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Production and Purification of Hantavirus Glycoproteins in *Drosophila melanogaster* S2 Cells

Annalisa Meola and Pablo Guardado-Calvo

Abstract

Hantaviruses, which are present worldwide, are rodent-borne viruses transmitted to humans through inhalation of contaminated excreta. These viruses can cause either a renal or pulmonary syndrome, depending on the virus, and no effective treatment is currently available for either syndrome. Hantaviral particles are covered by a protein lattice composed of two glycoproteins (Gn and Gc) that mediate adsorption to target cells and fusion with endosomal membranes, making them prime targets for neutralizing antibodies. Here we present the methodology to produce soluble recombinant glycoproteins in different conformations, either alone or as a stabilized Gn/Gc complex, using stably transfected *Drosophila* S2 cells.

Key words Hantavirus, Bunyavirus, Glycoproteins, Protein expression, S2, Insect cells

1 Introduction

1.1 Hantavirus Glycoproteins

Hantaviruses are rodent-borne viruses present worldwide and transmitted to humans by breathing contaminated aerosols. New-World hantaviruses, such as Andes (ANDV) and Sin Nombre (SNV) viruses, produce a respiratory disease termed hantavirus cardiopulmonary syndrome (HCPS) with up to 40% fatality rates [1]. Old-World hantaviruses, which include Puumala (PUUV) or Hantaan (HNTV) virus, produce a renal syndrome denominated hemorrhagic fever with renal syndrome (HFRS), less deadly but with a higher incidence. Neither of the two diseases has any approved treatments or vaccines, and patients only receive supportive care to ease their symptoms.

The surface of hantaviruses is covered by a protein lattice consisting of two glycoproteins termed Gn and Gc [2]. These proteins mediate virion assembly [3, 4], adsorption to the plasma membrane of susceptible cells [5], and fusion in the acidic lumen of endosomes [6]. They are the only targets of neutralizing antibodies produced

during infection [7–9]. Gc is a class-II fusion protein [6, 10] with three beta-structured domains and Gn is a companion protein with two globular regions denominated head (Gn^H) and base (Gn^B) [11]. This organization is strictly conserved throughout the family and the boundaries of Gn^H , Gn^B , and Gc are easily distinguishable in the amino acidic sequence (Figs. 1a and 2) [12]. During biogenesis, Gn and Gc fold together to form a metastable Gn/Gc heterodimer [13, 14], which further associates to form the spikes that make up the outer lattice mentioned above (Fig. 1b). Structural analysis of the recombinant glycoproteins revealed three different conformations of Gc: the pre-fusion conformation, which is acquired in complex with Gn on the viral surface [13, 15]; a transient intermediate conformation [6], which is formed after dissociation of the heterodimer at acidic pH; and a stable post-fusion conformation [6, 10], which is a homotrimer formed after the fusion reaction (Fig. 1c). In isolation, Gn^H is monomeric at neutral pH [13, 16] and tetrameric at acidic pH [17]. Gn^B forms a tetramer [13]. Several studies have identified and characterized neutralizing antibodies targeting Gn^H [8, 18–20], Gc [7, 15], and the Gn/Gc interface [7]. So far, no antibodies recognizing Gn^B have been reported.

1.2 Production of Hantavirus Glycoproteins

Hantavirus glycoproteins have been used as immunogens [19] or as tools to isolate and characterize monoclonal antibodies [7, 8, 15, 18]. To this end, the glycoprotein domains mentioned above have been produced recombinantly using a variety of expression systems. Gn^H from PUUV [17, 21] and HNTV [13] have been expressed using mammalian and Drosophila S2 cells, respectively. Gn^B from ANDV has been produced using S2 cells [13]. The complete ectodomain of Gc from PUUV has been obtained from insect cells infected with a baculovirus [10] and using mammalian cells [15] and Gc from HNTV purified from the supernatants of S2 cells [13]. The stabilized heterodimers from ANDV and Maporal virus (MPRV) [13] have been purified from stable transfected S2 cells.

In this chapter, we will detail how to express all the glycoprotein domains of hantaviruses by using *Drosophila melanogaster* S2 cells. Although other expression systems can also produce these proteins, we prioritize the S2 cell system due to its cost-effectiveness and glycosylation properties. S2 cells can grow at room temperature without CO₂ and do not require a dedicated incubator. They are semi-adherent and can be grown in suspension at high densities, making scaling up culture easy using spinners flasks or Erlenmeyer. Generating stable S2 cell lines is a slow process, but they are highly stable, allowing for long-term storage and no need for re-transfection. S2 cells secrete and accumulate

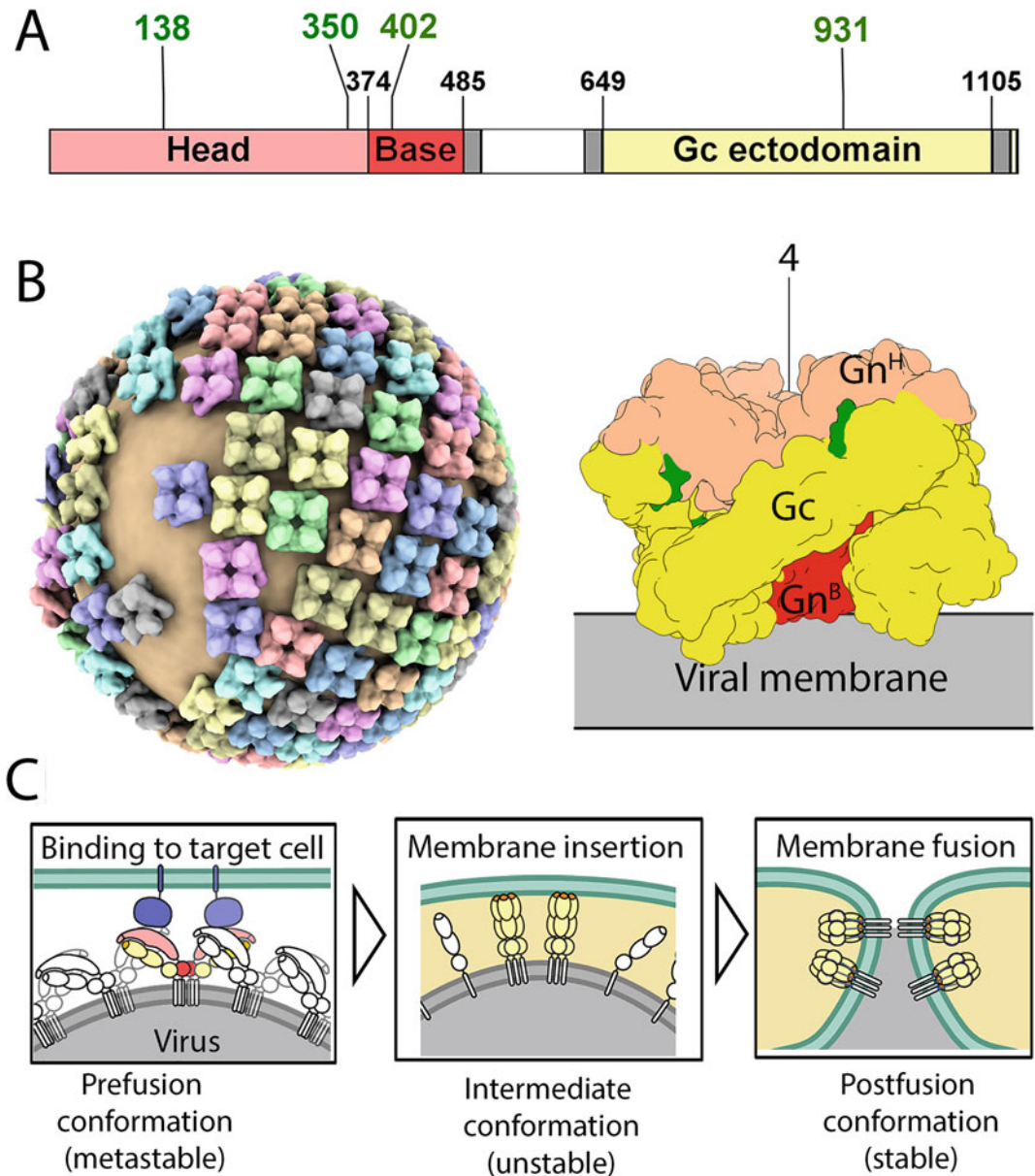


Fig. 1 The organization of the hantavirus glycoproteins. (a) Organization of the glycoprotein precursor. The different regions of Gn (Gn^H, Gn^B) are colored in light and dark red and Gc in yellow with the boundaries indicated on the diagram in black numbers. The TM segments are shown in gray and the conserved glycosylation sites are annotated above in green numbers. The numbering in this diagram belongs to the Andes virus sequence (ANDV, NP_604472.1). (b) The left panel is a reconstruction of the glycoprotein outer lattice on the virion surface. For clarity, each tetrameric spike is colored differently and the viral membrane is colored in wheat. The right panel shows the organization of a single spike in the side view colored as in a. The approximate position of the viral membrane is indicated. (c) Scheme showing the different conformations of Gc during viral entry. Gn^H, Gn^B, and Gc are colored as in a

glycoproteins in the supernatant without cell lysis, which reduces the release of proteases and proteolytic degradation. Finally, S2 cells produce glycoproteins with simple, high-mannose sugars [22], which recapitulates the *N*-glycans observed in authentic hantaviruses [23] and which plays an important structural role in the spike [13].

A few remarks are needed about the constructs. Gn^B is formed by a β -sandwich followed by an amphipathic α -helical hairpin (Fig. 2a). The latter is a membrane proximal region (Gn-MPER) that interacts with the viral membrane through a hydrophobic surface [13]. Both regions interact to make a compact Gn^B tetramer. In solution, the Gn^B tetramer is sticky and aggregates easily, complicating its purification and subsequent handling. An alternative is to remove the Gn-MPER region and produce a construct containing only the β -sandwich domain. This construct produces much better but is monomeric in solution. A similar consideration can be made for the Gc ectodomain. At its C-terminal end, between the transmembrane region and a conserved CX₄C motif, there is an amphipathic helix that interacts with the viral membrane on the viral particle. This region is known as Gc-MPER (Fig. 2b) and interacts with the viral membrane [13]. Although it is possible to produce the Gc ectodomain containing this region, when it is removed, a larger amount of protein is obtained. The heterodimer Gn/Gc is difficult to purify because the complex cannot be reconstituted *in vitro* from its components and the wild-type form is too labile to be purified. The approach that we have followed to purify the complex is to connect both subunits using a flexible linker joining the C-terminus of Gn^H (we have indicated the boundary between Gn^H and Gn^B in various hantaviruses in Fig. 2a) with the N-terminus of Gc. The sequence of the linker (GGSGLVPRG SGGGSGGGSWHPQFEKGGGTGGGTLVPRGSGTGG) contains two thrombin cleavage sites (underlined) at either end and a strep-tag sequence (in bold) in the middle, separated by flexible GGGs or GGGT repeats. We have introduced these motifs to facilitate the crystallization of the complex but for some applications, it may be necessary to remove them. Based on the structure of the complex, we estimate that the minimum linker length needed to join Gn^H and Gc in the prefusion configuration is about 25 amino acids, assuming that the linker is flexible and does not interfere with the folding of the complex. Using the linker showed above and the protocol detailed in this chapter, we have succeeded in purifying the Gn^H/Gc heterodimers from PUUV, ANDV, HNTV, SNV, Maporal (MPRV), and Choclo viruses (CHOV).

Purification of Hantavirus Glycoproteins in Insect Cells

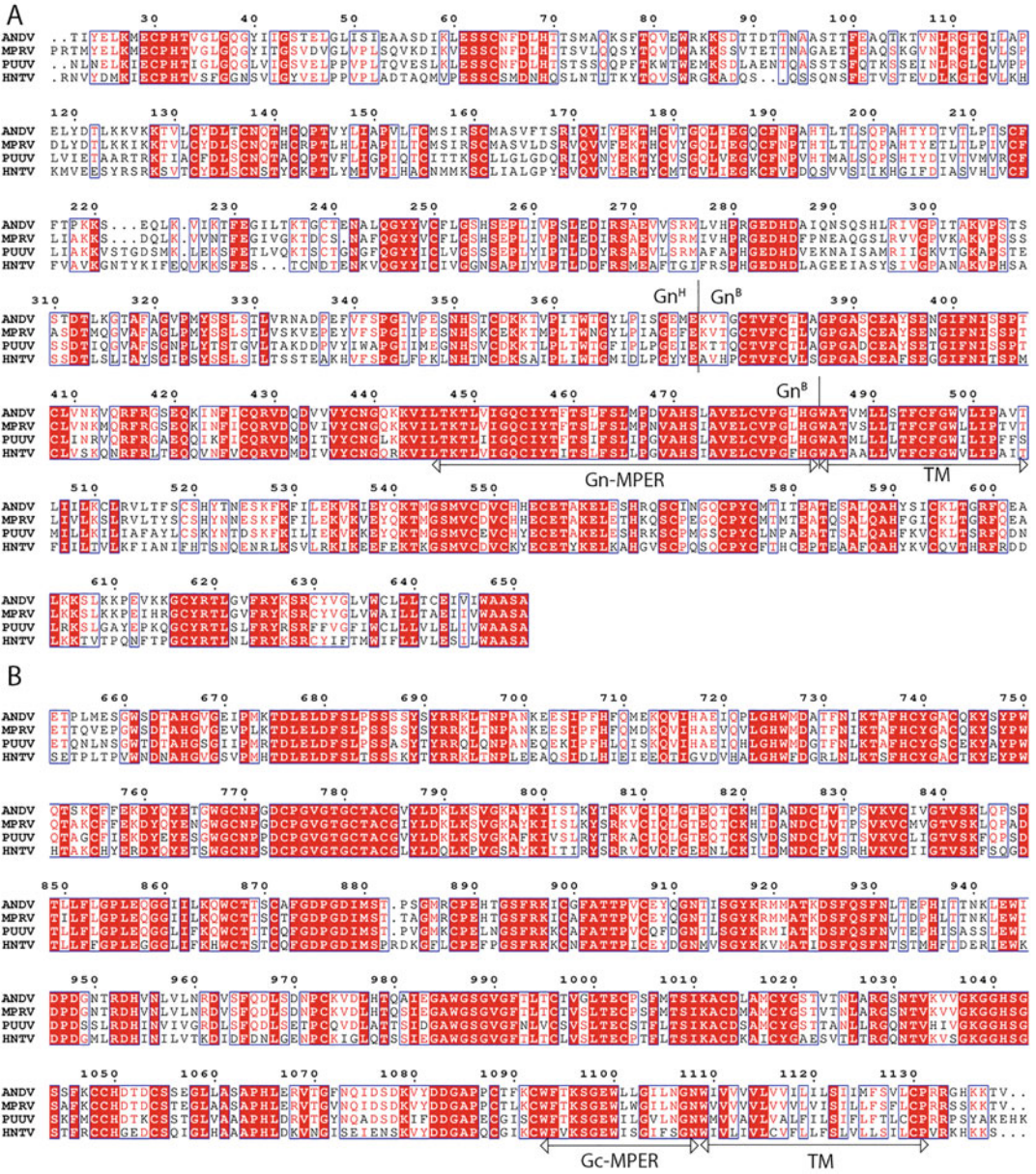


Fig. 2 Sequence analysis. Multiple sequence alignment of Gn (**a**) and Gc (**b**) from four representative hantaviruses: Andes virus (ANDV, NP_604472.1), Maporal virus (MPRV, YP_009362281), Puumala virus (PUUV, CAB43026.1), and Hantaan virus (HNTV, CAA68456.1). Residues are colored according to conservation: strictly conserved residues are colored in white on a red background, partially conserved in red on a white background and the non-conserved residues in black on a white background. Gn^H and Gn^B boundaries, transmembrane regions (TM), and the MPER regions of Gn and Gc are indicated

2 Materials		119
2.1 Plasmids	1. pMT/BiP vector series (<i>see Note 1</i>).	120
	2. pCoPURO plasmid (Addgene, #17533 [24]) for selection of stable cell lines (<i>see Note 2</i>).	121 122
2.2 Cells and Medium	1. Drosophila S2 cells.	123
	2. Growing medium: SFM4Insect medium supplemented with 1% penicillin/streptomycin (<i>see Note 3</i>).	124 125
	3. Selection medium: Growing medium supplemented with 8 µg/mL of puromycin.	126 127
	4. Freezing medium: 90% fetal bovine serum, 10% DMSO.	128
	5. Effectene transfection kit.	129
	6. Trypan Blue Solution 0.4%.	130
	7. 5 µM CdCl ₂ or 500 µM CuSO ₄ .	131
2.3 Other Materials	1. Cell culture flasks 25 cm ² , 75 cm ² , and 150 cm ² .	132
	2. Cell scrapers.	133
	3. Freezing container.	134
	4. Cryo tubes.	135
	5. Inverted light microscope.	136
	6. Automated cell counter.	137
	7. 1 L or 4 L glass spinner flasks or 5 L Erlenmeyer flasks with ordinary magnetic stirrers.	138 139
	8. Incubator at 28 °C without CO ₂ .	140
	9. Sterile disposable bottle top filter, membrane PES 0.2 µm filter.	141
	10. Tangential ultrafiltration devices.	142
	11. FPLC system.	143
	12. Class II laminar flow hood.	144
2.4 Protein Purification Buffers	1. Washing buffer: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA.	145 146
	2. Elution buffer: 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin.	147 148
	3. Gel filtration buffer: 10 mM Tris-HCl pH 8.0, 100 mM NaCl.	149

3 Methods

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3.1 Thawing S2 Cells

1. Thaw cells quickly in a 37 °C water bath and transfer them to a centrifuge tube containing 5 mL of growing medium. Centrifuge them for 5 min at 200 g. 151
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2. Resuspend the cell pellet in 15 mL of growing medium and transfer the cell suspension to a 75 cm² flask. Let the cells recover for 2–3 days. 154
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3.2 Passaging of S2 Cells

1. Use a pipette to wash down the surface of the flask and dislodge any adherent cells. Sometimes it can be useful to gently tap the side of the bottle with the palm of the hand to dislodge the cells before washing them with the pipette. 157
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2. Determine the cell density and viability using an automated cell counter (*see Note 4*). To determine viability, mix equal volumes of cells and trypan blue solution. As the membranes of dead cells are leaky, they will take up the dye and be colored blue. The device will be able to distinguish between live and dead cells and obtain a viability percentage. If an automatic cell counter is not available, it is possible to estimate the cell density and viability using a hemacytometer and the trypan blue dye method. Passaging of S2 cells should be performed when the culture density reaches about 10⁷ cells/mL. 161
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3. Dilute the cells in growing medium to a final concentration of 2 × 10⁶ cells/mL and seed them into a new flask (*see Note 5*). 171
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3.3 Transfection and Selection of Stable Cell Lines

1. Seed 15 × 10⁶ cells in 15 mL of growing medium into a 75 cm² flask (final density equivalent to 10⁶ cells/mL) and incubate them overnight at 28 °C (*see Notes 6 and 7*). 173
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2. Mix 2.0 µg of the expression plasmid with 0.1 µg of the selection plasmid (*see Notes 8 and 9*) into a sterile 1.5 mL eppendorf. Dilute the DNA mixture (minimal DNA concentration of 0.1 µg/µL) with Effectene Buffer EC, to a total volume of 150 µL. Add 16 µL Effectene Enhancer and mix by vortexing for 1 s. 176
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3. Incubate the mixture at room temperature for 5 min and then spin it down for a few seconds to remove drops from the top of the tube. 182
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4. Add 20 µL Effectene Transfection Reagent to the DNA–Enhancer mixture. Mix by pipetting up and down five times or by vortexing for 10 s. 185
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5. Incubate the samples for 15 min at room temperature (15–25 °C) to allow the formation of the transfection complex. 188
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6. Add 1 mL of growing medium to the tube containing the transfection complexes. Mix by pipetting up and down and add the transfection complexes drop-wise onto the cells. Do this gently to avoid dislodging the adhered cells. 190-193
7. Incubate cells at 28 °C for 24 h. 194
8. Carefully place the flask upright, trying not to dislodge the adhered cells. Transfer the supernatant into a fresh tube and add 10 mL of selection medium to the flask. Spin down the supernatant at 200 g for 3 min. Resuspend the cell pellet in 5 mL of selection medium and put the cells back into the same flask. 195-200
9. Incubate cells at 28 °C for at least 5 days. Monitor them using a microscope. At first, the cells should appear healthy and dense. Over time, non-transfected cells die resulting in a reduction of cell density (*see Note 10*). 201-204
10. Resuspend the cells tapping the flask and pipetting up and down. Transfer the 15 mL of the cell suspension to a 150 cm² flask and add 15 mL of selection medium. 205-207
11. Incubate cells at 28 °C for at least 3 days. Monitor them under the microscope. If the transfection has worked correctly, the number of cells should double every 3 days and the stable cell line would be established (*see Note 11*). 208-211
12. Transfer 10 ml of cell suspension into a 150 cm² flask containing 20 ml of selection medium. Repeat 2 times to prepare 2 more 150 cm² flasks. Incubate them at 28 °C for at least 3 days (*see Note 12*). 212-215

3.4 Expansion of Cell Culture

1. Determine the cell density and viability using an automated cell counter. When the cell density in the 150 cm² flasks reaches around $1-2 \times 10^7$ cells/mL, split the cells into nine 150 cm² flasks transferring 10 mL cell suspension and 20 mL fresh selection medium to each flask (270 mL in total). One flask will be used to prepare a stock of frozen cells as described in Subheading 3.6. The remaining flasks are dedicated to the preparation of the large culture in either spinner or Erlenmeyer flasks (*see Note 13*). 216-224
2. After 3–5 days, look at the cells under the microscope to confirm that they are growing healthy and measure cell density and viability. Resuspend the cells by tapping the flask and by pipetting up and down to wash the flask surface (*see Note 14*). 225-228
3. To maintain the cell line, transfer 5 mL of the cell suspension to a 150 cm² flask and add 25 mL of selection medium. 229-230

4. Transfer the remaining 235 mL of cell suspension into a spinner flask or an Erlenmeyer and add about 250 mL of growing medium to a total volume of about 500 mL (*see* **Notes 13 and 15**). Grow the cells at 28 °C. If an Erlenmeyer flask is used, use an agitation rate of 130 rpm. 231–235
5. After 3–5 days, count the cells. When the cell density reaches $3\text{--}5 \times 10^7$ cells/mL, add 500 mL of growing medium to a total volume of about 1 L (*see* **Note 16**). Induce the culture by adding 5 μM CdCl₂ or 500 μM CuSO₄ (*see* **Note 17**). 236–239
6. After 3–10 days, collect the cell solution and centrifuge at 6000 g for 30 min at 15 °C to separate the cells and supernatant (**Note 18**). Discard the cell pellet. 240–242

3.5 Protein Purification

1. Concentrate the supernatant fraction by using a tangential ultrafiltration device. Starting from a volume of 1 to 4 L, concentrate up to 50–100 mL. Adjust the pH with 1/10 of the volume of 1 M Tris-HCl (pH 8) and 120 μl of biotin-blocking solution for each 50 mL of concentrated supernatant. 243–247
2. Centrifuge the concentrated supernatant at 40,000 g for 30 min at 4 °C and filter it using a 0.22 μm top filter (*see* **Note 19**). Load the filtered supernatant into a 50 mL loop and plug it into an FPLC system. 248–251
3. Pass the supernatant through a StrepTrap chromatography column pre-equilibrated in washing buffer. 252–253
4. Wash the column with 10 bed volumes of washing buffer until the OD₂₈₀ reaches baseline and then elute the protein complex with 10 bed volumes elution buffer. 254–256
5. Concentrate the fractions containing the eluted protein to 0.5 mL using 20 mL concentrators (cut-off 10 kDa) (*see* **Note 20**). 257–259
6. Pre-equilibrate the size exclusion chromatography column (Superdex 200 10/300) with 60 mL of gel filtration buffer. 260–261
7. Load the sample using a 500 μL loop and run the gel filtration column at a suitable flow rate (0.4 mL/min). Monitor the absorbance signal at 280 nM and collect fractions. Hantavirus glycoproteins usually elute in two peaks, a minor dimeric fraction and a major monomeric fraction eluting respectively at about 11 and 12.5 mL (**Fig. 3**). 262–267
8. Analyze the fractions using SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) (**Fig. 3**) (*see* **Note 21**). 268–269
9. Pool the fractions of interest and concentrate using 6 mL concentrators (cut-off 10 kDa). Check concentration by measuring the optical density at 280 nM using a Nanodrop device (*see* **Note 22**). Prepare aliquots and flash freeze them in liquid nitrogen. 270–274

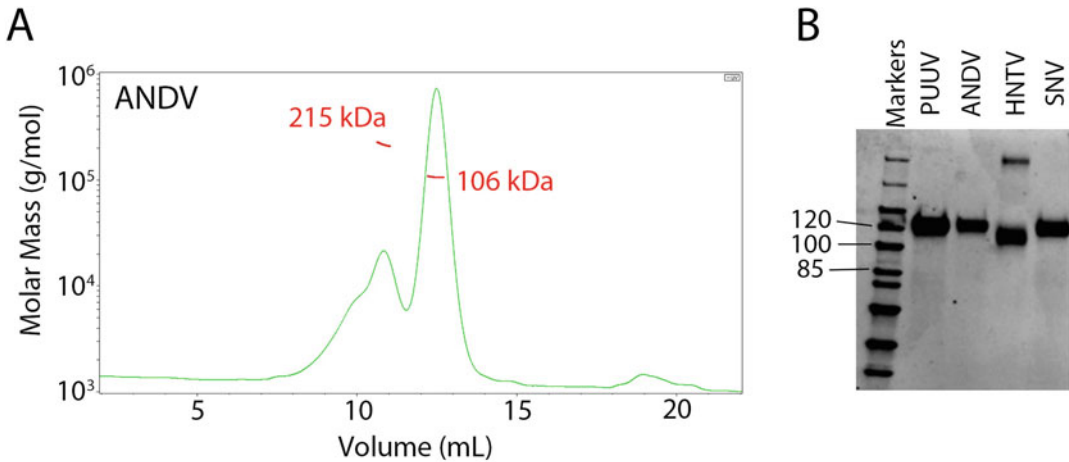


Fig. 3 Protein purification. (a) SEC elution volume profiles of Gn^H/Gc from ANDV at pH 8.0. The left axis indicates the molecular mass (kDa) determined by MALS, with the values for each species indicated on the corresponding peak. (b) SDS-PAGE of the monomeric peaks of Gn^H/Gc from PUUV, ANDV, HNTV, and SNV, as indicated

3.6 Freezing S2 Cells

1. Freezing of S2 cells should be performed when the culture density reaches about 10^7 cells/mL. 275
2. Dislodge any adherent cells by tapping the flask and pipetting up and down to wash down the surface of the flask. 276
3. Spin down the cells for 5 min at 200 g and resuspend the cell pellet in cold freezing medium. Cell density should be higher than 1.5×10^7 cell/mL in the freezing medium (*see Note 23*). 277
4. Aliquot resuspended cells in 1.5 mL aliquots in cryo vials and freeze them at -80 °C for 24–48 h in a “Mr. Frosty”. 278
5. Transfer cryovials to liquid nitrogen for long-term storage. 279

4 Notes

1. To optimize protein production and help in the purification, we cloned synthetic codon-optimized genes for expression in *Drosophila* cells into a modified pMT/BiP plasmid, which translates the protein in frame with a Thrombin cleavage site (underlined) and a double strep-tag (in bold) at the C-terminal end of the sequence (**G**SGLVPR**G**SGGSGGSAG WSHPQFEKGGGSGGGSGGG**WSHPQFEK**). 285
2. The pCoPuro plasmid confers resistance to puromycin. There is also the possibility of using pCoBlasto or pCoHygro, which confer resistance to blasticidin S hydrochloride and hygromycin B, respectively. 286

3. Another culture medium that can also be used is Insect Xpress. 297
4. The TC20 automated cell counter counts cells within a range 298
of 10^4 – 10^7 cells/mL. S2 cells can grow at densities above this 299
range, so it may be necessary to dilute the cells. For your 300
calculation note that the counter takes into consideration just 301
the 1:2 dilution in Trypan Blue solution. 302
5. As a rule of thumb, cells are diluted 1 to 5 or 6. Higher dilution 303
rates may decrease cell viability. 304
6. A quicker alternative that works in our hands is to seed 21×10^6 305
cells 1 h before transfection. 306
7. Under optimal working conditions, incubate the cells at 28 °C. 307
S2 cell incubations can also be performed at room temperature, 308
omitting the need for an incubator. However, the incubation 309
time between passages must be corrected accordingly. 310
8. Transfections should be made with DNA of the highest purity. 311
Contaminants may kill the cells and interfere with the forma- 312
tion of the transfection complexes, thus decreasing transfection 313
efficiency. However, the purer the DNA, the more expensive 314
the process. We generally purify the resistance plasmid using a 315
Midi-prep kit and the different expression plasmids using a 316
miniprep kit. Transfections performed with these plasmids 317
have good efficiencies. Purification of the expression plasmid 318
by the use of a Midi Kit can increase transfection efficiency in 319
difficult cases. 320
9. The number of inserted gene copies in the genome, and there- 321
fore the level of protein expression, can be manipulated by 322
varying the ratio of expression plasmid to resistance 323
plasmid [25]. 324
10. The next steps simultaneously allow the selection of the stably 325
transfected population and cell amplification. The presence of a 326
large number of cell clusters in suspension indicates that the 327
cells are in a good state and are ready to be amplified. The first 328
two passages after transfection are the most critical ones. If the 329
cells are too diluted, they will not survive. 330
11. In most cases, the stable S2 cell transfectants maintain their 331
expression level for a very large number of passages. However, 332
in some cases, the quantity or quality of secreted protein wor- 333
sens with the number of passages. We recommend freezing 334
aliquots of the stable S2 transfectants immediately after the 335
stable lines are generated. To do so, please follow the instruc- 336
tions in Subheading 3.6 (Freezing S2 cells). 337
12. S2 cells begin to grow by adhering to the bottom of the flask 338
and then, as density increases, they detach from the bottom 339
and form clusters in suspension. Depending on the cell line, 340
this process can be faster or slower. It may be possible to have 341

- many cells in suspension and still have many cells attached to the bottom of the plate. In this case, it is advisable to recover these cells as well using a cell scraper. 342
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13. Eight 150 cm² flasks give the right number of cells to start the culture in a 4 L spinner. Although for a 1 L spinner six 150 cm² flasks are sufficient, the use of eight flasks allows to reach the time of induction more quickly. 345
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 14. For S2 stable transfectants, 100% viability is not a prerequisite. A viability index above 90% is considered acceptable. 349
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 15. The presence of the selection antibiotic (*see Note 2*) is required for each passaging step to keep selection, but it can be omitted during amplification for protein production to save costs. However, if amplification is done immediately after the establishment of the cell line, we strongly advise using a selection medium during the first production. 351
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 16. If a larger final volume is needed, wait 3–5 days and dilute the cells 1:2 or 1:3. Repeat as many times as necessary until the desired volume is reached. The final amount of CdCl₂ or CuSO₄ used for the induction has to be corrected accordingly. 357
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 17. The expression of the proteins is under the control of the metallothionein (MT) promoter, which is inducible by adding either CdCl₂ or CuSO₄. Although the former is a more effective inducer, the latter is to be preferred for its lower toxicity. 361
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 18. The optimal induction time depends on each protein and the concentration and type of inductor (CdCl₂ or CuSO₄) and must be determined for each stable S2 cell transfectant. This can be done on a small scale by collecting 1 mL of supernatant every day for 10 days after induction. After centrifugation at 20,000 g for 30 min at 4 °C in a benchtop centrifuge, run a Western blot using streptactin-HRP for detection. We have found that the optimal induction conditions for hantavirus glycoproteins are 5 μM CdCl₂ for 5–6 days. 365
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 19. The filtration step is not compulsory but allows the preservation of the affinity purification columns for longer. 374
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 20. For concentration, we centrifuge at 4000 g for 15 min at 4 °C. For high volumes, multiple centrifugation cycles may be necessary. It is best to avoid prolonged centrifugation steps that might induce protein aggregation. In any case, it is advisable to resuspend the protein with a pipette between two successive centrifugations. 376
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 21. SDS-PAGE analysis is essential to check the purity and the quality of the eluted proteins. Usually, we run it in reducing conditions, i.e., adding 100 mM of fresh DTT in the loading buffer, but in some cases can be essential to run it in non-reducing conditions to detect the possible presence of disulfide-linked oligomers. 382
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22. To estimate the protein concentration more precisely, we 388
advise to determine the theoretical molar extinction coefficient 389
from the protein sequence. 390
23. From one 150 cm² flask it's possible to freeze between 3 and 391
4 cryo-vials. 392

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