Expression and Purification of SARS-related Spike Glycoproteins for Cryo-EM Analysis

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Published in the Microbiology section

Coronaviridae spike glycoproteins mediate viral entry and fusion to host cells through binding to host receptors (i.e., ACE2, DPP4) and are key components in determining viral host range, making them targets for antiviral research. Here, we describe the expression, purification, and characterization of recombinant spike proteins to aid in protein characterization and analysis. These protocols were used for the production of spike glycoproteins from civet, pangolin, and bat coronaviruses, as well as high-resolution cryo-electron microscopy (cryo-EM) structural analysis of bat and civet host coronavirus spike glycoproteins (Hills et al., 2024). © 2025 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Expression and purification of SARS-CoV spike protein from ExpiCHO cells
Basic Protocol 2: Preparation of SARS-CoV spike protein for visualization by negative-stain transmission electron microscopy and cryo-electron microscopy

Keywords: coronaviruses • cryo-electron microscopy • protein expression • protein purification • spike protein

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How to cite this article:
Hills, F. R., Jorge, F., Burga, L. N., & Bostina, M. (2025). Expression and purification of SARS-related spike glycoproteins for cryo-EM analysis. *Current Protocols*, *5,* e70115. doi: 10.1002/cpz1.70115

INTRODUCTION

The structural analysis of spike (S) glycoproteins from animal coronaviruses is essential to fully understand human coronavirus pathogenesis and evolution (Li, 2016). Both SARS-CoV and SARS-CoV-2 hail from the sarbecovirus subgenus, within which the only S proteins that have been structurally investigated are those from three nonhuman hosts: bats, civets, and pangolins (Schoch et al., 2020). Even in these hosts, only 11 S proteins have been structurally characterized (Buchanan et al., 2022; Hills et al., 2024; Lee et al., 2023; Ou et al., 2023; Qiao & Wang, 2024; Toelzer et al., 2022; Wrobel et al.,

CURRENT PROTOCOLS
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Current Protocols e70115, Volume 5
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.70115

2021; Xiong et al., 2022; Zhang et al., 2021). This lack of structural understanding of animal coronaviruses makes it more difficult to track the evolution of human coronaviruses, predict the emergence of zoonotic diseases, and develop pan-sarbecovirus neutralizing treatments.

To date, all high-resolution full ectodomain S protein structures have been solved through cryo-electron microscopy (cryo-EM; Buchanan et al., 2022; Hills et al., 2024; Lee et al., 2023; Ou et al., 2023; Qiao & Wang, 2024; Toelzer et al., 2022; Wrapp et al., 2020; Wrobel et al., 2021; Xiong et al., 2022; Zhang et al., 2021). Cryo-EM is a microscopy imaging technique used to visualize and analyze the three-dimensional structure of molecules and macromolecular complexes to near-atomic resolution (Chua et al., 2022). Cryo-EM requires the preparation of a highly pure protein sample that is vitrified in a thin amorphous layer of ice in multiple orientations so that it can be visualized in a three-dimensional near-native state. Successful high-resolution data collection through cryo-EM is highly protein dependent. However, once protein expression, purification, sample preparation, and microscopy have been specifically optimized, cryo-EM single particle analysis offers a reliable technique for producing high-quality S protein reconstruction.

Here, we provide detailed protocols for these workflows, compiled and optimized for the ectodomain portion of the sarbecovirus S glycoprotein. Optimization was performed on spike proteins from multiple animal hosts, including civet, pangolin, and bat, resulting in the successful production of novel S protein structures.

This article contains two basic protocols and their most common troubleshooting solutions. In Basic Protocol 1, we describe the expression and purification of S protein from ExpiCHO-S cells. This includes detailed descriptions of the plasmid design and preparation, S protein expression in ExpiCHO-S cells, and methods for the confirmation of protein expression, protein purification, buffer exchange, and concentration. Basic Protocol 2 outlines protein and grid preparation for microscopy visualization, including negative-stain grid preparation and analysis and cryo-electron microscopy grid preparation.

CAUTION: All protocols must be performed with appropriate PPE, including protective clothing (i.e., laboratory coat, closed-toe shoes) and reagent-impermeable gloves. Specific protocols call for the use of a fume hood, laminar flow cabinet, or eye protection. Where necessary, this is noted with a caution at the beginning of the protocol.

BASIC PROTOCOL 1

EXPRESSION AND PURIFICATION OF SARS-CoV2 SPIKE PROTEIN FROM ExpiCHO CELLS

The successful expression and purification of spike glycoproteins from ExpiCHO cells is a multi-step process beginning with plasmid design and bacterial transformation. These steps are crucial for achieving efficient protein expression in ExpiCHO cells by ensuring proper protein folding, the incorporation of a tag for purification and visualization, and sufficient levels of plasmid for cell transfection. Transfection of ExpiCHO cells, when performed correctly, should in soluble protein expressed in the cell supernatant, which can be purified through binding Ni-NTA beads and passing through a flow column. The purified fractions can be analyzed for protein via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by either immunostaining (western blotting) or Coomassie brilliant blue staining. Pure protein fractions can then be buffer exchanged and concentrated as desired for structural visualization through both negative-stain transmission electron microscopy (TEM) and cryo-EM.

Plasmid design

An efficient plasmid design was established by Tortorici et al. (2020) for the production of trimeric prefusion spike ectodomains. Spike glycoprotein gene sequences can be obtained from GenBank (Sayers et al., 2022) and should be codon-optimized for

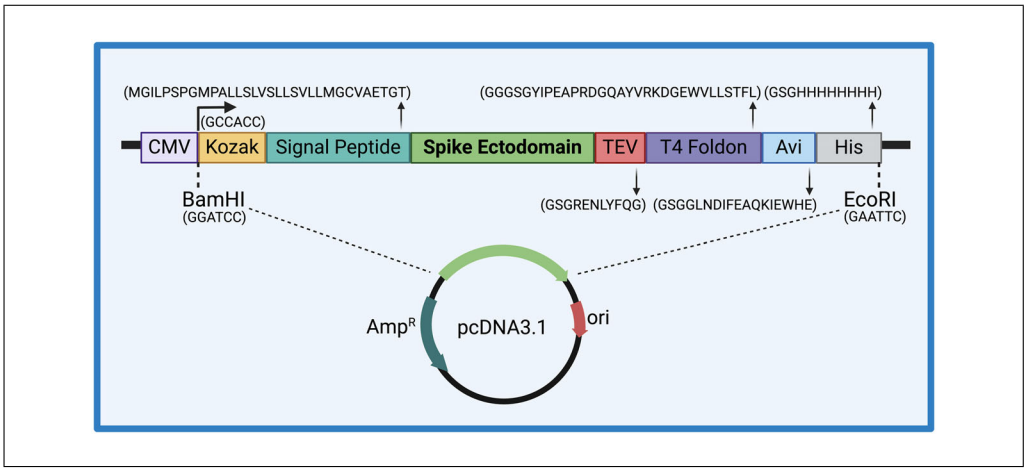

Figure 1 Schematic of appropriate plasmid design containing the spike ectodomain insert and 5′/3′ flanking sequences.

mammalian cells to ensure efficient protein expression. Codon optimization is available through plasmid ordering services such as GenScript (GenScript, 2024) and Twist-Bioscience (TwistBioscience, 2024). When designing plasmids for spike glycoprotein expression, gene inserts (spike ectodomain sequences) should begin after a ubiquitously observed hydrophobic N-terminal signal peptide sequence and end before the similarly ubiquitous transmembrane domain. These features can be identified using web-based servers such as SignalP (Teufel et al., 2022) and DeepTMHMM (Hallgren et al., 2022). An N-terminal 5′ flanking extension should also be added containing a BamHI restriction site (GGATCC), a Kozak consensus sequence (GCCACC), and a μ-phosphatase-derived signal peptide (MGILPSPGMPALLSLVSLLSVLLMGCVAETGT) to ensure extracellular protein secretion (Fig. 1). Additionally, a C-terminal 3′ flanking extension should be added containing: a TEV cleavage site (GSGRENLYFQG), a T4 foldon trimerization motif (GGGSGYIPEAPRDGQAYVRKDGEWVLLSTFL), an Avi tag (GSGGLNDIFEAQKIEWHE), an 8× His tag (GSGHHHHHHHH), a stop codon (TAA), and an EcoRI restriction site (GAATTC) (Fig. 1). Inserts should be ordered in a pcDNA3.1+ or equivalent plasmid manufactured to transfection grade (90% ± 5% supercoiled, ≤0.1 EU/μg endotoxin). Upon arrival from the supplier, the plasmids should be aliquoted into appropriate volumes for required usage and stored per the manufacturer's instructions.

Bacterial transformation

Plasmid DNA can be amplified and purified using standard bacterial transformation methods (Fig. 2). We use the competent cell line *Escherichia coli* DH5α for plasmid amplification. Plasmid purification is performed with Qiagen minprep and midiprep kits as per the manufacturer's instructions (Qiagen, 2016), eluting into TE buffer for long-term storage. The steps below follow the step-by-step instructions provided by Addgene (Addgene, 2024).

Materials

LB agar plates (see recipe)
LB broth (see recipe)
Electrocompetent E. coli cells (e.g., DH5α)
pCDNA3.1 plasmid containing the SARS-CoV spike ectodomain of interest
TE buffer (see recipe)
Glycerol (Thermo Fisher, cat no. A16205.0F)
Qiagen miniprep kit (Qiagen, cat. no. 12123)

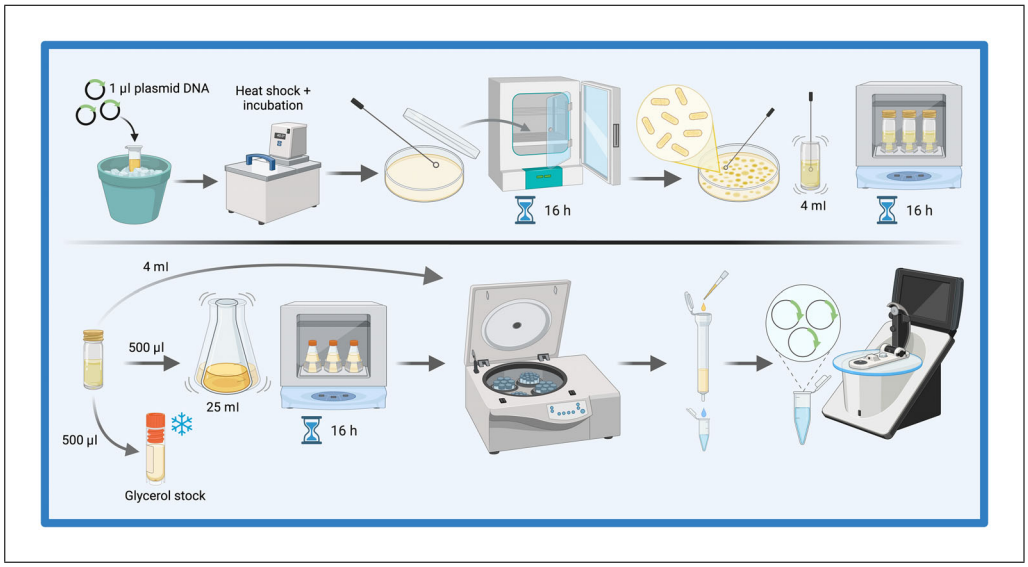

Figure 2 Methodology for bacterial transformation and purification of plasmid DNA. Plasmid DNA is added to *E. coli* DH5α and incubated before heat shock. Heat-shocked *E. coli* transformed with plasmid DNA are streaked onto an agar plate and incubated overnight. A single colony is collected from the overnight agar plate and added to LB broth, which is placed in a shaking incubator overnight. Part of the overnight culture is used to inoculate larger flasks for another overnight incubation and a glycerol stock, while the rest undergoes centrifugation, with the supernatant being harvested for a plasmid miniprep. The following day, the large overnight culture undergoes centrifugation, and the supernatant is harvested for a plasmid midiprep.

Qiagen midiprep kit (Qiagen, cat. no. 12143)
ExpiCHO Expression system (Thermo Fisher, cat. no. A29133)
Dimethylsulfoxide (DMSO; Sigma Aldrich, CAS no. 67-68-5)
OptiPRO SFM (Thermo Fisher, cat. no. 12309050)
8% SDS-PAGE gel (see recipe)
Tris-glycine running buffer (see recipe)
Sample loading buffer (see recipe)
Precision plus protein standards (Bio-Rad, cat no. 161-0374)
Cathode buffer (see recipe)
Anode buffer I (see recipe)
Anode buffer II (see recipe)
Nitrocellulose membrane (Bio-Rad, cat. no. 1620097)
Coomassie stain and destain (see recipes)
Blocking buffer (see recipe)
Primary antibody: 6× His tag monoclonal antibody (Thermo Fisher, cat. no. MA1-21315)
PBST (see recipe)
Secondary antibody: Goat anti-mouse IgG Dylight 800 (Thermo Fisher, cat. no. SA5-35521)
Ni-NTA agarose (Qiagen, cat. no. 30210)
Wash buffer (see recipe)
Elution buffer (see recipe)
Liquid nitrogen
Exchange buffer (see recipe)
RIPA buffer (Thermo Fisher, cat. no. J63306.AP)

Water bath, set to 42°C
Shaking incubator, 37°C
Laminar flow hood

Eppendorf 5810 R centrifuge equipped with S-A-462 rotor (Eppendorf)
Sorvall Lynx 6000 with F12-6 × 500 lex and F14-6 × 250y rotors (Thermo Fisher)
Avanti J-26 XPI equipped with F250 rotor (Beckman Coulter)
Tissue culture incubator with shaking, 37°C, 8% CO_2, ≥80% humidity
NanoDrop spectrophotometer or equivalent
125-ml Erlenmeyer screw-top flasks (Thermo Fisher)
Luna II automated cell counter (Logos Biosystems)
Cryovials
Controlled freezing system (e.g., Mr. Frosty Freezing Container)
Mini-PROTEAN Tetra cell kit (Bio-Rad)
Gel imager: e.g., GelDoc (Bio-Rad)
Trans-Blot Turbo Transfer system (Bio-Rad, cat. no. 1704150)
Affinity gravity column (Thermo Scientific, cat. no. 89898)
50,000-MWCO centrifugal concentrator column: e.g., Amicon Ultra-4 Centrifugal Filter Unit (Merck Millipore, cat. no. UFC905024)
Sorvall RT 7 centrifuge equipped with RTH-250 rotor (Sorvall)
Pico 21 microcentrifuge with 24 × 1.5/2.0 ml rotor

Before beginning

1. Prepare LB agar plates with 100 µg/ml ampicillin (see recipe).
2. Prepare liquid LB medium (LB broth; see recipe).

Day 1

3. Take electrocompetent *E. coli* cells out of –80°C freezer and thaw on ice (~20-30 min).
4. Remove LB agar plates (containing the appropriate antibiotics) from storage at 4°C and let them reach room temperature before placing them in a 37°C incubator.
5. Add 1 µl of 100 ng pCDNA3.1 plasmid containing the spike ectodomain into 50 µl of competent cells and mix by flicking the bottom of the tube a few times.
6. Incubate the competent cell/plasmid DNA mixture on ice for 20 min.
7. Heat-shock each transformation tube by submerging the bottom half to one-third of the tube in a 42°C water bath for 45 s.
8. Put the tubes back on ice for 2 min.
9. Add 500 µl liquid LB medium (without antibiotics) to the bacteria and place in a 37°C shaking incubator at 200 rpm for 45 min.
10. Place different volumes (50, 100, and 250 µl) of this transformation mixture onto 100-mm LB agar plates containing ampicillin and perform a streak plate.
11. Incubate plates at 37°C overnight.

Day 2

12. Using a sterile pipette tip or toothpick, select a single colony from the LB agar plate and drop the tip or toothpick into the liquid LB.
13. Loosely cover the culture with sterile aluminum foil or a cap that is not airtight.
14. Incubate bacterial culture at 37°C for 12-18 hr in a shaking incubator at 200 rpm.

Day 3

15. Check culture for growth, which is characterized by a cloudy haze in the medium.
16. Prepare glycerol stock solutions of bacteria by mixing 500 µl bacterial culture and 500 µl of 50% glycerol, and store these at –80°C.

17. Isolate plasmid DNA using the Qiagen miniprep kit as per manufacturer's instructions.

18. Redissolve plasmid DNA in TE buffer and determine plasmid concentration using a NanoDrop spectrophotometer or equivalent equipment.

19. Take 500 µl of the culture from step 15 and inoculate into 25 ml LB medium in a 125-ml flask. Incubate bacterial culture at 37°C for 12-18 hr in a shaking incubator at 200 rpm.

Day 4

20. Isolate plasmid DNA using a Qiagen midiprep kit as per manufacturer's instructions.

21. Redissolve plasmid DNA in TE buffer and determine plasmid concentration using a NanoDrop or equivalent.

22. In preparation for transfection into ExpiCHO cells, prepare a 20-µl aliquot of plasmid DNA at 1 µg/µl concentration in TE buffer and store it at –80°C.

Spike protein expression in ExpiCHO-S cells

For full methodology of seeding, propagating, and storing the ExpiCHO-S cell, see the detailed Thermo Fisher handbook (ThermoFisher, 2018). The steps below provide a brief description.

CAUTION: *All cell handling should be performed in a laminar flow cabinet to avoid contamination*.

CAUTION: *Frozen cell stocks may contain DMSO, which is a hazardous chemical. Extra caution, including the use of safety goggles, should be used when handling solutions containing DMSO. For further safety information, see the Sigma Aldrich Safety Data Sheet. Liquid nitrogen can cause significant injury when handled improperly, including frostbite, asphyxiation, and explosion. Appropriate training is required when handling and storing liquid nitrogen*.

Thawing

23. Remove the cryovial containing ExpiCHO-S cells from liquid nitrogen and thaw quickly for 1-2 min in a 37°C water bath.

24. Before cells have completely thawed, decontaminate and transfer the cryovial to a sterile laminar flow hood.

    If frozen cells contain DMSO, you will need to perform step 25b before incubation; otherwise, follow step 25a.

25a. *When no DMSO is present:* Transfer the cells from the cryovial to a labeled 125-ml screw-top Erlenmeyer flask containing 30 ml prewarmed (37°C) ExpiCHO expression medium.

    It is important that your shaker flask be of the correct diameter and sterile, and have a vented cap or loosened lid (for specific information, see Thermo Fisher handbook).

25b. *When working with frozen cells that contained DMSO:* Transfer the cells from the cryovial to a 15-ml Falcon tube containing 10 ml prewarmed ExpiCHO expression medium. Centrifuge tube for 5 min at 450 × *g,* room temperature, using an Eppendorf 5810R centrifuge, S-4-104 rotor, or equivalent. Discard the supernatant, resuspend cells in 10 ml prewarmed ExpiCHO expression medium, and transfer the entire solution to a shaker flask containing 20 ml prewarmed ExpiCHO expression medium.

26. Incubate the cells in a 37°C, 8% CO_2, ≥80% humidity shaking incubator at 120 rpm.

    Shake speed will vary depending on your specific flask diameter (for information, see Thermo Fisher handbook).

27. Three days after thawing the cells, check cell viability and density. Viability should be ≥90% for cells frozen correctly. Once the culture has reached between 4×10^6 and 6×10^6 viable cells/ml (typically 3-4 days after thawing), subculture the cells.

Subculture

The subculture method described in steps 28-32 differs from that detailed in the ExpiCHO Expression System handbook; you may wish to follow the handbook method first before trialling the following method.

28. In a laminar flow hood, transfer the incubated culture to a 50-ml Falcon tube, set aside a 10-µl aliquot, and centrifuge the 50-ml tube for 5 min at 450 × *g*, room temperature, using an Eppendorf centrifuge 5810R, S-4-104 rotor, or equivalent.
29. While the main culture is in the centrifuge, determine the viable cell density and use the results to calculate the number of cells required to seed a new flask, depending on when you wish to subculture them again. Generally, for a 125-ml flask containing 30 ml of culture, seeding at a density of 2×10^5 cells/ml will require the cells to be subcultured again in 3 days.
30. Discard supernatant from the 50-ml tube and resuspend the pellet in 10 ml prewarmed ExpiCHO expression medium.
31. Transfer the calculated volume of cells required to a fresh, sterile flask containing prewarmed ExpiCHO expression medium to a total volume of 30 ml.
32. Place flasks back in the 37°C, 8% CO_2, ≥80% humidity shaking incubator at 120 rpm, counting cells periodically until it reaches 4×10^6 to 6×10^6 cells/ml.

Freezing

33. Use cells grown to between 4×10^6 and 6×10^6 cells/ml with cell viability of 95%.
34. Centrifuge the culture for 5 min at 450 × *g,* room temperature, using an Eppendorf centrifuge 5810R, S-4-104 rotor, or equivalent, and then discard supernatant.
35. Resuspend the pellet with cold ExpiCHO expression medium containing 10% DMSO to a viable cell concentration of 1×10^7 cells/ml.
36. Aliquot 1 ml of cells into each cryovial and freeze cells in a controlled freezing system (e.g., Mr. Frosty Freezing Container).
37. Transfer frozen cryovials to liquid nitrogen for long-term storage.

Spike protein expression

For full methodology of the ExpiCHO Expression System, including troubleshooting, see the detailed Thermo Fisher handbook (ThermoFisher, 2018). A brief description of the specific protocol we followed for the expression of multiple spike glycoproteins follows (Fig. 3). ExpiCHO-S cells were maintained at 37°C in 8% CO_2 at ≥80% relative humidity and were not transfected until at least two passages post-thaw.

Day 1

38. Once the ExpiCHO-S culture is between 4×10^6 and 6×10^6 cells/ml with cell viability ≥90% (step 32), split the culture to a viable cell density of 4×10^6 cells/ml and incubate overnight.

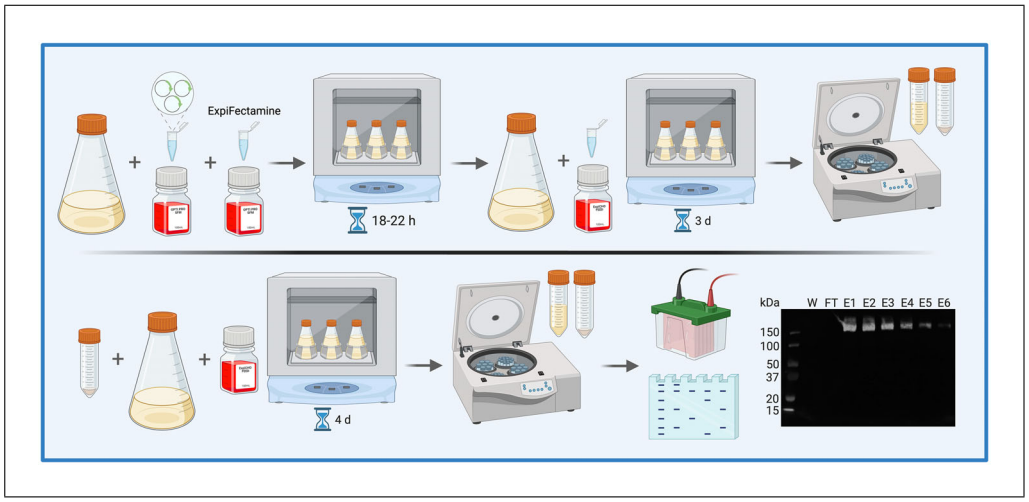

Figure 3 Methodology of the ExpiCHO expression system. OptiPRO diluted plasmid DNA and Expifectamine are added to the ExpiCHO cell culture and incubated overnight. Transfection reagent and Feed are added to the transfected ExpiCHO cells, which are incubated for a further 3 days. The supernatant is harvested, and ExpiCHO cells are replenished with fresh medium and Feed and incubated for a further 4 days. The supernatant is harvested and assessed for target protein through SDS-PAGE and western blot. Image at bottom right is a western blot image of bat coronavirus spike glycoprotein taken on an Odyssey Fc imaging system with channel 800.

Day 2

39. Check cell density has reached $\geq 7 \times 10^6$ cells/ml with cell viability $\geq 90\%$, and dilute the cells to 6×10^6 viable cells/ml with prewarmed ExpiCHO expression medium.
40. Take 25 ml diluted ExpiCHO culture, place in a prewarmed 125-ml Erlenmeyer shaker flask, and place back in the incubator while preparing the plasmid/ExpiFectamine complexes.
41. Add 20 µl plasmid DNA (1 µg/µl) from step 22 to 1 ml OptiPRO SFM in a microcentrifuge tube.
42. Add 80 µl ExpiFectamine CHO reagent (from ExpiCHO Expression System) to 920 µl of OptiPRO SFM in a microcentrifuge tube.
43. Add the diluted ExpiFectamine to the diluted plasmid DNA and invert the tube 4-5 times to mix. Incubate at room temperature for 5 min.
44. Add the ExpiFectamine/plasmid DNA complex to the 25 ml of ExpiCHO flask culture prepared in step 40, while gently swirling the flask.
45. Place the cells back in the incubator at 37°C, 8% CO_2, $\geq 80\%$ relative humidity.

Day 3

46. At 18-22 hr after infection, add 150 µl ExpiCHO enhancer and 4 ml ExpiCHO Feed (from ExpiCHO Expression System) to the flask of transfected ExpiCHO cells. Gently swirl the flask to mix. Enhancer and Feed do not have to be prewarmed before adding, but return the flask to the incubator immediately after adding the reagents.

Day 6

47. Harvest the supernatant by placing the culture in a 50-ml Falcon tube and centrifuge for 5 min at $450 \times g$, room temperature, using an Eppendorf 5810R centrifuge, S-4-104 rotor, or equivalent.
48. Check the cell viability, which should remain $>70\%$ for optimal protein expression. If it is $>70\%$, continue with the following steps; if $<70\%$, discard the cells and thaw

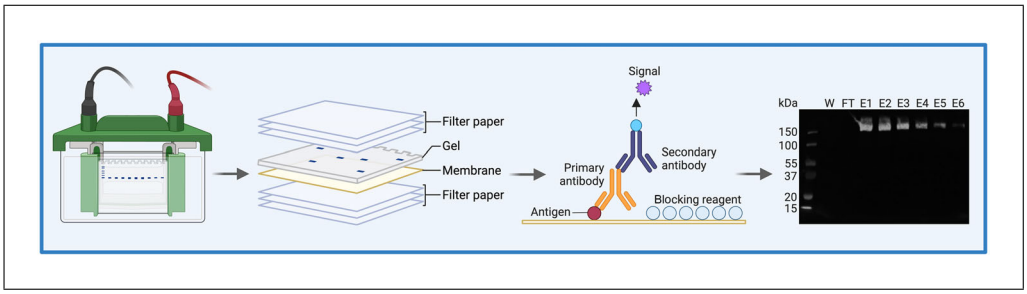

Figure 4 SDS-PAGE, membrane transfer, and western blotting. SDS-PAGE of the ExpiCHO supernatant is run before semi-dry membrane transfer. The membrane is incubated with a blocking reagent, primary antibody, and secondary antibody in that order before western blot visualization. Image at bottom right is a western blot image of bat coronavirus spike glycoprotein taken on an Odyssey Fc imaging system with channel 800.

a fresh batch for future protein expression. Check the supernatant for target protein expression.

Note that the protein yield may be low due to poor cell viability.

49. Keep the supernatant for confirmation protein expression in steps 55-59.
50. Resuspend the cell pellet with 25 ml prewarmed ExpiCHO expression medium and place it in a 125-ml Erlenmeyer shaker flask.
51. Add 4 ml ExpiCHO Feed to the flask and return to the incubator at 37°C, 8% CO_2, ≥80% relative humidity.

Day 10

52. Harvest the supernatant by placing the culture in a 50-ml Falcon tube and centrifuge for 5 min at 450 × *g*, room temperature, using an Eppendorf 5810R centrifuge, S-4-104 rotor, or equivalent.
53. Check the cell viability, which should still be >70% for optimal protein expression.
54. Keep the supernatant for confirmation of protein expression in steps 55-59; discard used cells.

Confirmation of spike protein expression

Confirm protein expression by SDS-PAGE and western blotting, probing for the His-tagged protein with an anti-His primary antibody, before proceeding with protein purification (Fig. 4). The most reliable results are seen when running a precast 4%-12% gradient protein gel. However, consistent results were produced with hand-cast 8% acrylamide gels containing stacking and resolving regions, with Tris-glycine running buffer (see recipe in the Reagents and Solutions section).

SDS-PAGE

55. Add 50 µl of ExpiCHO expression supernatant to a microcentrifuge tube.

    CAUTION: *2-Mercaptoethanol and sodium dodecyl sulfate are hazardous chemicals and toxic if inhaled, swallowed, or in contact with skin. Stock bottles should only be opened in a fume hood with reagent-impermeable gloves. For further information, see Bio-Rad Safety Data Sheet for 2-mercaptoethanol and ThermoFisher Safety Data Sheet for sodium dodecyl sulfate.*

56. Add 2 µl of 2-mercaptoethanol to 180 µl of 4× sample loading buffer (see recipe for buffer).
57. Add 20 µl of the loading buffer complex to the ExpiCHO sample and boil at 95°C for 5 min.

58. Take 20 µl of boiled sample and place it into the SDS-PAGE well. Store the remaining sample at –20°C for later use, if needed. Include the appropriate ladder for western blot visualization and positive His-tag control.

59. Run the gel at 80 V for 15 min until samples have stacked, and then run at 180 V for a further 60 min.

SDS-PAGE transfer

60. Remove the gel from the GelDoc and its glass case, and place it in a box containing just enough cathode buffer to cover the gel. Let stand for 10 min to remove SDS.

61. Prepare six pieces of filter paper the size of the gel; place three in a box containing cathode buffer, two in anode buffer I, and one in anode buffer II. There should be enough of each buffer in each box to soak the filter paper fully.

62. Prepare a piece of nitrocellulose membrane the size of the gel and place in cathode buffer so that it is fully submerged. Only handle the membrane very carefully with tweezers.

63. Using a semi-dry blot cassette, place the two pieces of filter paper from the anode buffer I down first, followed by the piece of filter paper soaked in anode buffer II, the nitrocellulose membrane, gel, and finally the three pieces of filter paper soaked in cathode buffer.

64. Using a roller and applying medium pressure, roll over the transfer sandwich to make sure that no air bubbles are trapped within it.

65. Place the lid on the cassette and run the transfer for 15 min at a voltage of 25 V.

66. Remove the membrane from the cassette to perform a western blot or Coomassie stain, discard the gel and filter papers, and clean the cassette.

Western blotting

67. Place the membrane in a box containing blocking buffer and place on a shaker at 200 rpm for 1 hr.

68. After removing the blocking buffer, add the primary antibody diluted 1/5,000 with PBST.

69. Shake on a shaker at 200 rpm for 1 hr at 4°C (this incubation time can be increased to overnight).

70. Remove the primary antibody solution. Add enough PBST to cover the membrane, allow to wash on a shaker at 200 rpm for 10 min, and remove the PBST. Repeat two more times.

71. After removing the PBST from the final wash, add the secondary antibody diluted to 1/20,000 with PBST.

72. Shake on a shaker at 200 rpm for 1 hr at 4°C.

73. Remove the secondary antibody solution and wash the membrane again as described in step 70.

74. After washing, the western blot is ready to be visualized.

Spike protein purification

His-tagged coronavirus spike proteins have been purified by immobilized affinity chromatography with Ni-NTA agarose using a gravity flow column (Fig. 5). Protein can be eluted from the column and assessed for continued protein presence and sample purity by SDS-PAGE and western blotting as described (Fig. 4; see steps 55-59). Before beginning

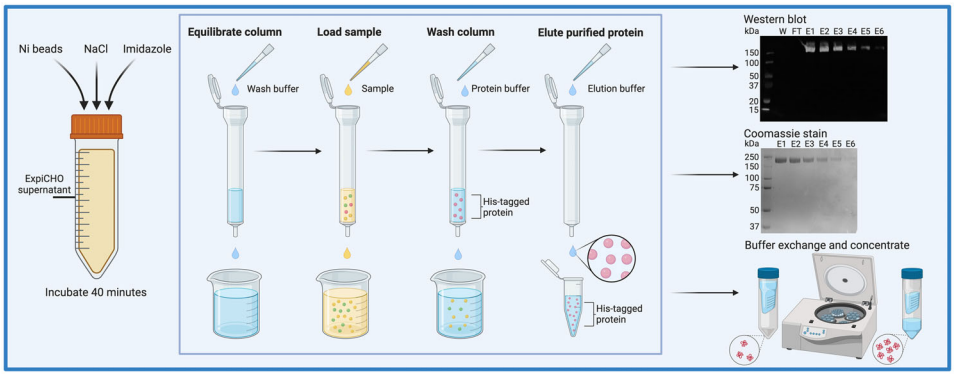

Figure 5 Purification of spike glycoproteins using nickel beads and a gravity-flow column. Nickel beads, NaCl, and imidazole are added to the ExpiCHO supernatant containing the His-tagged target protein and incubated at 4°C for 40 min. The gravity-flow column is equilibrated, loaded with sample, and washed before the target protein is eluted from the beads using competitive imidazole binding. The purified protein sample is then visualized through western blotting and Coomassie staining to confirm protein presence and purity before buffer exchange and concentration. Images at right are a western blot image of bat coronavirus spike glycoprotein taken on an Odyssey Fc imaging system with channel 800 and a Coomassie stain image of bat coronavirus spike glycoprotein taken on ChemiDoc Go imaging system.

the purification, prepare the wash, elution, and exchange buffers from sterile stocks (see recipes).

75. Add 1 ml Ni-NTA agarose resin (500 µl Ni beads) to a microcentrifuge tube and centrifuge for 1 min in a benchtop centrifuge at 4000 × *g*, room temperature, using a Pico 21 microcentrifuge with 24 × 1.5/2.0 Pico 21 microcentrifuge with 24 × 1.5/2.0 ml rotorml rotor. Once the beads have been separated from the storage solution, aspirate the solution.
76. Add 1 ml wash buffer, centrifuge again as in step 75, and decant wash buffer. Repeat twice more and then add the beads to the ExpiCHO expression supernatant from step 54.
77. Add 150 mM NaCl and 10 mM imidazole to the ExpiCHO supernatant and rotate tube for 40 min at 4°C (this can be increased to 2 hr if needed). If using 5 M NaCl and imidazole stocks, use 15 µl of NaCl and 10 µl imidazole per milliliter of supernatant instead.

    CAUTION: *Imidazole is toxic if inhaled, swallowed, or in contact with skin. If making imidazole solution from powder stock, the bottle should only be opened in a fume hood with reagent-impermeable gloves. For further information, see Sigma-Aldrich Safety Data Sheet.*

78. Equilibrate the gravity flow column by adding 3 ml wash buffer and allowing it to flow through.
79. Add the sample supernatant containing Ni-NTA beads to the column. Collect all fractions, including the flowthrough, in case protein flows off the column prior to elution.
80. Stop the column with the provided stopper, add 500 µl elution buffer, and incubate for 3 min.
81. Remove the stopper and collect the eluate in a microcentrifuge tube.
82. Repeat the elution process eight times.
83. Take 50-µl aliquots of each elution for SDS-PAGE.

84. If performing buffer exchange on the same day, keep fractions on ice. If performing buffer exchange on a separate day, flash freeze all fractions in liquid nitrogen and store in cryovials at –80°C.

85. Perform SDS-PAGE as described in steps 55-59, and carry out western blotting to confirm protein presence following purification and Coomassie blue staining to assess protein purity as described in steps 60-74 (also see steps 49 and 54).

Buffer exchange and protein concentration

Once protein presence and purity have been confirmed through western blotting and Coomassie staining, fractions containing target protein can be pooled, buffer exchanged, and concentrated to ensure optimal protein sample for microscopy.

86. Equilibrate a 50,000-MWCO centrifugal concentrator column (e.g., Amicon Ultra-4, Merck Millipore) by adding 4 ml exchange buffer and centrifuging at 2100 × *g*, 4°C, using a Sorvall RT 7 centrifuge, RTH-250 rotor, or equivalent, until ∼200 µl remains (∼8 min).

87. Pool together the elution fractions containing pure protein, add them to the concentrator column, and centrifuge at 2100 × *g*, 4°C, using a Sorvall RT 7 centrifuge, RTH-250 rotor, or equivalent, until ∼200 µl remains (∼8 min, dependent on the purity of the sample and amount of protein present).

88. Collect flowthrough in case the protein is present in it.

89. Top up the exchange buffer to 4 ml, and gently wash the buffer over the concentrator membrane to prevent protein precipitation. Repeat centrifugation as described in step 86.

90. Repeat steps 88 and 89 for a total of five buffer exchanges.

91. Add the pure, concentrated protein from the top of the column to a microcentrifuge tube

92. Check the protein concentration and aliquot sample into separate microcentrifuge tubes for negative-stain TEM and cryo-EM. If not performing microscope grid preparations on the same day, flash freeze the samples in liquid nitrogen and store in cryovials at –80°C.

BASIC PROTOCOL 2

PREPARATION OF SARS-CoV-2 SPIKE PROTEIN FOR VISUALIZATION BY NEGATIVE-STAIN TEM AND CRYO-ELECTRON MICROSCOPY

Multiple factors should be considered before moving to grid preparation for negative-stain transmission electron microscopy (TEM) and cryo-EM. The sample must be of sufficient purity and concentration for both techniques. Generally, protein concentration should be ∼0.05 mg/ml for negative-staining TEM of spike glycoproteins and ∼0.5 mg/ml for cryo-EM. It is also best practice to minimize the number of protein freeze-thaw cycles to prevent protein damage. For this reason, it is advisable to keep multiple aliquots of various protein concentrations for negative-stain TEM and cryo-EM while determining the best sample/grid preparation methods for each protein sample.

Materials

Filter paper (Thermo Fisher, cat. no. 09-920-115)
1% uranyl acetate (Thermo Fisher, cat. no. 18-607-644)

C-flat holey carbon-coated grid copper 300 mesh (MiTeGen)
UltrAuFoil R1.2/1.3 holey gold grid (Quantifoil)
GloQube discharge system (Quorum)

Fume hood
Parafilm (Sigma Aldrich, cat. no. P7793)
Liquid ethane
Liquid nitrogen
PELCO EasiGlow Glow Discharge Cleaning System (Ted Pella, Inc.)
JEOL 1400 Flash transmission electron microscope with LaB6 emitter and 120kV accelerating voltage, equipped with a Matataki CMOS camera
NanoSoft clipping station and tools (NanoSoft, SKU 25011002) or equivalent

Negative-staining protocol

1. Select a copper 300 mesh carbon-coated grid and negatively plasma discharge in a GloQube discharge system for 30 s at 15 mA (carbon side facing up).
2. While grids are undergoing plasma glow discharge, prepare the fume hood and staining reagents.

   CAUTION: *Uranyl acetate is a hazardous chemical that is weakly radioactive and toxic if inhaled, swallowed, or in contact with skin. Only use uranyl acetate in a fume hood with reagent-impermeable gloves. For further information, see the Chemwatch Safety Data Sheet for this reagent.*

3. On a piece of Parafilm, prepare 10-µl droplets in the following order: 10 µl of protein sample, 10 µl of deionized, distilled water (ddH_2O), 10 µl of 1% uranyl acetate, and 10 µl of ddH_2O.
4. With tweezers, carefully pick up the 'plasma-glowed' grid by its very edge.
5. Place the grid, carbon side down, on the drop of protein sample and incubate for 1 min.
6. Pick up the grid and blot by touching a piece of filter paper to the edge of the grid, being careful not to damage the carbon film.
7. Touch the grid, carbon side down, to the drop of ddH_2O and immediately blot with a piece of filter paper to wash the grid.
8. Place the grid, carbon side down, on the drop of uranyl acetate stain and incubate for 1 min.
9. Pick up the grid and blot by touching a piece of filter paper to the edge of the grid.
10. Touch the grid, carbon side down, to the drop of ddH_2O, and immediately blot with a piece of filter paper to wash the grid.
11. Leave the grid to air-dry for 1-2 min before placing it in a grid box for negative-stain TEM.

    Sample quality can be determined by visual inspection of the negative-stained images, looking for intact particle appearance, homogenous protein distribution, and lack of obvious specimen contaminants (Fig. 6). Samples determined to be of high quality should be selected for further microscopy analysis via cryo-EM.

Cryo-EM grid preparation

There are many variables that may be altered during the grid preparation process, including but not limited to grid type, sample buffer and concentration, and Vitrobot parameters. The following protocol was used for the collection of cryo-EM datasets of spike glycoproteins from bat and civet hosts.

12. Perform negative glow discharge of UltrAuFoil (Quantifoil) R1.2/1.3 300 mesh gold grids, carbon side up, in a Pelco EasiGlow machine for 30 s at 30 mA.

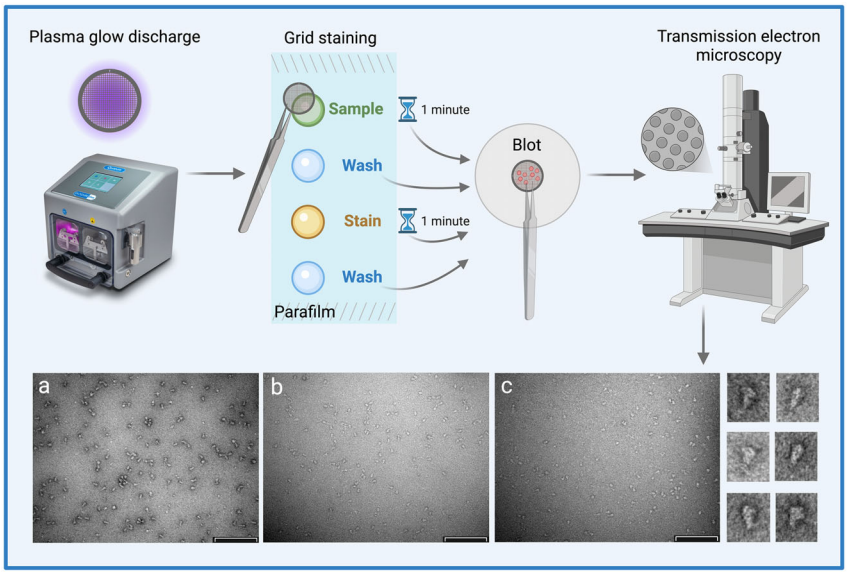

Figure 6 Negative-stain grid preparation protocol for protein visualization through cryo-EM. Clean 'carbon-coated' 300-mesh grids are plasma glow discharged ready for sample application. A grid is placed carbon side down on the protein sample for 1 min and then blotted with filter paper. The grid is then washed with ddH_2O, blotted, and placed carbon side down on the 1% uranyl acetate stain for 1 min before blotting. It is then washed with ddH_2O and blotted. The grid containing the stained protein sample is allowed to dry before negative-stain transmission electron microscopy imaging is performed. Images at bottom are negative-stain TEM images of (**a**) bat, (**b**) civet, and (**c**) host coronavirus spike glycoproteins taken on a Philips CM-100 transmission electron microscope (TEM); scale bars, 200 nm. Full-field-of-view negative-stain images are followed by zoomed-in images of six representative spike glycoprotein particles at right.

13. Load the 'plasma-glowed' grid into an FEI Mark IV Vitrobot set to 4°C and 100% humidity, using a wait time of 30 s, blot force of 0 and blot time of 5 s.

14. Apply 3 µl of purified glycoprotein sample at a concentration of 0.5 mg/ml and plunge-freeze in liquid ethane cooled with liquid nitrogen.

    CAUTION: *Liquid nitrogen can cause significant injury when handled improperly, including frostbite, asphyxiation, and explosion. Appropriate training is required when handling and storing liquid nitrogen. Ethane is a highly flammable gas and can also cause frostbite and displace oxygen. Wear cold-insulating gloves and either a face shield or eye protection when working with ethane. Keep away from heat, hot surfaces, sparks, open flames, and other ignition sources.*

15. Clip the grids using a Nanosoft clipping station and tools or similar, and store the grids in a grid puck until cryo-EM screening and data collection.

    Handle the grids with both speed and care while moving from the liquid ethane to the grid box to avoid the formation of frost on the grid. Frost may form if the grid is not kept as cold as possible during the transfer process.

REAGENTS AND SOLUTIONS

Anode buffer I, pH 10.4

Stir the following together in 500 ml ddH_2O until dissolved:
0.3 M Tris base (Tocris Bioscience, CAS no. 77-86-1)
100 ml methanol (Sigma Aldrich, CAS no. 67-56-1)
Dilute to 1 liter with ddH_2O
Adjust pH to 10.4
Store up to 1 month at 4°C

Anode buffer II

Stir the following together in 500 ml ddH_2O until dissolved:
25 mM Tris base (Tocris Bioscience, CAS no. 77-86-1)
100 ml methanol (Sigma Aldrich, CAS no. 67-56-1)
Dilute to 1 liter with ddH_2O
Store up to 1 month at 4°C

Blocking buffer (PBS ± 0.1% [w/v] casein)

200 mg casein (Sigma Aldrich, CAS no. 9000-71-9)
200 ml PBS (Gibco, cat. no. 20012027)
Prepare immediately before use, or store up to 1 week at 4°C

Cathode buffer, pH 9.4

Stir the following together in 500 ml of ddH_2O until dissolved:
25 mM Tris base (Tocris Bioscience, CAS no. 77-86-1)
10 mM glycine (Sigma Aldrich, CAS no. 56-40-6)
100 ml methanol (Sigma Aldrich, CAS no. 67-56-1)
Dilute to 1 L with ddH_2O
Adjust pH to 9.4
Store up to 1 month at 4°C

Coomassie destain

100 ml absolute (100%) ethanol (Lab Supply, cat. no. 111727)
150 ml glacial acetic acid (Thermo Fisher, cat. no. 9526-33)
Dilute to 1 liter with ddH_2O
Store up to 1 year at room temperature

Coomassie stain

Add 2.5 g Coomassie brilliant blue G250 powder (Sigma Aldrich, CAS no. 6104-58-1) to 600 ml absolute (100%) ethanol (Lab Supply, cat. no. 111727) and swirl to mix. Once fully dissolved, add 75 ml glacial acetic acid (Thermo Fisher, cat. no. 9526-33) and dilute to 1 liter with ddH_2O. Store up to 1 year at room temperature.

Elution buffer

50 mM Tris·Cl, pH 8.0
200 mM NaCl
300 mM imidazole
Prepare fresh

See stock solutions recipe for Tris·Cl, NaCl, and imidazole stocks.

Exchange buffer

20 mM Tris·Cl, pH 8.0
100 mM NaCl
Prepare fresh

See stock solutions recipe for Tris·Cl and NaCl stocks.

Luria-Bertani (LB) agar plates

Stir the following with 500 ml ddH_2O until dissolved:
7.5 g agar (Thermo Fisher, cat. no. 400400050)
5 g NaCl (Sigma Aldrich, CAS 9002-18-0)
5 g tryptone (Condalab, cat. no. 1612)
2.5 g yeast extract (BD Biosciences, cat. no. 212750)
50 mg ampicillin (Thermo Fisher, cat. no. 11593027)

Autoclave solution to sterilize. When cool enough to touch (~ 55°C), working inside a laminar flow cabinet, pour ~20 ml each into 100- × 15-mm petri dishes (Sigma Aldrich, cat. no. P5731; ~20-25 plates). Leave plates to set, and then cover and store upside down for no more than 1 month at 4°C.

Luria-Bertani (LB) broth

Stir the following with 500 ml ddH_2O until dissolved:
4 g NaCl (Sigma Aldrich, CAS no. 9002-18-0)
4 g tryptone (Condalab, cat. no. 1612)
2 g yeast extract (BD Biosciences, cat. no. 212750)
Autoclave solution to sterilize
Allow to cool before use (to ~37°C)
Store up to 1 year at 4°C

Phosphate-buffered saline with 0.1% Tween 20 (PBST)

200 ml phosphate-buffered saline (Gibco, cat. no. 20012027)
0.2 ml Tween 20 (Sigma Aldrich, CAS no. 9005-64-5)
Prepare immediately before use or store for no more than 2 weeks at 4°C.

SDS-PAGE gel, 8% acrylamide

Prepare resolving gel solution by placing the following in an appropriately sized tube (scaling the volume depending on how many gels are required; this recipe makes 5 ml of gel):

1 ml 30% acrylamide/bisacrylamide solution (Bio-Rad, cat. no. 1610158)
1.3 ml 1.5 M Tris, pH 8.8 (Tocris Bioscience, CAS no. 77-86-1)
50 µl 10% sodium dodecyl sulfate (SDS; Fisher BioReagents, cat. no. 15875308)
50 µl 10% APS (Thermo Fisher, cat. no. 17874)
Dilute to 5 ml with ddH_2O

Prepare stacking gel as follows (per milliliter of gel):

170 µl 30% acrylamide/bisacrylamide solution (Bio-Rad, cat. no. 1610158)
130 µl 1 M Tris·Cl, pH 6.8 (Tocris Bioscience, CAS no. 77-86-1)
10 µl 10% SDS (Fisher BioReagents, cat. no. 15875308)
10 µl 10% APS (Thermo Fisher, cat. no. 17874)
1 µl TEMED (Sigma Aldrich, CAS No. 110-18-9)
Dilute to 1 ml with ddH_2O

Immediately before the gel is needed, add 4 µl *N,N,N′,N′*-tetramethylethylenediamine (TEMED; Sigma Aldrich, CAS no. 110-18-9) to the stacking gel solution; the gel will begin to set as soon as this is added. Pour the resolving gel into the mold and wait for it to set (~30 min), and then add the stacking gel and a gel comb. Once the stacking gel has set, either use the gel immediately or store for no longer than 2 weeks at 4°C.

CAUTION: *Sodium dodecyl sulfate is a hazardous chemical and toxic if inhaled, swallowed, or in contact with skin. Acrylamide/bisacrylamide solution is also a hazardous chemical, a probable carcinogen, and toxic if inhaled, swallowed, or in contact with skin. Stock bottles should only be opened in a fume hood with reagent-impermeable gloves. For further information, see ThermoFisher Safety Data Sheet for sodium dodecyl sulfate.*

Sample loading buffer

Stir the following with 50 ml ddH_2O until dissolved:
5 ml 1 M Tris·Cl, pH 7.0 (see recipe for stock solutions)
25 ml 20% SDS (Fisher BioReagents, cat. no. 15875308)
20 ml glycerol (Thermo Fisher, cat. no. A16205.0F)

2 mg bromophenol blue (BDH laboratory supplies, CAS no. 115-39-9)
Dilute to 100 ml with ddH_2O
Store up to 1 year at –20°C

Immediately before use, add 50 µl of 2-mercaptoethanol (Bio-Rad, cat. no. 161-0710) per milliliter of buffer; do not store after this point, but make up fresh for each use.

CAUTION: *Sodium dodecyl sulfate and 2-mercaptoethanol are hazardous chemicals and toxic if inhaled, swallowed, or in contact with skin. Stock bottles should only be opened in a fume hood with reagent-impermeable gloves. For further information, see ThermoFisher Safety Data Sheet for sodium dodecyl sulfate and Bio-Rad Safety Data Sheet for 2-mercaptoethanol.*

Stock solutions for wash, elution, and exchange buffers

Prepare 1 M Tris·Cl stock solution from solid Tris base (Tocris Bioscience, CAS no. 77-86-1), adjust pH to 8.0 with HCl (Thermo Fisher, cat. no. 450560050), and autoclave to sterilize.

Prepare 1 M NaCl stock solution from solid NaCl (Sigma Aldrich, CAS no. 9002-18-0) and autoclave to sterilize. Do not store-make up as needed.

Prepare 5 M imidazole stock solution from solid imidazole (Sigma Aldrich, CAS no. 288-32-4). Wrap the bottle in aluminum foil or store in a dark place, as imidazole is light sensitive.

Use these stock solutions to prepare the needed volumes of wash, elution, and exchange buffers on the day of use by diluting with ddH_2O—do not store those buffers once prepared.

CAUTION: *Imidazole is toxic if inhaled, swallowed, or in contact with skin. When making imidazole solution from powder stock, the bottle should only be opened in a fume hood with reagent-impermeable gloves. For further information, see Sigma-Aldrich Safety Data Sheet.*

TE buffer

Stir the following with 50 ml ddH_2O until dissolved:
10 mM Tris·Cl, pH 8.0 (see stock solutions recipe)
1 mM ethylenediaminetetraacetic acid (EDTA; Sigma Aldrich, cat. no. ED2SS)
Dilute to 100 ml with ddH_2O
Autoclave solution. Stored up to 3 months at room temperature or 1 year at 4°C.

Tris-glycine running buffer (10 ×)

Stir the following with 500 ml ddH_2O until dissolved:
30.2 g Tris base (Tocris Bioscience, CAS no. 77-86-1)
144 g glycine (Sigma Aldrich, CAS no. 56-40-6)
10 g SDS (Fisher BioReagents, cat. no. 15875308)
Dilute to 1 L with ddH_2O

Store up to 1 year at room temperature

Before use, aliquot 100 ml of the 10× buffer into 900 ml ddH_2O.

CAUTION: *Sodium dodecyl sulfate is a hazardous chemical and toxic if inhaled, swallowed, or in contact with skin. Stock bottles should only be opened in a fume hood with reagent-impermeable gloves. For further information, see ThermoFisher Safety Data Sheet for sodium dodecyl sulfate.*

Wash buffer

50 mM Tris·Cl, pH 8.0
200 mM NaCl
Prepare fresh

See stock solutions recipe for Tris·Cl and NaCl stocks.

Critical Parameters

Limitations

This article describes the specific protocols carried out for the expression and purification of spike glycoproteins from bat and civet host species, resulting in successful cryo-EM protein analysis. Each individual protein requires specific conditions for successful expression, purification, negative-stain TEM imaging, and cryo-EM imaging. Therefore, alterations to the protocols outlined here, or substitutions for more effective protocols based on protein-specific requirements, may be needed.

Basic Protocol 1: Spike protein expression and purification from ExpiCHO cells

Plasmid design

It is essential to remove both the hydrophobic N-terminal signal peptide sequence (replacing it with the μ-phosphatase-derived signal peptide to ensure extracellular protein secretion) and the transmembrane domain. If these features are not removed, the expression and folding of the protein will be greatly hindered. For further information on plasmid design, please refer to the supplementary material found in (Tortorici et al., 2020).

ExpiCHO cell handling

When thawing ExpiCHO cells, we have found it critical to both thaw and centrifuge the cells swiftly before replenishing with prewarmed (37°C) medium. This is to minimize any cellular damage caused by DMSO (if present) and allows faster seeding times following thawing.

When subculturing ExpiCHO, we found that centrifuging the cells for 5 min at 450 × *g* and resuspending them in fresh prewarmed (37°C) medium maintains the cell viability at ≥90% for a larger number of passages.

Although we have outlined some critical parameters, many factors may affect the performance of ExpiCHO cells. For full detailed information on the thawing, subculturing, and freezing of ExpiCHO cells, see (ThermoFisher, 2018).

Spike protein expression

For optimal protein expression, it is critical that the cell viability remains >70% for the entirety of the transfection incubation. We have found that a key determining factor for success is to ensure that the cell viability prior to transfection is ≥90%. Transfecting ExpiCHO cells with viability <90% resulted in diminished protein yield.

Spike protein purification

When purifying spike glycoproteins for microscopy, the most critical parameter is to swiftly place the pure protein fractions into an ice bath and store at 4°C until grid preparation. If buffer exchange or grid preparation is not being performed on the same day, flash freeze the protein fractions in liquid nitrogen and store at –80°C. Keeping the protein at low temperatures helps to prevent both protein degradation and protein aggregation, both of which make the sample unsuitable for microscopy.

Buffer exchange and concentration

Equilibrating and washing the concentrator column membrane is critical to prevent both protein accumulation on the membrane and protein aggregation. Gently washing the concentrator column membrane between centrifuge steps with exchange buffer (20 mM Tris·Cl, pH 8.0/100 mM NaCl; see recipe) will help prevent the protein from precipitating in the column. Once the protein has aggregated, it is not suitable for microscopy techniques and must be reproduced.

Another critical recommendation to prevent protein aggregation at this step is to check the protein concentration before beginning. If the concentration is sufficient for both negative-stain TEM and cryo-EM grid preparation, perform a buffer exchange without concentrating the protein. For specific solutions to this issue, see Table 1 and Fig. 7.

Basic Protocol 2: Spike protein preparation for visualization by negative-stain TEM and cryo-EM

Negative-stain TEM protocol

We found the most critical parameter for optimal negative-stain TEM images of spike glycoproteins was ensuring that the fresh 1% uranyl acetate staining solution was free from precipitate. Precipitate in the staining solution causes artifacts on the grids that can obscure the protein from view and makes determining the protein concentration for grid preparations far more difficult.

NOTE: To avoid artifacts from the staining solution, do not mix the solution before use, and be sure to remove the solution from the top of the bottle to avoid disturbing and collecting and stain precipitates.

Table 1 Common Problems Experienced with Basic Protocols 1 and 2, with Causes and Potential Solutions

Common problems	Potential cause	Potential solutions
Protein is not being expressed in the ExpiCHO supernatant	Cell viability was too low before or during transfection	Check the viability and general growth of the ExpiCHO cells. Transfections performed when the cell viability is <90% or before cells have recovered from thawing (2 growth cycles) are likely to result in poor protein yield.
		Check cell viability throughout the transfection protocol. ExpiCHO culture cell viability should not be <70% at any point throughout the transfection protocol, as this is likely to result in poor protein yield.
		Refer to the Thermo Fisher ExpiCHO Expression System handbook to confirm that all protocols are followed exactly.
	The protein is expressed but not secreted by the ExpiCHO cells	Protein may be expressed but not secreted from the cells to the supernatant. Resuspend the cell pellet in RIPA buffer (Thermo Fisher, cat. no. J63306.AP; 1 ml per 5×10^7 cells). Shear the lysate with a 22- to 26-G needle and centrifuge 10 min at 14,000 × *g*, 4°C. Collect the supernatant and check for protein through SDS-PAGE and western blotting. If protein is present at the correct size in the cell lysate, continue with the purification as described.
A significant amount of protein remains on the Ni beads after elution	The number of elution steps or amount of imidazole is not enough to sufficiently remove the protein from the beads	Increasing the number of fractions performed with the 50 mM Tris·Cl/200 mM NaCl/300 mM imidazole buffer. If protein is not eluted with further fractions and incubations with elution buffer, increase the amount of imidazole in the elution buffer. If the protein is not eluted with increased imidazole, it is likely aggregated and will not be suitable for grid preparation.
A significant amount of protein is flowing through the purification column	There may not be enough Ni beads in the supernatant incubation prior to purification	Increase the amount of Ni beads and recheck the flow through. If a significant amount of protein is still present in the flow through, check if there is any protein bound to the Ni beads through SDS-PAGE and western blotting. If the protein is not binding to the Ni beads, the His tag on the protein may be inaccessible or cleaved.
Protein is precipitating during the buffer-exchange protocol or is 'lost'	The protein may be aggregating due to high concentration or accumulation at the centrifuge step; multiple techniques can be used to potentially resolve this issue	Double-check that the concentration column membrane is being gently washed between centrifugations to prevent protein from aggregating on the membrane.
		Perform centrifugations at a lower speed.
		Incubate the column with 1 ml buffer at 4°C for 30 min, and gently wash the membrane with the buffer to try to regain protein from the membrane.
		If the purified protein concentration is high enough to not require concentrating for TEM or cryo-EM, elute protein from the purification column in elution buffer containing 20 mM Tris·Cl, 100 mM NaCl, and 300 mM imidazole and skip the buffer-exchange step.

(*Continued*)

Table 1 Common Problems Experienced with Basic Protocols 1 and 2, with Causes and Potential Solutions, *continued*

Common problems	Potential cause	Potential solutions
Poor quality negative-stain TEM images	Assuming that the microscope is correctly aligned and operated, the most common cause of poor-quality negative stain TEM images is sub-optimal sample and grid preparation	There are published review articles that compare the various negative-stain TEM grid preparation methods, which may be useful in this instance (Booth et al., 2011; Harris, 2007; Harris & de Carlo, 2014; Ohi et al., 2004; Scarff et al., 2018). Regarding the protocols outlined here, grid preparation may be optimized by changing parameters including, but not limited to, protein sample concentration, salt concentration in sample buffer, glow-discharge, on-grid sample incubation time, number of wash steps (if any), stain type, and stain incubation time.
When screening the protein sample by negative-stain TEM, the protein is aggregated (Fig. 7)	Protein aggregation can occur when sample preparation is suboptimal	You may need to optimize the sample preparation. See potential solutions to the previous problem for advice on sample preparation optimization. Low levels of protein aggregation may be rescued through sonication or the addition of detergent, which is suitable for cryo-EM. First, attempt sonication by placing the tube of protein sample in a sonicating water bath for 15 s and then on ice for 15 s, repeating this process 4-5 times. If this doesn't work, try adding a detergent suitable for cryo-EM, such as CHAPS (0.05%-0.2%). Protein aggregation interferes with negative-stain TEM or cryo-EM data collection and is also often very difficult to reverse; therefore, it may be easier to repeat the protein expression and purification protocols in the case of protein aggregation.
Poor-quality cryo-EM images	Assuming that the microscope is correctly aligned and operated, a major cause of poor-quality cryo-EM images is sub-optimal grid preparation.	As with the preparation of negative-stain TEM grids, there are a plethora of articles outlining and comparing various methods for cryo-EM grid preparation (Chua et al., 2022; Drulyte et al., 2018; Kampjut et al., 2021; Palovcak et al., 2018; Passmore & Russo, 2016; Sgro & Costa, 2018; Weissenberger et al., 2021). Regarding the protocols outlined here, grid preparation may be optimized by changing the protein sample preparation (e.g., protein concentration, salt concentration), glow-discharge parameters, grid type (grid material, grid coating, coating type), and Vitrobot parameters (humidity, multiple sample additions, wait time, blot force, blot time, back blotting, etc.).

Cryo-electron microscopy grid preparation

Cryo-EM grid preparation is highly specific and variable between even closely related proteins; however, a universal critical parameter is the protein concentration for cryo-EM data collection. Most spike glycoproteins have been successfully reconstructed through data collection at 0.5 mg/ml. However, we suggest preparing grids with various concentrations upon initial screening. We found a successful concentration range for initial screening to include 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, and 1 mg/ml.

Troubleshooting

Although some critical parameters for success have been discussed in the previous section, for a list of common problems and proposed solutions for the protocols outlined here, see Table 1.

Understanding Results

Basic Protocol 1

The success of Basic Protocol 1 can be assessed through SDS-PAGE and transfer into Coomassie blue stain or onto a nitrocellulose membrane for western blotting. The

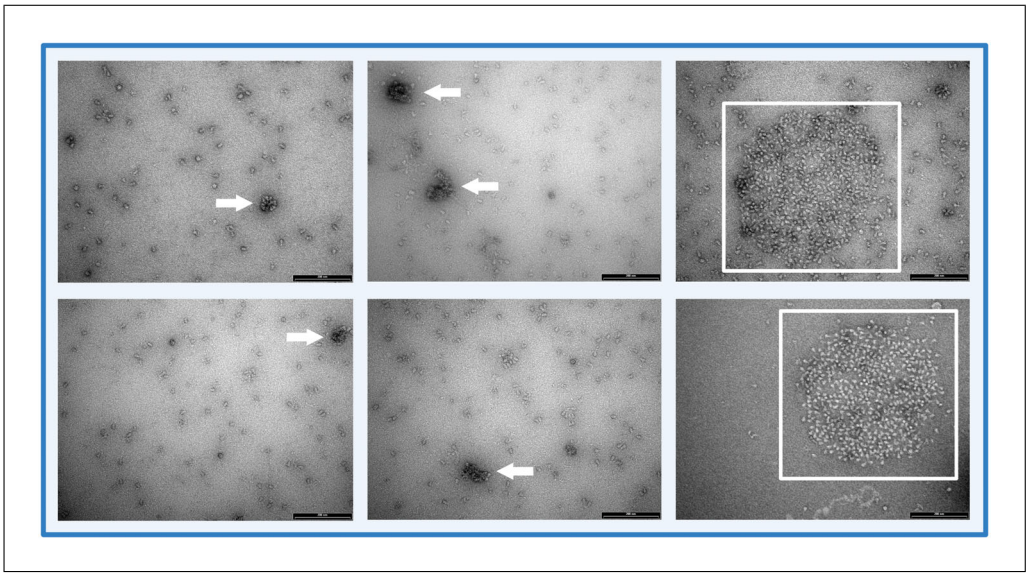

Figure 7 Representative negative-stain TEM images of various spike glycoprotein aggregates, indicated by white arrows and white boxes. Scale bars, 200 nm. Images were taken on a JEOL 1400 Flash TEM.

protein samples must be run with an appropriate molecular mass ladder (an upper range of 200 kDa is recommended) to assess whether correctly sized protein is present. For spike glycoproteins that have been reduced, a single monomer will be present at ~180 kDa.

Coomassie blue stain will show the purity of the protein sample; a single protein band at ~180 kDa indicates successful purification (Fig. 5). Subsequent western blot analysis will confirm the correct protein presence through the use of antibody binding and fluorescence, showing the protein of interest as a single ~180 kDa band (Figs. 3-5). The abundance of the protein of interest may also be assessed during western blotting based on the brightness and thickness of the protein band. For Basic Protocol 1, we recommend using commercially available antibodies directed against the His tag, which has been added to the C-terminus of the protein of interest during plasmid design.

The presentation of this data includes labelling the gel image clearly. Each lane of the gel must be labelled from left to right to describe what samples are in each lane. The lane containing the molecular mass ladder must be labelled with the value of each band in molecular mass (kDa). Bands in the sample lanes should be compared to the corresponding bands in the ladder lane to estimate protein mass and confirm the presence of the protein of interest.

Basic Protocol 2

As with the results from Basic Protocol 1, the goal of negative-stain TEM visualization in Basic Protocol 2 is to assess the presence of the protein of interest and the purity of the protein sample. In addition, negative-stain TEM visualization can be used to ensure the protein sample is free from artifacts, is at the correct concentration for cryo-EM grid preparation, is homogenous on the grid, is non-aggregated, and is stably folded. The microscopy images from both negative-stain TEM and cryo-EM should show a homogenous distribution of correctly sized and shaped protein of interest. The ideal concentration should be such that as many individual proteins as possible are within the field of view while a monodisperse distribution is maintained (Fig. 6). Images should include a scale bar to estimate the size of the protein of interest, which should be ~15 nm in height and ~10 nm in width.

Time Considerations

Time considerations for key steps within Basic Protocols 1 and 2 have been listed in order of occurrence as well as represented in Figure 8.

Basic Protocol 1: Spike protein expression and purification from ExpiCHO cells

Plasmid design
1 day

Bacterial transformation
Day 1: 2-3 hr
Overnight incubation: 12-18 hr
Day 2: 15 min
Overnight incubation: 12-18 hr
Day 3: 4 hr
Overnight incubation: 12-18 hr
Day 4: 4 hr

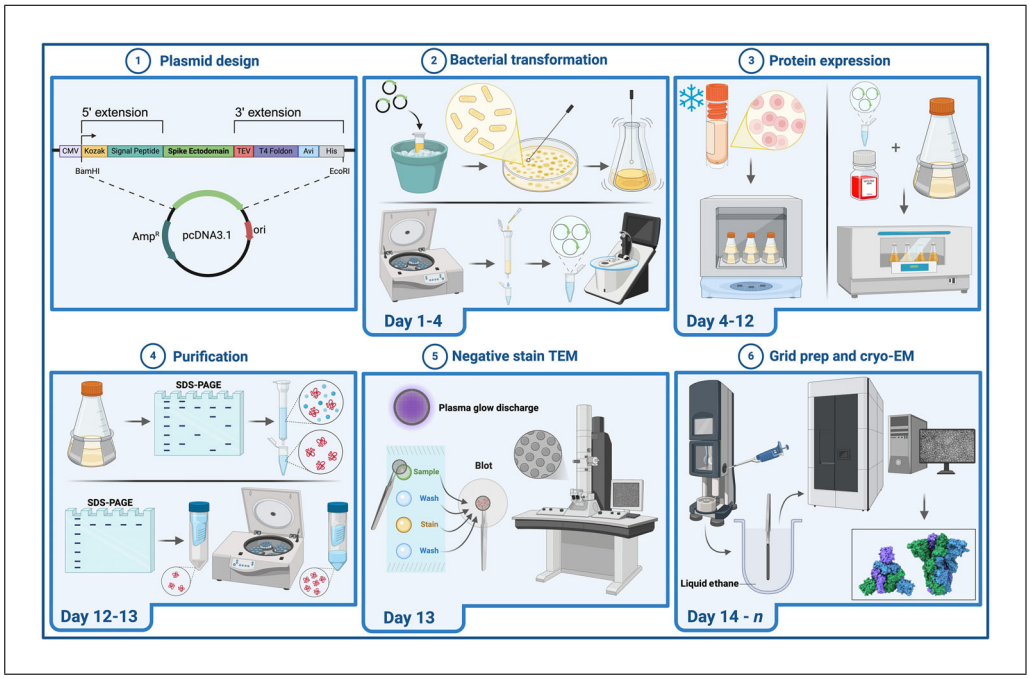

Figure 8 Workflow overview and time considerations for the main methodologies outlined in Basic Protocols 1 and 2. Plasmid design, bacterial transformation, and protein expression and purification relate to Basic Protocol 1 and take 13 days to complete. Negative stain TEM, grid prep, and cryo-EM take a minimum of 1-2 days to complete, depending on the number of grids being prepared.

ExpiCHO cell handling

Thawing: <1 hr laboratory work plus ~3 days of incubation

Subculture: <1 hr plus 2-4 days of incubation (dependent on seeding density)

Freezing: <1 hr

Spike protein expression

Day 1: 15 min

Overnight incubation: 12-18 hr

Day 2: 1 hr

Overnight incubation: 18 – 22 hr

Day 3: 15 min

Day 6: 30 min

Day 10: 15 min

Confirmation of spike protein expression

SDS-PAGE and transfer: 2 hr 30 min

Western blotting: 4-5 hr

Coomassie staining: 2-3 hr

Spike protein purification: – 3 hr

Buffer exchange and protein concentration: – 3 hr

Basic Protocol 2: Spike protein preparation for visualization by negative stain and cryo-electron microscopy

Negative stain protocol

Grid staining: <1 hr

Transmission electron microscopy: 1-3 hr (dependent on sample quality)

Cryo-EM grid preparation

Grid preparation and plunge freezing: 1 hr

Grid clipping and storage: 1 hr

Acknowledgments

This work was supported by fellow researchers at the University of Otago. Of note: Professor Alexander McLellan and Lachlan Dobson for discussions in the troubleshooting of laboratory protocols, and Dr Richard Easingwood and Dr Alice-Roza Eruera for discussions in the troubleshooting of grid preparation. This work was supported by Maurice Wilkins Centre for Molecular Biodiscovery New Zealand–China Catalyst program. All figures were created using BioRender.com.

Open access publishing facilitated by University of Otago, as part of the Wiley - University of Otago agreement via the Council of Australian University Librarians.

Author Contributions

Francesca Hills: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing—original draft; writing—review and editing. **Fátima Jorge:** Methodology; resources; writing—review and editing. **Laura Burga:** Investigation; methodology; writing—review and editing. **Mihnea Bostina:** Conceptualization; funding

acquisition; supervision; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

All source methodology has been referenced in the text where appropriate and is listed in the Literature Cited section. All variations from the source methodology have been noted in the text. No further novel resources were generated as a result of this study.

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