at RT before being stopped by the addition of 50 µl of 3 M HCl. Plate was read at 492 nm using a SpectraMax iD5 plate reader (Molecular Devices).

Spike ELISA for Serological Testing

The ELISA protocol was adapted from Stadlbauer et al. (2020) using spike protein with >95% purity. Microtiter plates (96-well) were coated overnight with 50 µL of spike per well (2 µg/mL in PBS pH 7.4) at 4°C. The coating solution was removed and 300 µL of blocking solution (3% non-fat milk in 0.1% PBS-T) was added for 1 h and washed 3 times (with 0.1% PBS-T). Samples were added at 1/20 dilution and incubated for 2 h at RT. Plate was washed 3 times and 100 µL of anti-human IgG conjugate was added to the wells and incubated for 1 h at RT. Plate was washed 3 times and 100 µL of substrate was added and incubated in the dark for 45 minutes. The reaction was stopped by the addition of 50 of µl 3 M HCl and the plate was read at 490 nm using the Agility ELISA system (Dynex Technologies). The index value was calculated as follows:

$$\text{Index value} = \frac{\text{Sample OD}}{\text{Mean of negative controls}+3\text{SDs}} \quad \text{(Eq. 2)}$$

The cut-off value was calculated with 100% specificity using ROC curves of calculated index values.

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LIST OF FIGURES

Figure 1. Transient production of recombinant spike in HEK and CHO cells. HEK Expi293F cells and CHO-S cells were transfected with plasmids encoding spike gene using PEI under optimized conditions and cultured at 37°C or 32°C. CHO cultures were fed every two days with 5% v/v EfficientFeed B. (A) Viable cell density and (B) cell viability post-transfection (>70%). (C) IVCD, recombinant qP and purified spike

titer, quantified using Bradford assay. We note that quantification using the A280 method produced ~25% higher titers. (D) Coomassie-stained reducing SDS-PAGE gel of purified HEK and CHO cell-derived spike (~200 kDa) based on the improved imidazole gradient elution method.

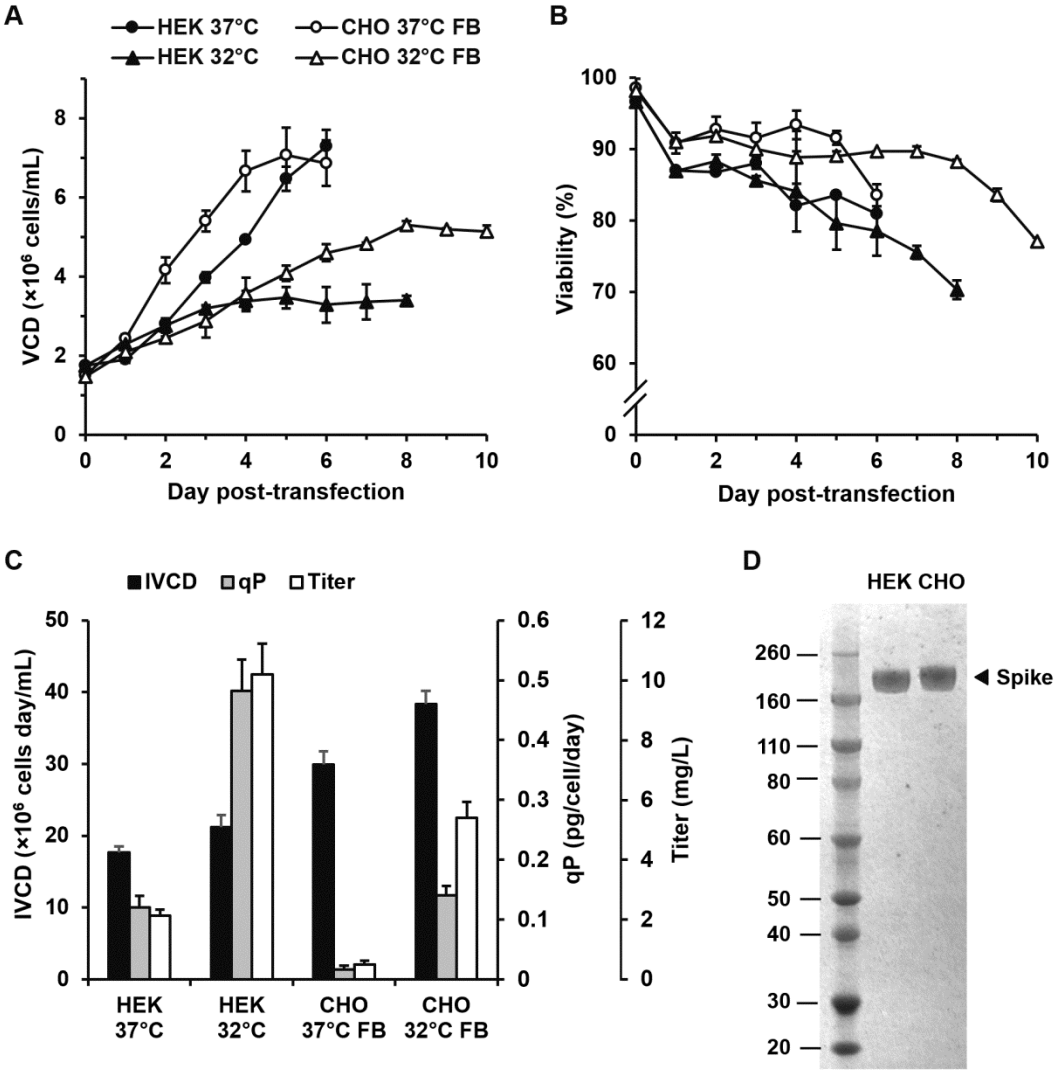

Figure 2. Development of a stable production platform for SARS-CoV-2 spike in CHO cells. (A) Generation and analysis of CHO stable transfectant pools expressing recombinant spike under the control of synthetic promoters. CHO-S cells were electroporated in duplicate with plasmids containing a GS gene driven by an SV40 promoter and a spike gene driven by either a 40RPU or 100RPU synthetic promoter, followed by selection in glutamine-free media containing 25 μM or 50 μM MSX under suspension condition. Recovered cell pools were assessed for their ability to express spike in a 3-days batch culture by Western blot. Figure shown is a representative Western blot of two technical replicates. (B) Cells from the best performing pools in A were inoculated and cultured at 37°C, 32°C, or 37°C with a shift to 32°C at Day 3. Cultures were fed every two days with 5% v/v EfficientFeed B. (C) Comparison of the fed-batch culture production performance without or with a chemical addition (chemical screening data is shown in Supplementary Figure S4). 1 mM VPA and/or 12.5 mM betaine were added at Day 4 for the biphasic cultures or at Day 6 for the 32°C cultures. Purified spike titer was quantified using Bradford assay. Data are normalized with respect to culture at 37°C without any chemical addition. Data shown are the mean value ± standard deviation of two independently generated stable pools each performed in duplicate.

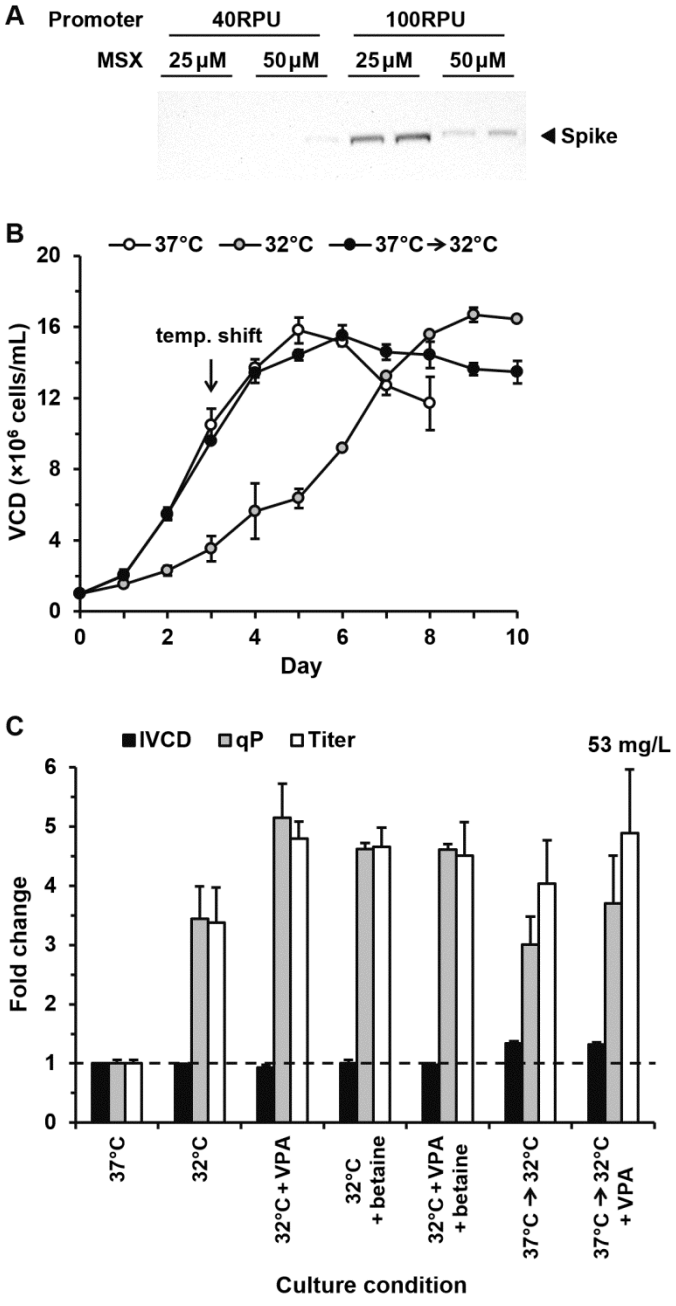

Figure 3. Optimization of affinity chromatography purification strategies for spike protein using HisTrap columns. (A) Coomassie stained gel of the initial purification strategy of spike utilizing the method from Stadlbauer et al. (2020) with associated impurities identified using tandem mass spectrometry. (B) Assessment of spike sample shown in A by Western Blot. (C) Gradient elution of spike protein starting from 10 mM imidazole up to a final concentration of 250 mM imidazole. Lanes 1–6: 115 mM, 125 mM, 135 mM, 145 mM, 180 mM and 190 mM imidazole, respectively. (D) Purified spike from optimized step elution affinity chromatography.

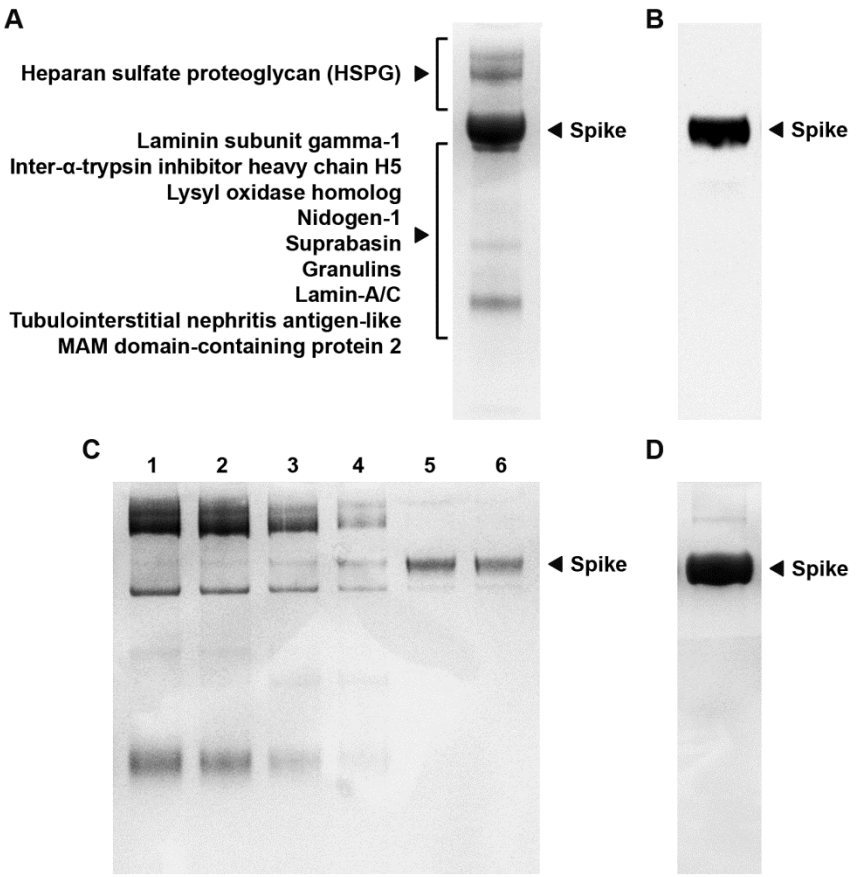

Figure 4. Evaluation of CHO-spike anti-SARS-CoV-2 ELISA. (A) 234 negative serum samples (taken pre-COVID-19 outbreak) and 26 positive serum samples (taken ≥15 days post-positive PCR test) were used to evaluate the assay performance, yielding an overall sensitivity of 92.3% anti-SARS-CoV-2 antibodies. (B) To determine the assay precision, one serum sample was assayed in triplicates at five separate times over two days (n = 15).