



**HAL**  
open science

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

Annalisa Meola, Pablo Guardado-Calvo

► **To cite this version:**

Annalisa Meola, Pablo Guardado-Calvo. Production and Purification of Hantavirus Glycoproteins in *Drosophila melanogaster* S2 Cells. *Recombinant Glycoproteins*, 2762, Springer US, pp.3-16, 2024, *Methods in Molecular Biology*, 10.1007/978-1-0716-3666-4\_1 . hal-04582090

**HAL Id: hal-04582090**

**<https://hal.science/hal-04582090>**

Submitted on 20 Jun 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## 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.

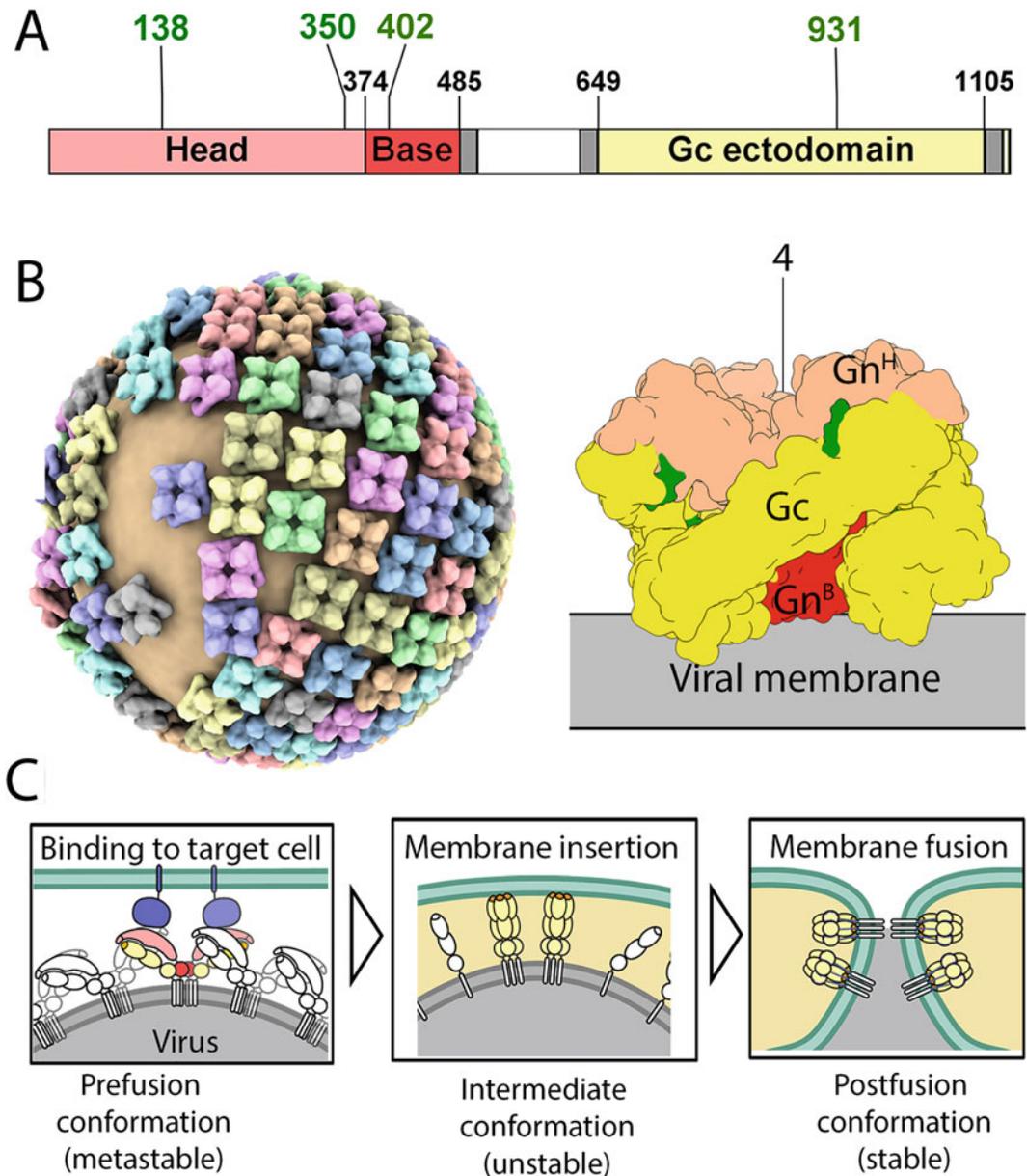
The surface of hantaviruses is covered by a protein lattice consisting of two glycoproteins termed Gn and Gc [2]. These proteins mediate virion assembly [3, 4], adsorption to the plasma membrane of susceptible cells [5], and fusion in the acidic lumen of endosomes [6]. They are the only targets of neutralizing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

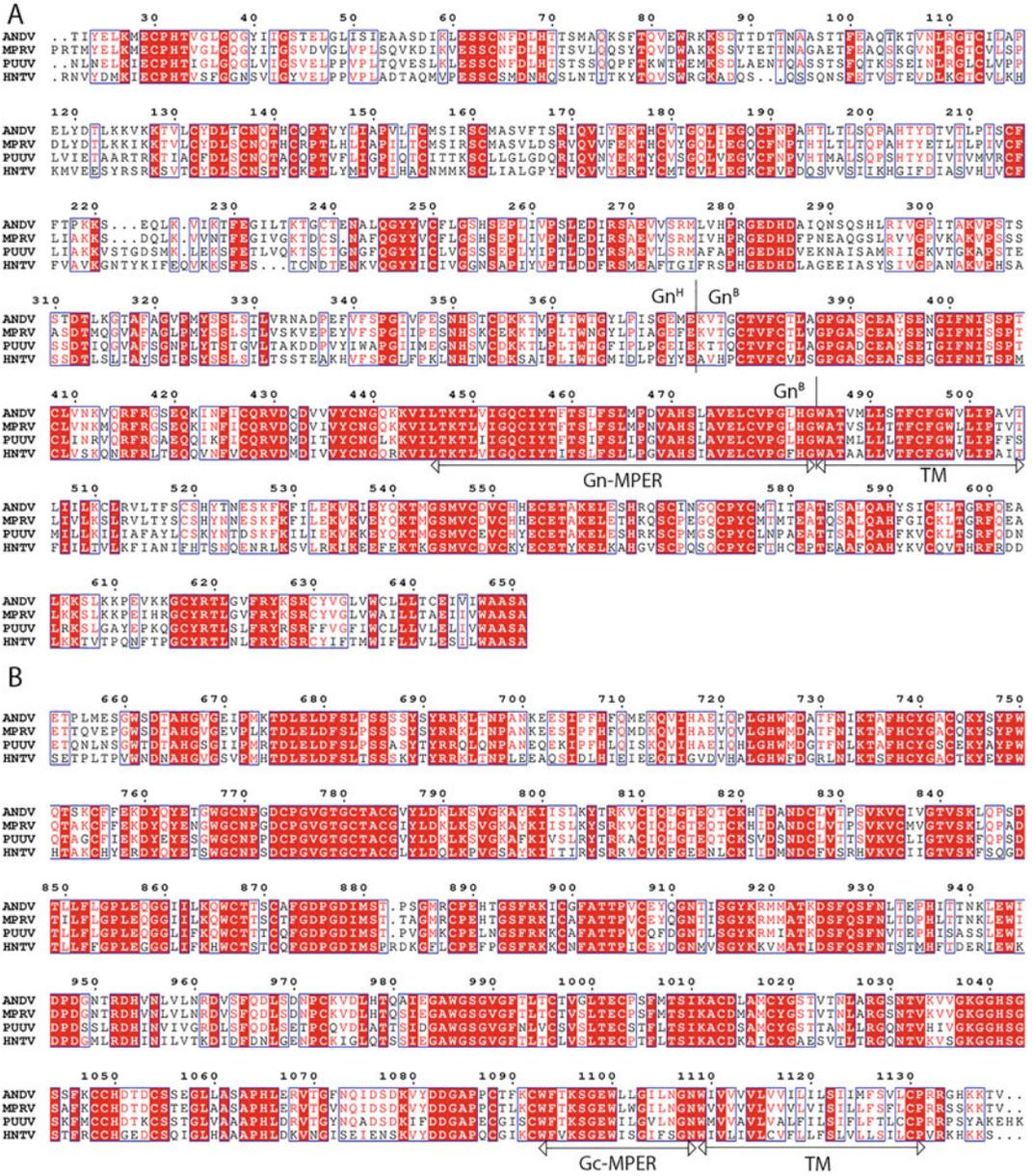


**Fig. 1** The organization of the hantavirus glycoproteins. (a) Organization of the glycoprotein precursor. The different regions of Gn (Gn<sup>H</sup>, Gn<sup>B</sup>) are colored in light and dark red and Gc in yellow with the boundaries indicated on the diagram in black numbers. The TM segments are 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<sup>H</sup>, Gn<sup>B</sup>, 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<sup>B</sup> is formed by a  $\beta$ -sandwich followed by an amphipathic  $\alpha$ -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<sup>B</sup> tetramer. In solution, the Gn<sup>B</sup> 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  $\beta$ -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 CX<sub>4</sub>C 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<sup>H</sup> (we have indicated the boundary between Gn<sup>H</sup> and Gn<sup>B</sup> 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<sup>H</sup> 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<sup>H</sup>/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<sup>H</sup> and Gn<sup>B</sup> boundaries, transmembrane regions (TM), and the MPER regions of Gn and Gc are indicated

---

<b>2 Materials</b>		119
<b>2.1 Plasmids</b>		
	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
<b>2.2 Cells and Medium</b>		
	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 <sub>2</sub> or 500 µM CuSO <sub>4</sub> .	131
<b>2.3 Other Materials</b>		
	1. Cell culture flasks 25 cm <sup>2</sup> , 75 cm <sup>2</sup> , and 150 cm <sup>2</sup> .	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 <sub>2</sub> .	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
<b>2.4 Protein Purification Buffers</b>		
	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

150

#### 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  
152  
153
2. Resuspend the cell pellet in 15 mL of growing medium and transfer the cell suspension to a 75 cm<sup>2</sup> flask. Let the cells recover for 2–3 days. 154  
155  
156

#### 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  
158  
159  
160
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 10<sup>7</sup> cells/mL. 161  
162  
163  
164  
165  
166  
167  
168  
169  
170
3. Dilute the cells in growing medium to a final concentration of  $2 \times 10^6$  cells/mL and seed them into a new flask (*see Note 5*). 171  
172

#### 3.3 Transfection and Selection of Stable Cell Lines

1. Seed  $15 \times 10^6$  cells in 15 mL of growing medium into a 75 cm<sup>2</sup> flask (final density equivalent to 10<sup>6</sup> cells/mL) and incubate them overnight at 28 °C (*see Notes 6 and 7*). 173  
174  
175
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  
177  
178  
179  
180  
181
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  
183  
184
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  
186  
187
5. Incubate the samples for 15 min at room temperature (15–25 °C) to allow the formation of the transfection complex. 188  
189

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 cm<sup>2</sup> 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 cm<sup>2</sup> flask containing 20 ml of selection medium. Repeat 2 times to prepare 2 more 150 cm<sup>2</sup> flasks. Incubate them at 28 °C for at least 3 days (*see Note 12*). 212-215

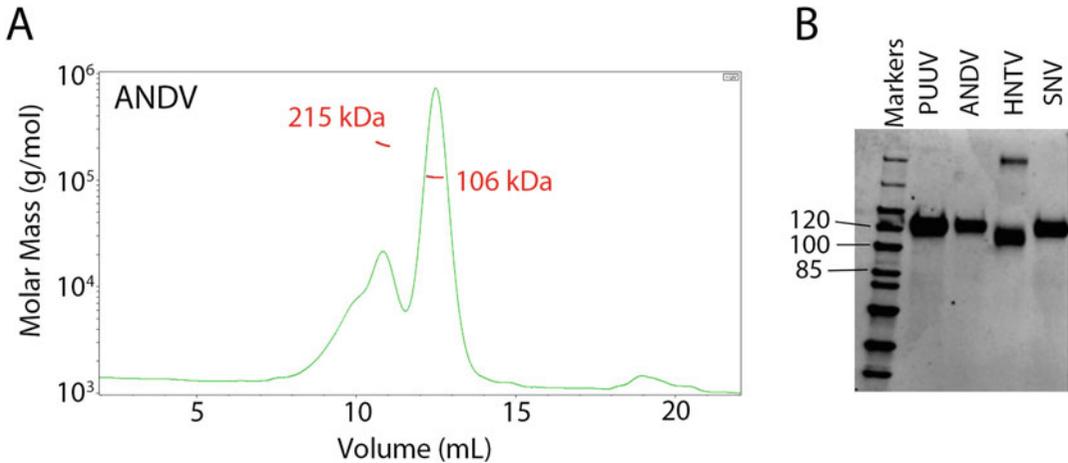
### 3.4 Expansion of Cell Culture

1. Determine the cell density and viability using an automated cell counter. When the cell density in the 150 cm<sup>2</sup> flasks reaches around  $1-2 \times 10^7$  cells/mL, split the cells into nine 150 cm<sup>2</sup> 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 cm<sup>2</sup> 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  $\mu\text{M}$  CdCl<sub>2</sub> or 500  $\mu\text{M}$  CuSO<sub>4</sub> (*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  $\mu\text{l}$  of biotinylation 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  $\mu\text{m}$  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 OD<sub>280</sub> 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  $\mu\text{L}$  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<sup>H</sup>/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<sup>H</sup>/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  $1.5 \times 10^7$  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**SGG**S**GG**S**AG WSHPQFEKGGGSGGGSGGG**S**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  $21 \times 10^6$  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  
343  
344
13. Eight 150 cm<sup>2</sup> flasks give the right number of cells to start the culture in a 4 L spinner. Although for a 1 L spinner six 150 cm<sup>2</sup> flasks are sufficient, the use of eight flasks allows to reach the time of induction more quickly. 345  
346  
347  
348
  14. For S2 stable transfectants, 100% viability is not a prerequisite. A viability index above 90% is considered acceptable. 349  
350
  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  
352  
353  
354  
355  
356
  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 CdCl<sub>2</sub> or CuSO<sub>4</sub> used for the induction has to be corrected accordingly. 357  
358  
359  
360
  17. The expression of the proteins is under the control of the metallothionein (MT) promoter, which is inducible by adding either CdCl<sub>2</sub> or CuSO<sub>4</sub>. Although the former is a more effective inducer, the latter is to be preferred for its lower toxicity. 361  
362  
363  
364
  18. The optimal induction time depends on each protein and the concentration and type of inductor (CdCl<sub>2</sub> or CuSO<sub>4</sub>) 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 CdCl<sub>2</sub> for 5–6 days. 365  
366  
367  
368  
369  
370  
371  
372  
373
  19. The filtration step is not compulsory but allows the preservation of the affinity purification columns for longer. 374  
375
  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  
377  
378  
379  
380  
381
  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  
383  
384  
385  
386  
387

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 cm<sup>2</sup> flask it's possible to freeze between 3 and 391  
4 cryo-vials. 392

394 **References**

- 396 1. Watson DC, Sargianou M, Papa A, Chra P, 444  
397 Starakis I, Panos G (2014) Epidemiology of 445  
398 Hantavirus infections in humans: a compre- 446  
399 hensive, global overview. *Crit Rev Microbiol* 447  
400 40(3):261–272. [https://doi.org/10.3109/](https://doi.org/10.3109/1040841X.2013.783555) 448  
401 [1040841X.2013.783555](https://doi.org/10.3109/1040841X.2013.783555) 449
- 402 2. Guardado-Calvo P, Rey FA (2017) The enve- 450  
403 lope proteins of the bunyavirales. *Adv Virus* 451  
404 *Res* 98:83–118. [https://doi.org/10.1016/bs.](https://doi.org/10.1016/bs.avir.2017.02.002) 452  
405 [avir.2017.02.002](https://doi.org/10.1016/bs.avir.2017.02.002) 453
- 406 3. Acuna R, Cifuentes-Munoz N, Marquez CL, 454  
407 Bulling M, Klingstrom J, Mancini R, Lozach 455  
408 PY, Tischler ND (2014) Hantavirus Gn and Gc 456  
409 glycoproteins self-assemble into virus-like par- 457  
410 ticles. *J Virol* 88(4):2344–2348. [https://doi.](https://doi.org/10.1128/JVI.03118-13) 458  
411 [org/10.1128/JVI.03118-13](https://doi.org/10.1128/JVI.03118-13) 459
- 412 4. Mittler E, Dieterle ME, Kleinfelter LM, Slough 460  
413 MM, Chandran K, Jangra RK (2019) Hantavi- 461  
414 rus entry: perspectives and recent advances. 462  
415 *Adv Virus Res* 104:185–224. [https://doi.](https://doi.org/10.1016/bs.avir.2019.07.002) 463  
416 [org/10.1016/bs.avir.2019.07.002](https://doi.org/10.1016/bs.avir.2019.07.002) 464
- 417 5. Jangra RK, Herbert AS, Li R, Jae LT, Kleinfel- 465  
418 ter LM, Slough MM, Barker SL, Guardado- 466  
419 Calvo P, Roman-Sosa G, Dieterle ME, Kuehne 467  
420 AI, Muenza NA, Wirchnianski AS, Nyakatura 468  
421 EK, Fels JM, Ng M, Mittler E, Pan J, 469  
422 Bharrhan S, Wec AZ, Lai JR, Sidhu SS, Tischler 470  
423 ND, Rey FA, Moffat J, Brummelkamp TR, 471  
424 Wang Z, Dye JM, Chandran K (2018) 472  
425 Protocadherin-1 is essential for cell entry by 473  
426 new world hantaviruses. *Nature* 563(7732): 474  
427 559–563. [https://doi.org/10.1038/s41586-](https://doi.org/10.1038/s41586-018-0702-1) 475  
428 [018-0702-1](https://doi.org/10.1038/s41586-018-0702-1) 476
- 429 6. Guardado-Calvo P, Bignon EA, Stettner E, Jef- 477  
430 fers SA, Perez-Vargas J, Pehau-Arnaudet G, 478  
431 Tortorici MA, Justin JL, England P, Tischler 479  
432 ND, Rey FA (2016) Mechanistic insight into 480  
433 bunyavirus-induced membrane fusion from 481  
434 structure-function analyses of the hantavirus 482  
435 envelope glycoprotein Gc. *PLoS Pathog* 483  
436 12(10):e1005813. [https://doi.org/10.1371/](https://doi.org/10.1371/journal.ppat.1005813) 484  
437 [journal.ppat.1005813](https://doi.org/10.1371/journal.ppat.1005813) 485
- 438 7. Mittler E, Wec AZ, Tynell J, Guardado-Calvo- 486  
439 P, Wigren-Bystrom J, Polanco LC, O'Brien 487  
440 CM, Slough MM, Abelson DM, Serris A, 488  
441 Sakharkar M, Pehau-Arnaudet G, Bakken RR, 489  
442 Geoghegan JC, Jangra RK, Keller M, Zeitlin L, 490  
443 Vapalahti O, Ulrich RG, Bornholdt ZA, 491
- Ahlm C, Rey FA, Dye JM, Bradfute SB, 444  
Strandin T, Herbert AS, Forsell MNE, Walker 445  
LM, Chandran K (2022) Human antibody 446  
recognizing a quaternary epitope in the Puu- 447  
mala virus glycoprotein provides broad protec- 448  
tion against orthohantaviruses. *Sci Transl Med* 449  
14(636):eab15399. [https://doi.org/10.](https://doi.org/10.1126/scitranslmed.abl5399) 450  
1126/scitranslmed.abl5399 451
8. Engdahl TB, Kuzmina NA, Ronk AJ, Mire CE, 452  
Hyde MA, Kose N, Joselyn MD, Sutton RE, 453  
Mehta A, Wolters RM, Lloyd NM, Valdivieso 454  
FR, Ksiazek TG, Hooper JW, Bukreyev A, 455  
Crowe JE Jr (2021) Broad and potentially neu- 456  
tralizing monoclonal antibodies isolated from 457  
human survivors of new world hantavirus 458  
infection. *Cell Rep* 36(3):109453. [https://](https://doi.org/10.1016/j.celrep.2021.109453) 459  
[doi.org/10.1016/j.celrep.2021.109453](https://doi.org/10.1016/j.celrep.2021.109453) 460
9. Engdahl TB, Crowe JE Jr (2020) Humoral 461  
immunity to hantavirus infection. *mSphere* 462  
5(4). [https://doi.org/10.1128/mSphere.](https://doi.org/10.1128/mSphere.00482-20) 463  
[00482-20](https://doi.org/10.1128/mSphere.00482-20) 464
10. Willensky S, Bar-Rogovsky H, Bignon EA, 465  
Tischler ND, Modis Y, Dessau M (2016) Crys- 466  
tal structure of glycoprotein C from a hantavi- 467  
rus in the post-fusion conformation. *PLoS* 468  
*Pathog* 12(10):e1005948. [https://doi.org/](https://doi.org/10.1371/journal.ppat.1005948) 469  
[10.1371/journal.ppat.1005948](https://doi.org/10.1371/journal.ppat.1005948) 470
11. Guardado-Calvo P, Rey FA (2021) The viral 471  
class II membrane fusion machinery: divergent 472  
evolution from an ancestral heterodimer. 473  
*Viruses* 13(12). [https://doi.org/10.3390/](https://doi.org/10.3390/v13122368) 474  
[v13122368](https://doi.org/10.3390/v13122368) 475
12. Guardado-Calvo P, Rey FA (2021) The surface 476  
glycoproteins of hantaviruses. *Curr Opin Virol* 477  
50:87–94. [https://doi.org/10.1016/j.coviro.](https://doi.org/10.1016/j.coviro.2021.07.009) 478  
[2021.07.009](https://doi.org/10.1016/j.coviro.2021.07.009) 479
13. Serris A, Stass R, Bignon EA, Muenza NA, Man- 480  
uguerra JC, Jangra RK, Li S, Chandran K, 481  
Tischler ND, Huiskonen JT, Rey FA, 482  
Guardado-Calvo P (2020) The hantavirus sur- 483  
face glycoprotein lattice and its fusion control 484  
mechanism. *Cell* 183(2):442–456 e416. 485  
<https://doi.org/10.1016/j.cell.2020.08.023> 486
14. Bignon EA, Alborno A, Guardado-Calvo P, 487  
Rey FA, Tischler ND (2019) Molecular orga- 488  
nization and dynamics of the fusion protein Gc 489  
at the hantavirus surface. *elife* 8. [https://doi.](https://doi.org/10.7554/eLife.46028) 490  
[org/10.7554/eLife.46028](https://doi.org/10.7554/eLife.46028) 491

- 492 15. Rissanen I, Stass R, Krumm SA, Seow J, Huls- 530  
 493 wit RJ, Paesen GC, Hepojoki J, Vapalahti O, 531  
 494 Lundkvist A, Reynard O, Volchkov V, Doores 532  
 495 KJ, Huiskonen JT, Bowden TA (2020) Molec- 533  
 496 ular rationale for antibody-mediated targeting 534  
 497 of the hantavirus fusion glycoprotein. *elife* 535  
 498 9. <https://doi.org/10.7554/eLife.58242>
- 499 16. Li S, Rissanen I, Zeltina A, Hepojoki J, 536  
 500 Raghwani J, Harlos K, Pybus OG, Huiskonen 537  
 501 JT, Bowden TA (2016) A molecular-level 538  
 502 account of the antigenic hantaviral surface. 539  
 503 *Cell Rep* 16(1):278. <https://doi.org/10.1016/j.celrep.2016.06.039>
- 505 17. Rissanen I, Stass R, Zeltina A, Li S, Hepojoki J, 542  
 506 Harlos K, Gilbert RJC, Huiskonen JT, Bowden 543  
 507 TA (2017) Structural transitions of the con- 544  
 508 served and metastable hantaviral glycoprotein 545  
 509 envelope. *J Virol* 91(21). <https://doi.org/10.1128/JVI.00378-17>
- 511 18. Rissanen I, Krumm SA, Stass R, Whitaker A, 549  
 512 Voss JE, Bruce EA, Rothenberger S, Kunz S, 550  
 513 Burton DR, Huiskonen JT, Botten JW, Bow- 551  
 514 den TA, Doores KJ (2021) Structural basis for 552  
 515 a neutralizing antibody response elicited by a 553  
 516 recombinant hantaan virus Gn immunogen. 554  
 517 *mBio* 12(4):e0253120. <https://doi.org/10.1128/mBio.02531-20>
- 519 19. Duehr J, McMahon M, Williamson B, 555  
 520 Amanat F, Durbin A, Hawman DW, 556  
 521 Noack D, Uhl S, Tan GS, Feldmann H, Kramer 557  
 522 F (2020) Neutralizing monoclonal anti- 558  
 523 bodies against the Gn and the Gc of the 559  
 524 Andes virus glycoprotein spike complex protect 560  
 525 from virus challenge in a preclinical hamster 561  
 526 model. *mBio* 11(2). <https://doi.org/10.1128/mBio.00028-20>
- 528 20. Garrido JL, Prescott J, Calvo M, Bravo F, 562  
 529 Alvarez R, Salas A, Riquelme R, Riosco ML, 563  
 Williamson BN, Haddock E, Feldmann H, 564  
 Barria MI (2018) Two recombinant human 565  
 monoclonal antibodies that protect against 566  
 lethal Andes hantavirus infection in vivo. *Sci*  
*Transl Med* 10(468). <https://doi.org/10.1126/scitranslmed.aat6420>
21. Li S, Rissanen I, Zeltina A, Hepojoki J, 536  
 Raghwani J, Harlos K, Pybus OG, Huiskonen 537  
 JT, Bowden TA (2016) A molecular-level 538  
 account of the antigenic hantaviral surface. 539  
*Cell Rep* 15(5):959–967. <https://doi.org/10.1016/j.celrep.2016.03.082>
22. Kim YK, Shin HS, Tomiya N, Lee YC, Beten- 542  
 baugh MJ, Cha HJ (2005) Production and 543  
 N-glycan analysis of secreted human erythro- 544  
 poietin glycoprotein in stably transfected Dro- 545  
 sophila S2 cells. *Biotechnol Bioeng* 92(4): 546  
 452–461. <https://doi.org/10.1002/bit.20605>
23. Shi X, Elliott RM (2004) Analysis of N-linked 549  
 glycosylation of hantaan virus glycoproteins 550  
 and the role of oligosaccharide side chains in 551  
 protein folding and intracellular trafficking. *J*  
*Virol* 78(10):5414–5422. <https://doi.org/10.1128/jvi.78.10.5414-5422.2004>
24. Iwaki T, Figuera M, Ploplis VA, Castellino FJ 555  
 (2003) Rapid selection of Drosophila S2 cells 556  
 with the puromycin resistance gene. *BioTechniques* 35(3):482–484, 486. <https://doi.org/10.2144/03353bm08>
25. Johansen H, van der Straten A, Sweet R, 560  
 Otto E, Maroni G, Rosenberg M (1989) Regu- 561  
 lated expression at high copy number allows 562  
 production of a growth-inhibitory oncogene 563  
 product in Drosophila Schneider cells. *Genes*  
*Dev* 3(6):882–889. <https://doi.org/10.1101/gad.3.6.882>