

Expression and Ni-NTA-Agarose Purification of Recombinant Hepatitis C Virus E2 Ectodomain Produced in a Baculovirus Expression System

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[Abstract] In this protocol, we describe the production and purification of the ectodomain of the E2₆₆₁ envelope protein (amino acids 384-661) of the Hepatitis C virus, which plays a fundamental role in the entry of the virus into the host cell. This protein has been expressed in both prokaryotic and eukaryotic systems but in small quantities or without native protein characteristics. In our case, we use the Baculovirus expression system in insect cells. E2₆₆₁ is secreted into the extracellular medium and purified by means of affinity chromatography a Ni-NTA-column because the protein has a tag of six histidines at its amino terminal end. The purified protein possesses a native-like conformation and it is produced in large quantities, around 5-6 mg per liter.

Keywords: Hepatitis C virus, Envelope protein, Affinity chromatography, Baculovirus expression system, Recombinant proteins, Ectodomain

[Background] Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (Major *et al.*, 2001; Alter, 2006). At this moment, there is no vaccine for HCV and antivirals are used to treat the HCV infection (Imran *et al.*, 2014). However, treatments are expensive and not 100% effective (Kohli *et al.*, 2014). The HCV envelope glycoprotein E2 is responsible for the interaction with cellular receptors, thus it is a major candidate to study the first steps of the infective cycle of the virus. Previous expression systems produce low levels of heterogeneous protein due to glycosylation and aggregation, and it is difficult to distinguish between molecules that undergo productive and non-productive folding (Flint *et al.*, 2000). In this protocol, we describe the production of the recombinant ectodomain of E2 tagged with a 6xHis extension at N-terminal end of the protein in a baculovirus/insect cell system. The gp67 signal peptide fused to the E2 ectodomain mediates the forced secretion of the recombinant protein. The protein is secreted to the cell supernatant and purified by means of affinity chromatography with a Ni-NTA-Agarose column. The yield of the process was 5-6 mg of protein per liter of media. This protein possesses a native-like conformation as determined by different spectroscopic techniques such as circular dichroism or fluorescence spectroscopy, as well as by its recognition in an enzyme immunoassay by a conformation specific antibody (Rodríguez-Rodríguez *et al.*, 2009). The use of this independent folding domain that is able to acquire its proper folding in absence of the E1 glycoprotein, may contribute to shed light on the biology of HCV (three-dimensional or

secondary structure of the protein and its role in the fusion of the HCV virus and the host cell membranes). Also, it could also be used as a vaccine in the prevention of HCV infection.

Materials and Reagents

1. Pipette tips 200 μ l (Sigma-Aldrich, catalog number: P5161)
2. Tissue culture flasks F75 (75 cm² surface area) (TPP Techno Plastic Products, catalog number: 90075)
3. Tissue culture flasks F150 (150 cm² surface area) (TPP Techno Plastic Products, catalog number: 90150)
4. Cell culture flasks F25 (25 cm² surface area) (Corning, catalog number: 3055)
5. Tissue culture dish 35 mm (SARSTEDT, catalog number: 83.3900)
6. Sterile tube 50 ml (SARSTEDT, catalog number: 62.547.254)
7. Sterile tube 15 ml (Fisher Scientific, catalog number: 05-539-12)
8. Serological pipette 10 ml (SARSTEDT, catalog number: 86.1254.001)
9. Dialysis membrane Spectra/Por[®] 6 (VWR, Spectrum, catalog number: 734-0646)
10. Insect cell line *Spodoptera frugiperda* (Sf9) (Oxford Expression Technologies, catalog number: 100201)
11. Insect cell line *Trichopulsia ni* (High Five) (Tni) (generously donated by PhD J. Pérez-Gil, Dpt. Biochemistry and Molecular Biology, Faculty of Biology, University Complutense of Madrid, Madrid, Spain)
12. Baculovirus transfer vector pAcGP37A (BD, BD Pharmingen[™], catalog number: 21220P)
13. Recombinant transfer vector pAcGP67A-E2₆₆₁, obtained according to the procedure described in Rodriguez-Rodriguez *et al.*, 2009)
14. *FlashBAC* GOLD kit (Oxford Expression Technologies, catalog number: 100201) composed of:
 - a. *flashBAC*[™] DNA
 - b. Positive control transfer plasmid DNA (expressing *lacZ*)
 - c. Baculofectin II
15. Insect-XPRESS[™] Protein-free Insect Cell Medium with L-glutamine (Lonza, catalog number: 12-730Q)
16. Neubauer's counting camera (VWR, MARIENFELD, catalog number: 631-0696)
17. Gentamicin (Sigma-Aldrich, catalog number: G1264)
18. TC100 Insect Medium (Lonza, catalog number: BE02-011F)
19. Ni²⁺-nitrilotriacetic acid agarose (Ni-NTA agarose) resin (QIAGEN, catalog number: 30230)
20. Tris-Base (Sigma-Aldrich, catalog number: T1503)
21. NaCl (Merck, catalog number: 106404)
22. Imidazole (Sigma-Aldrich, catalog number: 56750)
23. EDTA (Sigma-Aldrich, catalog number: 03695)
24. Dodecyl sulfate sodium salt (Merck, catalog number: 1.13760.1000)

25. Glycerol (Sigma-Aldrich, catalog number: G5516)
26. Glycine (Sigma-Aldrich, catalog number: G8898-1KG)
27. Bromophenol blue (United States Biological, catalog number: 12370)
28. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
29. Acrylamide (Sigma-Aldrich, catalog number: A8887-500G)
30. N,N'-Methylenebis(acrylamide) (Sigma-Aldrich, catalog number: M7279-250G)
31. Ammonium persulphate (Sigma-Aldrich, catalog number: A3678-100G)
32. N,N,N',N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad Laboratories, catalog number: 1610801)
33. Coomassie Brilliant Blue R-250 (Sigma-Aldrich, catalog number: B8647)
34. Methanol (Sigma-Aldrich, catalog number: 322415-1L)
35. Acetic acid (Merck, catalog number: 1000063)
36. 3x Protein electrophoresis application buffer (see Recipes)
37. Running electrophoresis buffer (see Recipes)
38. Blue staining solution (see Recipes)
39. Bleaching solution (see Recipes)
40. Separating gel (see Recipes)
41. Concentrating gel (see Recipes)

Equipment

1. Pipettes
2. -80 °C freezer
3. CO₂ Water-jacketed Incubator (NuAire, model: NU-2700 IR Autoflow)
4. Laminar flow chamber (Azbil Telstar, model: BV-100)
5. Laboratory centrifuge (MPW MED. INSTRUMENTS, model: MPW-223e)
6. Upright microscope (Nikon Instruments, IZASA, model: H550S)
7. 5 ml plastic syringe
8. Mini-PROTEAN® Tetra Electrophoresis System (Bio-Rad Laboratories, catalog number: 165-8001)
9. Plastic bucket (Labotienda, catalog number: BTL006)
10. UV-Spectrophotometer (Shimadzu, model: UV-1800)
11. 15 L Plastic bucket for dialysis

Procedure

A. Generation of recombinant baculoviruses by cotransfection

1. Seed the 35 mm tissue culture dishes with 1.5×10^6 Sf9 cells in 2 ml of Insect-XPRESS™ medium with gentamicin at a final concentration of 10 µg/ml to form a sub-confluent monolayer.

The number of insect cells is determined by using a Neubauer's camera.

2. Incubate for 1 h at 27 °C in the CO₂ incubator to allow cell attachment to the plate.
3. Add the following reagents to prepare the co-transfection mix of DNA and transfection reagent in a 1.5 ml polystyrene tube:
 - a. 100 µl of serum-free medium (TC100)
 - b. 100 ng of virus DNA from the *flashBAC* kit
 - c. 500 ng of transfer plasmid (pAcGP67A-E2₆₆₁ recombinant plasmid or *lacZ* positive control transfer vector from the *flashBAC* kit)
 - d. 1.2 µl of baculoFECTIN II from the *flashBAC* kit

Mix and leave at room temperature for 15 min.
4. Wash the cell monolayers twice by pouring 5 ml of TC100 medium without serum onto monolayer, shake gently and decant into waste. Add 1 ml of TC100 medium without serum to each 35 mm dish. If the cells are maintained in serum-free medium, it is not necessary to wash the monolayer. In this case, remove and discard 1 ml of medium from the 35 mm dishes.
5. Add the transfection mix (111.2 µl) to the 35 mm dish.
6. Incubate overnight at 27 °C in the CO₂ Incubator without shaking.
7. Add 1 ml of Insect-XPRESS™ medium with gentamicin at 10 µg/ml to the 35 mm dish.
8. Incubate at 27 °C for 4 days in the CO₂ Incubator without shaking.
9. Harvest the culture medium that contains the recombinant virus by decanting the medium into a 15 ml sterile container. Centrifuge in the laboratory centrifuge for 15 min at 325 x *g*. Collect the supernatant and store in the dark in a refrigerator at 4 °C. For a long-term storage, save the container at -80 °C.

B. Amplification of recombinant viruses

Note: Once the purified recombinant baculovirus is available, it must be amplified in order to obtain recombinant protein.

1. Seed 2 x 10⁶ Tni insect cells per 25 cm² of surface area and incubate for 1 h at 27 °C.
2. Add 1 ml of the supernatant containing the recombinant viruses for every 25 cm² of surface area (Flask F25, 1 ml; Flask F75, 3 ml; Flask F150, 6 ml).
3. Incubate for one hour at 27 °C in the CO₂ incubator with very slow agitation (rock the flask every 10 min) to maintain the hydration of the cell monolayer with the minimum volume of inoculum.
4. Add the relevant volume of Insect-XPRESS™ medium with gentamicin at 10 µg/ml to the flask (Flask F25, until 5 ml; Flask F75, until 10 ml; Flask F150, until 30 ml).
5. Incubate at 27 °C for 5 days in the CO₂ Incubator without agitation.
6. Collect the supernatant in a 15 or 50 ml sterile tube in order to recover the amplified recombinant virus and centrifuge in the laboratory centrifuge for 15 min at 325 x *g*.
7. Use the supernatant for a second round of amplification. Transfer the supernatant to a separate sterile tube and repeat Steps B2 to B6. Usually, two or three rounds of amplification are sufficient for large-scale infection to allow expression of the recombinant protein of interest.

C. Production and Purification of HCV E2₆₆₁ protein

1. Prepare 10 flasks of 150 cm² surface area with 8-10 x 10⁶ Tni insect cells in 20 ml medium with gentamicin at 10 mg/ml and incubate for one hour at 27 °C in the CO₂ incubator without agitation.
2. Remove the medium and add 6 ml of supernatant containing the recombinant E2₆₆₁ baculovirus per flask.
3. Incubate for one hour at 27 °C in the CO₂ incubator with very slow agitation (rock the flask every 10 min) to maintain the hydration of the cell monolayer with the minimum volume of inoculum.
4. Add 24 ml of Insect-XPRESS™ medium with gentamicin at 10 µg/ml per flask.
5. Incubate at 27 °C for 5 days.
6. Collect the supernatants and centrifuge for 15 min at 325 x g.
7. Dialyze the supernatant (300 ml, 10 flasks of 30 ml) against 20 L (10 L, 2x) of 50 mM Tris-HCl, pH 8.0, 300 mM NaCl buffer in a 15 L plastic bucket.
8. Load the supernatant onto a 2 ml Ni-NTA-Agarose column previously equilibrated with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl buffer.
9. Wash the column by adding 20-40 ml of 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM Imidazole buffer to the top, let the solution flow into a beaker to collect the waste. Wash until optical density at 280 nm is below 0.03. The adsorption at 280 nm is determined by using an UV-spectrophotometer.
10. Wash the column by adding 10-30 ml 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 30 mM Imidazole until optical density at 280 nm is below 0.03.
11. Elute the E2₆₆₁ protein by adding 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 200 mM Imidazole. Collect fractions of 1-1.2 ml. either in 1.5 ml Eppendorf or in glass tubes. Usually 10-12 tubes are enough for elution; thus, manual collection with the operator changing the tube after 1 ml is recommended.
12. The protein is detected by measuring the optical density at 280 nm of each fraction in the UV-spectrophotometer.
13. Check the purification steps of the recombinant E2₆₆₁ protein by SDS-PAGE. In order to check the purity of the protein, load 20 µl of sample per well from each 200 mM Imidazole fraction. After Coomassie blue staining, the purity of recombinant E2₆₆₁ is tested by the appearance of a unique, isolated band at 48 kDa.
14. Collect the fractions containing significant amounts (optical density at 280 nm higher than 0.3) of pure E2₆₆₁ protein in a single fraction.
15. Dialyze the E2₆₆₁ protein against 50 mM Tris-HCl, pH 8.0, 300 mM NaCl buffer.

A scheme to obtain the pure recombinant protein E2₆₆₁ is shown in the Figure 1. **Note that step A is not included in the protocol.**

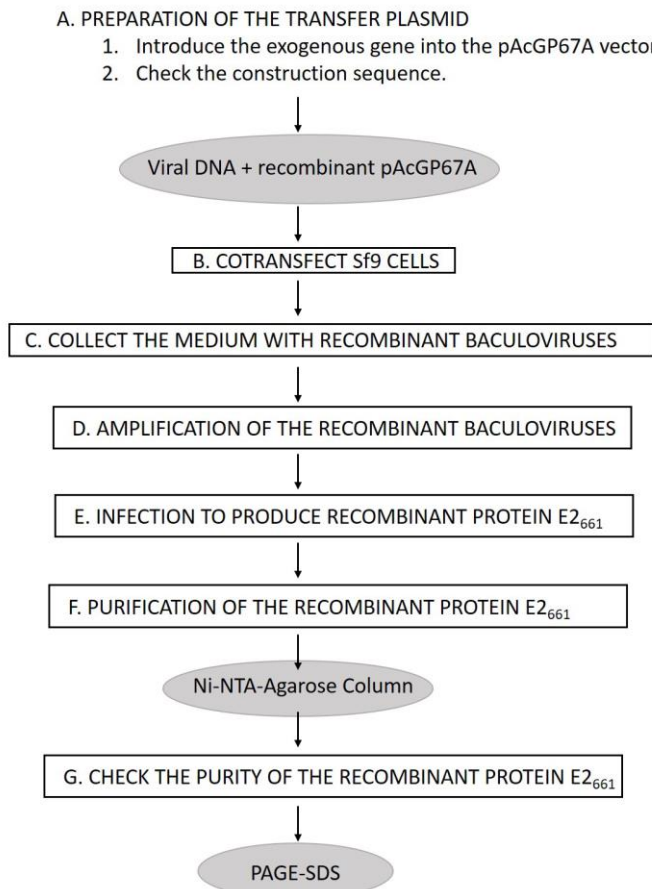


Figure 1. Scheme to produce the recombinant protein E2₆₆₁

D. SDS-PAGE (Protein electrophoresis)

Note: A more detailed protocol to carry out SDS-PAGE is described in He (2011) and Yan (2011).

1. Prepare the electrophoresis system.
2. Prepare the separating gel (see Recipe 5).
3. Mix with a Pasteur pipette and deposit into the gel stand.
4. Prepare the concentrating gel (see Recipe 6).
5. Mix with a Pasteur pipette and deposit on top of the separating gel.
6. Add 10 μ l of 3x Protein electrophoresis application buffer (see Recipe 1) to 20 μ l of sample.
7. Heat at 95 $^{\circ}$ C for 5 min.
8. Load into the concentrating gel.
9. Develop the electrophoresis in the Running electrophoresis buffer (see Recipe 2) at room temperature at 25 mA per gel until the marker reached the end of the gel.
10. Incubate the gel in a Coomassie blue staining solution (see Recipe 3) at room temperature for 10 min.
11. Incubate the gel in the bleaching solution (see Recipe 4) to detect the proteins.

Data analysis

The results of a standard purification process, corresponding to a PAGE-SDS gel, are shown in Figure 2. Lanes 2, 3 and 4 show washing steps with 10 mM (lane 2) and 30 mM (lanes 3 and 4) Imidazole buffer, where some contaminant proteins weakly attached to the Ni-NTA-agarose column are eliminated. As can be seen, the protein E2₆₆₁ remains in the column and is only eluted when the 200 mM Imidazole buffer is used (Figure 2, lanes 5 and 6). As shown in the gel, the recombinant protein is pure.

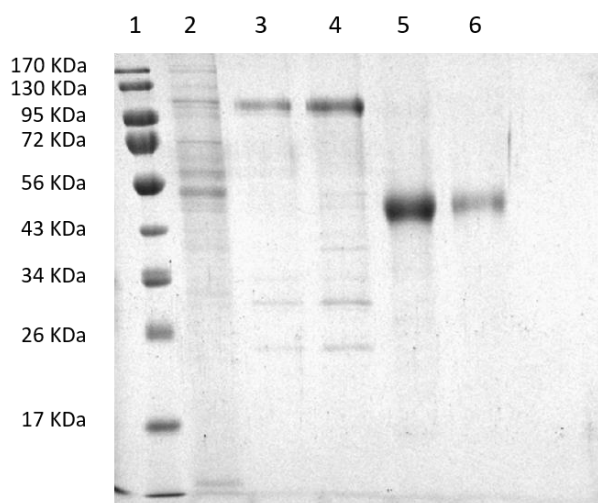


Figure 2. SDS-PAGE of the purification steps of the recombinant protein E2₆₆₁. (1) Molecular weights ladder; (2) Wash with 10 mM Imidazole buffer; (3, 4) Wash with 30 mM Imidazole buffer; (5, 6) E2₆₆₁ protein eluted with 200 mM Imidazole buffer.

Recipes

1. 3x Protein electrophoresis application buffer
 - Tris 150 mM, pH 7.6
 - EDTA 6 mM
 - 3% (w/v) SDS
 - 30% (v/v) Glycerol
 - 0.06% (w/v) Bromophenol blue
 - 15% (v/v) β -mercaptoethanol
2. Running electrophoresis buffer
 - Tris 0.025 M, pH 8.3
 - Glycine 0.192 M with 0.1% SDS
3. Blue staining solution
 - 0.3% (w/v) Coomassie Brilliant Blue R-250
 - 45% (v/v) Methanol

- 10% (v/v) Acetic acid
4. Bleaching solution
 - 7.5% (v/v) acetic acid
 - 20% (v/v) methanol
 5. Separating gel
 - 650 μ l H₂O
 - 1.82 ml Tris 1 M, pH 8.8
 - 2.5 ml acrylamide-bisacrylamide at 30%
 - 55 μ l SDS 10%
 - 12 μ l of 0.075% TEMED
 - 14 μ l 0.02% (w/v) ammonium persulfate
 6. Concentrating gel
 - 2.0 ml acrylamide-bisacrylamide at 4%
 - 55 μ l SDS 10%
 - 8 μ l of 0.075% TEMED
 - 8 μ l of 0.02% (w/v) ammonium persulfate

Acknowledgments

This work was supported by grant BFU 2006-13033 from the Ministerio de Educación y Ciencia, Spain and the research project SANTANDER/COMPLUTENSE PR26/16-20271.

Competing interests

Authors declare that there are any conflicts of interest or competing interests.

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