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Expression and purification of Class 7 Semaphorin and its receptor PlexinC1 using baculovirus-mediated mammalian cell gene transduction

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Abstract

Semaphorins and their receptor Plexins are large glycoproteins that are difficult to express using regular recombinant methods, and the widely used *E. coli* and baculovirus-insect cell systems have been inadequate for Semaphorins and Plexins which contain a large number of domains and are heavily modified by glycosylation. Here, we describe the expression of class 7 Semaphorin (Sema7A) and the extracellular domain of its receptors PlexinC1, using the baculovirus-mediated mammalian cell gene transduction (BacMam) method. A robust mammalian cell expression gene cassette, including a highly efficient secretion signal peptide, is introduced into the baculovirus which subsequently enters mammalian cells for efficient expression in suspension cell culture. Large amount of high-infectivity BacMam viruses are needed for infecting suspended mammalian cells in large scale, to generate Semaphorin and Plexin proteins at an amount sufficient for binding experiments and crystallographic studies. The inclusion of serum in expression ensure the robustness of cell culture, but introduces substantial amount of contaminant proteins interfering with immobilized metal ion affinity purification, which can be overcome with a two-step purification scheme.

Keywords

Glycoprotein; Semaphorin; Plexin; Baculovirus; Mammalian cell expression; BacMam; Suspension mammalian cell culture; Cell-surface receptor

1. Introduction

Bacteria (such as *E. coli*) and Baculovirus-infected insect cell systems (such as Hi5, sf9, and sf21 cells) are the most widely used recombinant expression systems for the generation of materials for biophysical characterization. However, they have severe limitations in producing large glycoproteins such as Semaphorins and the ligand binding extracellular domains of their receptors Plexins, especially when large amounts of proteins are needed for solution binding measurements and biophysical characterization. Bacterial expression is convenient and versatile, but is incapable of most post-translational modifications including

glycosylation that are required for mammalian protein stability and functionality. Insect cells are capable of glycosylation, and add hi-mannose type or pauci-mannose type of simple glycans to the N-linked glycosylation sites of secreted proteins (1), but the glycans added are highly heterogeneous and often incomplete, resulting in frequent protein aggregation and intracellular retention. It is often desirable to produce the difficult human glycoproteins that are refractory to *E. coli* and insect cell expression using mammalian cells, which are natural hosts to these proteins. Although small-scale expression transient transfection in adherent format is routinely used in cell biology experiments, scaling up this type of expression by using larger plates and increasing the number of plates is labor-intensive and impractical. Methods that combine the convenience and versatility of the *E. coli*/insect cell systems and the authentic glycosylation of mammalian cells are extremely desirable for studies of human proteins such as Semaphorins and Plexins, and the newly emerged baculovirus-mediated mammalian cell gene transduction (BacMam) system is one of the methods that can achieve these goals.

BacMam is one of the transient methods for expression in mammalian cells, which takes advantage of the capability of baculoviruses, although naturally only infecting selected types of insect cells, to deliver its genomic content into many types of mammalian cells. Although baculoviruses do not propagate in mammalian cells, the baculovirus DNA entering mammalian cells can be recognized for transcription (2, 3). Given a strong expression cassette and appropriate amount of viruses for transduction per cell, the transduction rate can approach completeness (4–6). The major advantages of the BacMam method are the convenience and the scalability. Once the primary BacMam virus is generated, it can be stored permanently and scaled up at any time. There is no need of keeping multiple cell lines specific for each glycoprotein. The amenability of the BacMam method for suspension cell culture also allows easy large scale preparation. The BacMam method has been exploited and developed for diverse applications, including the rapid production of pharmaceutical proteins (7), protease high-throughput screens (8), and the production of soluble and membrane glycoproteins for structural biology (9) and assay development (10). A particularly interesting example is the large scale BacMam method combined with a powerful mammalian expression cassette developed in the Garcia laboratory, which supports the production of not only ligand-binding domains of glycoprotein receptors, but also membrane proteins such as G-protein-coupled receptors (9).

Here we describe how the Garcia method is slightly modified and utilized to generate recombinant proteins of the class 7 Semaphorin Sema7A and its receptor PlexinC1, at a level that is amenable for structural and biophysical characterization (11). In particular, strong secretion signals, such as the Gaussia luciferase signal peptide, are utilized for strong expression (12), and a specific scheme is designed to overcome the interference of serum proteins with metal ion affinity purification.

2. Materials

2.1 Constructing the expression plasmids

1. Full-length human Plexin C1 cDNA (reference ID: NM_005761).

2. Full-length human Sema7A cDNA (reference ID: BC101647).
3. PVLAD6 plasmid [9] (ADDGENE Plasmid 41850).
4. Primer SPfor1: 5' atgtagccgccaccatgggagtgcaagtctgtttgccctgatctgcctgctgtggcagaggca-3'.
5. Primer PlexinC1for2: 5'-atctgcatcgctgtggcagaggca gcggacgagcccgtgtg-3'.
6. Primer PlexinC1rev: 5'-atcgggccgc tgtgaaggaactgactcctgc.
7. Primer Sema7Afor2: 5'-atctgcatcgctgtggcagaggca cagggccacctaaggagc.
8. Primer Sema7Arev: 5'-atcgggccgc gccgtcctcgggcagc.
9. NheI restriction enzyme.
10. NotI restriction enzyme.
11. DH5alpha competent cells.

2.2 Generating the primary viral stock using co-transfection

1. Sf9 cells (frozen in Gibco Sf-900 II Serum Free Medium [SFM]).
2. Gibco Sf-900 II SFM (Invitrogen).
3. Fetal Bovine Serum.
4. Gentamicin Sulfate solution: 50 mg/mL in deionized water, sterile-filtered, BioReagent, suitable for cell culture (Sigma-Aldrich).
5. BacVector-3000 Triple Cut Virus DNA (EMD Millipore).
6. Cellfectin II Reagent (Invitrogen).
7. Fetal Bovine Serum (FBS) and gentamycin supplemented Sf-900 II SFM media: 10% (v/v) FBS and 20ug/ml gentamycin sulfate in Sf-900 II SFM media.
8. Cellfectin-Sf-900 II SFM Media: 10% Cellfectin and 90% Sf-900 II SFM media, with no antibiotics or serum.
9. Serum supplemented Sf-900 II SFM media: 10% FBS in Sf-900 II SFM media.

2.3 Amplify the BacMam virus

- 1 1000ml Triangular/Erlenmeyer flasks.
- 3 500ml Sterile Conical bottom centrifuge Bottles (Nalgene).
- 4 Tight-sealing sterile bottles, such as 500ml Nalgene filter unit receivers, for virus storage.
- 5 Inverted microscope for observing cytopathic effects of infected cells.

2.4 Adapt and maintain HEK293 cells in suspension culture

1. Frozen HEK293 cells such as 293F (FreeStyle 293-F Cells, Invitrogen), 293H (Gibco 293H cells in CD 293, Invitrogen) and 293S GnTI-(ATCC CRL-3022).

2. HyClone CDM4HEK293 Media (Thermo Scientific).
3. DMEM media (Life Technologies).
4. 200 mM Glutamine-S (L-alanyl-L-glutamine) Solution: 43.44 mg/mL in NaCl.
5. T-75 tissue culture flasks
6. Sterile 250ml and 1000ml triangular/Erlenmeyer flasks.
7. Sterile 2.8 liter Fernbach flasks.
8. Serum and Alanyl-glutamine supplemented CDM4HEK293 media: 3% FBS, 4mM Alanyl-glutamine in CDM4HEK293 media.
9. DMEM media with heat-inactivated FBS: 10% FBS in DMEM media.

2.5 Large scale BacMam transduction of HEK293 cells in suspension culture

1. Sterile sodium butyrate: 2M sodium butyrate in water.
2. Large-capacity 37°C CO₂ incubator compatible with shakers inside.
3. Shaker (Thermo Scientific Barnstead/Lab-Line Max CO₂ Resistant Open-Air Shaker)
4. T-175 tissue culture flasks.
5. Serum supplemented CDM4HEK293 media: 3% FBS in CDM4HEK293 media.

2.6 Two-step purification of His-tagged proteins using Ni-NTA

1. Ultralab Systems & Ultraservoir Containers (PALL).
2. Hepes-Buffered Saline (HBS): 10mM Hepes, pH 7.5, 150mM sodium chloride in water.
3. Ni-NTA Agarose resin 50% slurry (QIAGEN).
4. Poly-Prep Chromatography Columns (Bio-Rad).
5. 5mM Imidazole, pH 7.5: add 0.34g of Imidazole to water, adjust pH to 7.5 and filter sterilize.
6. 200mM Imidazole, pH 7.5: add xxx13.6g of Imidazole to water, adjust pH to 7.5 and filter sterilize.
7. 1M Imidazole, pH 7.5: add 68xxxg of Imidazole xxx to water, adjust pH to 7.5 and filter sterilize.
8. Human Rhinovirus 3C Protease (HRV 3C) (Pierce).
9. Superdex-200 Chromatography column (GE Healthcare).
10. Vivaspin Centrifugal Concentrators (Cole-Parmer).
11. vacuum filter (Kimax, 60ml-40C).
12. 500ml Sterile Conical bottom centrifuge Bottles (Nalgene).

3. Methods

3.1 Constructing the expression plasmids

The expression vector PVLAD6 (9) is used to generate transfer plasmids that can be recombined into the baculovirus genome. The PVLAD6 already has a complete mammalian expression cassette, including the CMV immediate early promoter, the synthetic intron, a multiple cloning site, cleavable His-tag, the 3'-WPRE enhancer, and the late SV40 poly(A) signal. However, for secreted proteins, if the native signal peptide is undesired, alternative signal peptides can be added before the mature protein sequences. Here we use the Gaussia luciferase signal peptide (MGVKVLFALICIAVAEA) (12) to replace the native signal peptides of PlexinC1 and Sema7A.

1. The entire extracellular domain of PlexinC1, excluding its signal peptide and right before the transmembrane segment, and the entire Sema7A sequence excluding its signal peptide and right before the GPI anchor sequence (Figure 1), are attached to the Gaussia luciferase signal peptide (*see Note 1*) using polymerase chain reaction (PCR), with the full length cDNAs as templates respectively.
2. In each PCR reaction, two forward primers and one reverse primer are used. For PlexinC1, the SPfor1, PlexinC1for2, and PlexinC1rev primers are used at a 100:1:100 ratio (*see Note 2*). The sequence of Gaussia luciferase signal peptide is included in the SPfor1 primer. The PlexinC1for2 primer contains an overlapping sequence from Gaussia luciferase signal peptide and the starting sequence of PlexinC1 mature sequence. From the initial DNA template, the PlexinC1for2 and PlexinC1rev primers generate the secondary template, which is then used by SPfor1 and PlexinC1rev to generate the full PCR product. The same scheme is used for Sema7A.
3. An NheI site (GCTAGC) is added to the 5'-end of the primers before the Kozak sequence (GCCGCCACC) (13) (and is present in primer SPfor1; *see Note 3*) and the start codon (ATG); an Not I site (GCGGCGCC, coding for Ala-Ala-Ala) site is added to the 3' end downstream to coding sequences (and is present in primer PlexinC1rev).
4. The resulting PCR products are digested with NheI and NotI, and gel purified. The fragments are ligated into the NheI/NotI sites of PVLAD6. The 3' end is fused in-frame with HRV 3C protease and the 8-His tag through the NotI restriction site. The ligation mixture is transformed into DH5alpha competent cells. The correct clones are verified by restriction digestion and sequencing.

¹. The human Interleukin-2 (IL-2) signal peptide and the human Growth Hormone (hGH) signal peptide are also very strong secretion signals that are recognized and processed efficiently in almost any mammalian cell expression system. These alternative signal peptides can be incorporated to the transfer plasmid in a similar fashion.

². Of the two forward primers SPfor1 and PlexinC1for2, the first primer can be synthesized in large quantity and aliquotted for long-term storage. It can be used repeatedly for cloning and expressing a variety of target glycoproteins. The second primer needs to be used in minimal amount during the PCR, so that the predominant PCR product is the full extension product determined by the SPfor1 primer and the reverse primer.

³. The first two bp of the KOZAK sequence GCCGCCACC originate from the last two bp of the NheI site GCTAGC. If a shorter primer is desired, a slightly shorter Kozak sequence, GCCACC can be used.

3.2 Generating the primary viral stock using co-transfection

The BAcMam virus is generated by co-transfecting the *Spodoptera frugiperda* ovary sf9 cells with the above constructed baculovirus transfer plasmid and the linearized baculovirus DNA.

1. Grow sf9 cells in log phase at 27°C, with densities between 1×10^6 cells/ml and 6×10^6 cells/ml using FBS and gentamycin supplemented Sf-900 II SFM media (for instructions on how to grow and maintain sf9 cells, see http://tools.lifetechnologies.com/content/sfs/manuals/insect_man.pdf).
2. Add a total 1×10^6 cells to each well of a 6-well tissue culture plate, e.g., if the cell density is 2×10^6 cells/ml, add only 0.5ml. Bring the total volume in each well to 2ml with fresh Sf-900 II SFM media. Let the cells attach to the bottom for more than two hours (*see* Note 4).
3. In a clean sterile 2ml microcentrifuge tube, mix 2µl bacVector-3000 baculovirus DNA and 2.5µg Sema7A-PVLAD6 or PlexinC1-PVLAD6 construct plasmids. Add 100µl pre-mixed Cellfectin-Sf-900 II SFM media (with no antibiotics or serum). Mix gently by pipetting up and down. Incubate for 30 minutes before adding to cells (*see* below).
4. While incubating the mixture in Step #3, gently pipet out the 2ml of Sf-900 II SFM media from the attached cells in the 6-well tissue culture plate from Step #2, and place 1ml fresh Sf-900 II SFM without serum back to each well containing the cells.
5. Repeat step 4 to ensure that the serum and antibiotics content in each well is minimal.
6. Add 1ml fresh Sf-900 II SFM media to the incubated mixture from step 3. Mix gently by pipetting up and down, then add the entire mixture to each well containing the attached cells in the 6-well plate. Mix immediately by swirling gently.
7. Cover the 6-well plate, wrap it with plastic film, and incubate for 6–8 hours.
8. Replace the transfection media covering the attached cells with 3ml fresh serum supplemented Sf-900 II SFM media. Wrap the 6-well plate with plastic wrap and place in 27°C incubator for 6 days (*see* Note 5).
9. Pipet all the media from each well into a 15ml conical tube, centrifuge at 1000g for 5 minutes. Pipet the supernatant, which contains the recombinant BacMam virus into the 2ml cryo-vials for storage (*see* Note 6).

⁴. It is important for the sf9 cells to attach tightly to the bottom of the 6-well plate, because multiple exchanges of the media covering the cells are undertaken in the following steps. To ensure tight attachment, sf9s adapted to suspension need to be used. We have observed that sf9 cells freshly sloughed from adherent culture do not attach as well as the suspension-adapted sf9 cells. Alternatively, one can plate the 6-well plate at low density 1–2 days before transfection, so that at the day of transfection, the confluency is around 30%.

⁵. Although the ideal volume of media for each well in the 6-well plate is 2ml, slightly more media is needed in this case because over the 6 days of incubation, evaporation will reduce the volume and increase the osmolality of the media, which sometimes is not well tolerated by sf9 cells. Wrapping the 6-well plate will slow but not completely prevent evaporation during incubation.

3.3 Amplify the BacMam virus

The key to obtaining high infectivity BacMam virus is to start the amplification process when the sf9 cells are exposed to larger percentage of fresh media than spent media, and the initial multiplicity of infection is very low.

1. Three days before the start of the amplification, begin to maintain sf9 cells between 3×10^6 cells/ml and 6×10^6 cells/ml, by feeding the cells everyday with approximately equal volumes of Sf-900 II media. Use 1000ml triangular flasks to shake the cells at 27°C. The volume of culture in the flask should be no less than 50ml and no more than 200ml.
2. On the day of infection, feed the sf9 cells at a density around 6×10^6 cells/ml back to 2×10^6 cells/ml (*see* Note 7). Shake the newly fed cells for 4 hours.
3. Add the initial Sema7A and PlexinC1 BacMam virus freshly harvested from co-transfection to the culture at 1:1000 ratio, *e.g.*, for every 100ml of cell culture, add 100ul virus. If the initial virus has been stored in 80°C and newly thawed, the ratio should be 1:100, and for every 100ml of cell culture, add 1ml initial virus. Shake at 27°C overnight.
4. The next day, add an equal volume of fresh Sf-900 II media to the cells in Step 3 (in which the virus is being amplified).
5. After shaking the cells for another 2 days, check the sf9 cells under inverted microscope for cytopathy everyday. When all the cells are enlarged and the cell density starts to decrease, harvest the amplified virus by centrifuging the cell culture at 1000g for 20 minutes, using 500ml conical bottom centrifuge bottles (*see* Note 8). Collect the supernatant and discard the pellet. The supernatant containing the amplified virus can be stored at 4°C for approximately a month (*see* Note 9).

3.4 Adapt and maintain HEK293 cells in suspension culture

Most types of HEK293 cells are adaptable to suspension culture, including at least the 293F, 293H, and the GnTI-1 293S cells. Typically when these cells are thawed from liquid nitrogen, they need to recover from freezing shock in adherent culture for approximately 2 weeks. So even if the cells were pre-adapted to suspension before being frozen, the cells will

⁶. It is advisable to immediately freeze the majority of the primary viral stock resulted from transfection to -80°C freezer for permanent storage in a sterile 2ml cryo tube for later use. Only leave a small volume in the 4°C refrigerator for virus amplification. 4°C storage reduces the infectivity of the BacMam virus, and does so more profoundly to the primary viral stock than to the amplified viral stocks.

⁷. Feeding sf9 cells between 3×10^6 cells/ml and 6×10^6 cells/ml, roughly the higher end of the log phase, prior to virus amplification is advantageous because it allows the cells to be exposed to as much fresh media as possible at the time of infection. When cells are fed back to 2×10^6 cells/ml from 6×10^6 cells/ml, the cells are in 67% fresh media-33% spent media. The larger percentage of fresh media enables the cells to grow at faster rate, which benefits viral production. Another approach to achieve this is to spin down sf9 cells and re-suspend the cells in 100% fresh media. If the latter approach is taken, the cells need 3–4 hours extra time to recover from the stress before virus is added.

⁸. The length of amplification should be determined on an individual basis. The viral stock should be harvested when it reaches the highest infectivity. During virus amplification, the cells should be observed under microscope daily from the third day. The infected cells show cytopathic effect ~ 24 hours after being infected. If nearly all the cells appear enlarged, it is time to harvest the amplified virus immediately. When the cell numbers start to decline, a large percentage of the viruses will be damaged by continued shaking.

⁹. The amplified virus should be stored at 4°C with minimal light exposure. At room temperature the virus lose activity much faster than at 4°C. It is advisable to not generate the amount of amplified virus beyond immediate use.

need to be re-adapted to suspension culture when they are thawed. Newly thawed 293 cells recover better in adherent culture using DMEM media, but they can be gradually exposed to an increasing percentage of the CDM4HEK293 media in static culture. When the cells begin to adapt to suspension culture, they are already fully adapted to the CDM4HEK293 media. The elimination of media adaptation during suspension adaptation significantly reduces the stress on the cells and ensures the success of the adaptation process.

1. The newly thawed 293 cells should be centrifuged at 1000g for 5 minutes, and re-suspended in DMEM media with heat-inactivated FBS. Place the re-suspended cells in a T-75 flask with 100% confluency ($1.0\text{--}1.5\times 10^6$ cells/ml). Keep the T-flask at 37°C in a 5%-CO₂-controlled incubator. The high density of the initial adherent culture increases the survival rate of the newly thawed cells. At lower density, a large percentage of cells die within the first 12 hours (*see* Note 10).
2. The next day add an equal volume of DMEM media to the T-flask and split the culture into 2 T-flasks. Keep at 37°C.
3. Maintain the adherent cell culture between 20% confluency and 100% confluency for 7 days at 37°C, then start to feed the cells with serum and Alanine-glutamine supplemented CDM4HEK293 media (*see* Note 11). The DMEM media will be gradually changed to CDM4HEK293 media over the next 7–10 days. The cells are now floating near the bottom, instead of being attached to the bottom.
4. When the cells are in mostly CDM4HEK293 media and reach 100% confluency, transfer the cells to a 250ml triangular flask, shake at 120–130rpm at 37°C for 2 days.
5. Centrifuge the cells gently at 1000g for 5 minutes, discard the supernatant, and re-suspend the cells in fresh HEK293CDM media at a density of 1.5×10^6 cells/ml. Shake for 2 days at 37°C (*see* Note 12).
6. Repeat step 5 until no dead cell clumps are formed during shaking (*see* Note 13).

¹⁰. A suspension of cultured 293 cells needs to be re-started every 3 months to ensure reproducible protein expression results from low passage number cells. Given the frequent need to thaw new vials of frozen 293 cells, it is important to have high success rate at this step. The largest obstacle for the newly thawed cells to recover is the low starting cell density. At low starting density, a large percentage of cells die within the first few hours, and the remaining cells take a long time to re-start growth. In comparison, if the newly thawed cells are seeded at near confluency level, very few cells die at the recovery stage, and exponential growth typically starts within the first days. To help the recovery of 293 cells, antibiotics such as penicillin/streptomycin should not be used in the first a few days, but can be added from the second passage if this is desired for preventing bacterial contamination.

¹¹. Although CDM4HEK293 media is designed to be serum-independent, we found supplementing it with 3–5% serum is advantageous for the long term stability of 293 cells in suspension culture. If completely devoid of serum, the cell culture is more prone to a sudden drop of viability which is often difficult to reverse. The disadvantage of adding serum is the introduction of bovine serum albumin as well as other contaminant proteins that competes with affinity purification. This disadvantage can be overcome by the specific purification scheme described in method 3.6, or with the complete exchange of media after virus transduction. When serum is present in the media, a fraction of 293 cells tend to form small clusters in a grape-like shape, although the majority of cells are still mono-dispersed. If each cluster contains less than 20 cells, each cell is still well-exposed to oxygen, nutrients, and BacMam virus transduction. The slight tendency of clustering is even a good sign that the cells are healthy. If the clusters are undesired, the concentration of serum can be decreased.

¹². During the first day of being adapted to suspension, some cells will die from the mechanical stress. If the death rate of the cells exceeds the growth rate, the cell density will decrease instead of increase. This does not mean the adaptation process has failed. By centrifuging and then re-suspending the cells to the appropriate density, the adaptation process will continue.

7. Transfer the cells to a 1000ml triangular flask. Maintain the cells between 1×10^6 and 3×10^6 cells/ml. If dead cell clumps form, repeat centrifugation and re-suspension procedures as in step 5.
8. The cells can now be scaled up to 2.8 liter Fernbach flasks (see Method 3.5), which can hold as much as 1000ml cell culture (see Note 14).

3.5 Large scale BacMam transduction of HEK293 cells in suspension culture

The method described below is particularly suitable for large glycoproteins, which are expressed at low level. For small-to-medium sized and high expression proteins, transducing adherent cells using a few T-175 tissue culture flasks can be sufficient and much more convenient (see Note 15).

- 1 Per every 1000ml preparation, 100–200 ml freshly amplified BacMam virus should be used (see Note 16), and here 150ml is used as an example. The virus is ideally within one week from harvest. Pre-warm the virus to room temperature before transduction (see Note 17).
- 2 In each 2.8l Fernbach flask, grow 850ml 293 cells to a density of $\sim 1.5 \times 10^6$ cells/ml in Serum supplemented CDM4HEK293 media at 37°C (see Note 18).
- 3 Add 150ml Sema7A or PlexinC1 BacMam virus to the 850ml 293 cells, to make a total volume of 1000ml. Shake at 37°C for 6–8 hours.
- 5 Add sterile sodium butyrate (see Note 19). Continue shaking for 3 days.

¹³. The dead cells typically have shattered their membranes and released their intracellular debris. The debris can stick together and form large, cotton-like clumps. Centrifugation does not reduce the content of these clumps. To get rid of these clumps, let the flask sit for 1–2 minutes, the clumps will drop to the bottom, and can be pipetted out gently.

¹⁴. Routine maintenance of the suspension 293 cells should be in the 1000ml triangular/Erlenmeyer flask, which can hold between 50ml to 300 ml cell culture. Smaller flasks are not advised for maintenance because the cells gradually lose mechanical tolerance in small-diameter flasks. The cells can also be maintained in 2.8liter Fernbach flasks if a large volume of cells are routinely needed. In the Fernbach flask, it is advised to grow either 200–400ml cells or 600–1000ml culture. If 400–600ml culture is used, the orbiting liquid will lose synchronization with the orbital shaker, and the culture will be insufficiently oxygenized. The shaker flasks for mammalian cell culture should be autoclaved and completely dried before being used. They can be stored in enclosed cabinets for less than a month in a dust-free environment.

¹⁵. For transduction in T-flasks, it is advised to use regular DMEM media, but not HEK293CDM or other types of suspension media. The cells should be tightly attached to the bottom of the tissue culture flasks, to ensure it is convenient to exchange the media covering the cells.

¹⁶. The volume of the amplified virus should not exceed 20% of the total volume in a large scale transduction. This is because both the composition and pH of the Sf-900 II media are dramatically different from CDM4HEK293 or any other mammalian cell media. Insect cell media has a pH near 6.3 whereas the mammalian cell media is near 7.3. When >20% of amplified virus with mammalian cell culture, the viability of the cell culture drops, and the 293 cells tend to form large clumps which are resistant to BacMam transduction. The titer of the amplified virus can be measured using a variety of methods which are not described in this protocol. Since the virus loses infectivities during storage, the measured titer of the amplified virus is only of relative value. One way to assess the effective tier of the amplified virus for protein production is to use 2ml cultures in 6-well plates, with various amount of amplified virus added. The supernatant are collected after 3 days and the expression level is checked. The amount of virus near but not reaching expression plateau should be used for large scale expression experiments.

¹⁷. The virus taken out directly from 4°C refrigerator should not be directly added to 293 cells. A large sudden decrease of the cell culture temperature will shock the 293 cells so that they need additional hours to recover from stress. Although the virus can also be warmed up in 37°C, the higher temperature is harsh for the BacMam virus. Limit the exposure of the BacMam viruses to 37°C to only the course of transduction to ensure high transduction rate.

¹⁸. The best way to prepare 850ml 293 cells at 1.5×10^6 cells/ml at the time of transduction, is to feed the 293 cells to $\sim 1.0 \times 10^6$ approximately 20 hours before transduction, and let the cells grow to approximately 1.5×10^6 cells/ml. This will reduce the procedures at transduction and minimize the shocks to the cells, and ensure the cells are at their fastest growing rate when they are exposed to the BacMam virus.

¹⁹. The addition of the sodium butyrate boosts the expression level. However, it is toxic to 293 cells, therefore it is better to add it after 6–8 hours of transduction, when the majority of the BacMam virus has already been absorbed into cells.

- 6 Centrifuge the cell culture at 2000g; collect the supernatant, which contains the secreted Sema7A or PlexinC1 protein.

3.6 Two-step purification of His-tagged proteins using Ni-NTA

The procedure is specifically designed to purify His-tag protein from the conditioned media containing large amounts of bovine serum albumin (BSA), which competes with recombinant protein in binding to the metal-affinity. One way to reduce the serum level in the culture and simplify the process is to spin down the transduced cells and re-suspend the cells in fresh serum-free media. The cells in the serum-free culture are not as viable as the cells in the 3% serum, but can survive the short three days post transduction. If the level of BSA is insignificant in the culture, Steps #6–8 can be omitted (*see* Note 20).

1. Concentrate the supernatant from Method 3.5 to 200ml using a tangential flow ultrafiltration system. Bring the volume up to 1000ml with HBS (*see* Note 21), then concentrate it down to 200ml again. Repeat this step 3 times (*see* Note 22).
2. Collect the 200 ml concentrated and buffer-exchanged sample into 500ml centrifuge bottles. Centrifuge the sample at 12000g for 30 minutes.
3. Collect the supernatant in 500ml bottles, add 5ml Ni-NTA resin, and 1ml 1M imidazole to get a final concentration of 5mM imidazole (*see* Note 23). Shake gently at 4°C for more than 4 hours.
4. Collect the Ni-NTA resin by passing the mixture from step 3 through a vacuum filter. Wash the resin 3 times with HBS (*see* Note 24), and load the resin into a Poly-Prep chromatography (gravity-flow) column.
5. Wash the column with 10ml 5mM imidazole (*see* Note 25). Then elute the column with 5ml 200mM imidazole.

²⁰. During transduction, the total serum content of the cell culture is approximately 4%, the majority coming from the serum-supplemented HEK293CDM media and a minor fraction from the amplified viral stock. Even if serum-free mammalian cell media is used for culturing 293 cells, there is still more than 1% serum in the transduction media coming from the amplified viral stock which contains 10% serum. If the transduction media is not exchanged, its content of BSA still interferes with metal affinity purification.

²¹. 150mM NaCl in HBS is usually sufficient to keep the recombinant protein soluble. For hydrophobic proteins, a higher concentration of NaCl, such as 0.3–1M, may be needed.

²². The buffer exchange step is completed concomitantly with the concentrating step. The exchange of buffer is necessary because the spent media contains small molecules that interfere with the binding of recombinant proteins to the Ni-NTA resin, and these small molecules can be exchanged in ultrafiltration. The buffer exchange usually precipitates out some native proteins produced by 293 cells and generates a milky or cloudy appearance of the sample. The precipitant is pelleted out in the next centrifugation step so that it will not interfere with Ni-NTA affinity resin. Most recombinant protein stays in solution. However, if the target protein is highly hydrophobic, in addition to using a raised concentration of NaCl (0.3–1.0M), 1–5% glycerol or 0.1–0.5M arginine, or even a low concentration of detergents such as 0.01% n-Dodecyl β -D-maltoside, can be added in the buffer exchange step, to minimize the loss of target proteins due to precipitation.

²³. 5ml Ni-NTA resin (50% slurry) is appropriate in most cases. If the protein is expressed at a very low level, the amount of slurry should be reduced. A low concentration of imidazole is used to suppress the competition of contaminant protein binding to Ni-NTA. 5mM imidazole works in most cases, and no more than 10mM imidazole can be used for monomeric proteins. For tightly associated dimeric or oligomeric proteins, 20mM imidazole can be used because the target proteins bind the Ni-NTA resin much more tightly with the presence of two or more His-tags.

²⁴. A common mistake at this step is to use the imidazole-containing washing buffer, *e.g.*, 5mM imidazole in HBS, to wash the collected resin in the filter. This will result in a significant loss of the protein to be purified. Instead, only use only HBS without imidazole to wash off only unbound proteins. The proteins loosely bound to the resin and competing with the target proteins should be washed off in the column rather than in the filter.

²⁵. More stringent washing using higher concentrations of imidazole (*e.g.*, 10–20mM) is not necessary because this step is not targeting the elimination of all the competing contaminants. If the imidazole concentration is too high at this step, more target protein will be lost.

6. Collect the 5ml eluent, add 100U HRV 3C protease (*see* Note 26). Incubate at 4°C overnight.
7. Prepare a Poly-Prep gravity column loaded with 5ml Ni-NTA resin. Wash the column with 20ml HBS.
8. Add 200ml HBS to the mixture from step 6. Pass the mixture through the pre-washed Ni-NTA gravity column from step 7. Add additional 5ml HBS to the column after all the mixture has passed through. Collect all fractions. Concentrate the pooled fractions down to 1–2ml using a centrifugal concentrating device (*see* Note 27).
9. Use a Superdex-200 column to polish the protein sample if necessary (Figure 2) (*see* Note 28).

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²⁶. The amount of HRV3C protease and the incubation time can be adjusted to be consistent with the level of expression. HRV3C is highly efficient at 4°C, and the protein can be incubated with HRV3C in the refrigerator for several days if a lower amount of HRV3C is to be used.

²⁷. Many modification steps can be done at this step prior to gel filtration purification, such as deglycosylation with endoglycosidases or limited proteolytic digestion (*see* (14) for general instructions), so that the undesired reaction products can be purified away along with small molecules, salts, minor contaminant proteins and aggregates.

²⁸. The use of a size exclusion/gel filtration column with a fast-flow chromatography system, such as the FPLC system from GE Healthcare, is best used as a final polishing step in protein purification. It can separate the aggregated target protein from the same, but conformationally correct target protein. Typically the biggest difference between an “un-cleaned up” sample and a gel-filtration-cleaned up protein sample is the presence versus the absence of a large-molecular weight peak at the void volume. The sample to be applied to the gel filtration column should be clarified with high-speed centrifugation first (>16000g), to reduce the clogging of the column and the pre-column filters. See http://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314807262343/litdoc18102218_20140915112126.pdf and related handbooks for detailed instructions.

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Fig. 1. Construct design for the expression of Sema7A and PlexinC1. **(A)** Schematic representation of the region of Sema7A that is subcloned. **(B)** Schematic representation of the region of PlexinC1 that is subcloned. **(C)** Nucleotide and translated protein sequences around the secretion signal peptide for the PlexinC1-PVLAD6 construct.

Fig. 2.

A demonstration of the quality of the full extracellular segment of PlexinC1 as expressed and purified using the described methods. The concentrated PlexinC1 proteins after the two-step Ni-NTA purification is loaded to a Superdex-200 column and the fractions are analyzed with SDS-PAGE shown at the upper right of the gel filtration elution curve. It indicates that the extracellular segment of PlexinC1 exists as a mixture of dimeric and monomeric forms, as shown by two adjacent peaks in the gel filtration elution curve but a single band in denatured electrophoresis.