

Chapter 2

Isolating Fc-Tagged SEMA4D Recombinant Protein from 293FT Cells

Joanne Soong and Glynis A. Scott

Abstract

Recombinant proteins are widely used in biomedical sciences, and in pharmaceutical research. Through genetic recombination, specific DNA sequences are engineered and inserted into a biological host. Once inside the host, the DNA is transcribed and translated into the target protein and is ready to be purified. Here, we describe the transfection, purification, and visualization of Fc-tagged SEMA4D (semaphorin 4D) recombinant protein.

Key words Lipofection, Lipofectamine, Protein affinity purification, Affinity chromatography, Silver stain, SDS-PAGE, Recombinant protein

1 Introduction

Semaphorins, the main ligands for plexin receptors, are a class of secreted and transmembrane proteins that are crucial for neuronal pathfinding and neuronal development, as well as axon growth cone regulation [1]. Semaphorins also mediate migration and motility in several nonneuronal cell types. There are eight different classes of semaphorins: 1–7, and class V (expressed in viruses). Each class has its own subclass, and interacts with the plexin family of receptors through their “sema” domain. Semaphorin 4D (SEMA4D) binds to plexin B1 and plexin B2 receptors, which function as Ras GTPase-activating proteins (GAPs) [2]. SEMA4D has several different functions in the body; the two main roles are inducing growth cone collapse in the central nervous system [3] and stimulating B cell and dendritic cell aggregation via binding to the CD72 receptor [4].

In this chapter, we present techniques used in our laboratory to produce purified recombinant SEMA4D (also known as CD100) protein, suitable for cell culture use. The first method described is transfection of plasmid DNA into mammalian cells using Lipofectamine. This is an efficient and relatively nontoxic technique

that is used to introduce plasmid DNA into a cell via lipid bilayer vesicles or liposomes [5]. Lipofectamine contains a positively charged lipid subunit that readily forms liposomes with negatively charged DNA or RNA in an aqueous environment. This technique can be used for several transfection applications, i.e., stable, transient, or co-transfection [5].

The second technique described is protein purification, more specifically, protein affinity purification also known as affinity chromatography. Protein affinity purification is a process that isolates a specific target protein from a heterogeneous mixture using a separation technique based on the molecular structure of the target protein [6]. This method uses resins or gel matrixes (i.e., agarose or sepharose beads) that possess highly specific surfaces that entrap the target protein, allowing separation from the rest of the mixture [7, 8]. For the procedure described in this chapter, the target protein is Fc tagged. The Fc tag is the Fc domain sequence of amino acids that are present in immunoglobulins [9], which readily binds to protein A or G, but not L [10].

The final technique we will describe is visualization and quantitation of the purified recombinant protein through the highly sensitive procedure of silver staining. This technique uses silver to detect target proteins after electrophoretic separation on a sodium dodecyl sulfate (SDS) polyacrylamide gel. Proteins bind to the introduced silver ions and are reduced to silver metal under certain conditions (typically with formaldehyde at an alkaline pH) [11]. There are several silver staining methods; the one used in this manual utilizes silver nitrate, which binds to particular amino acids on proteins under weakly acidic or neutral pH conditions [12]. Quantitation of the purified protein is through an estimated concentration using bovine serum albumin (BSA) as a standard.

2 Materials

Prepare all solutions at room temperature and store solutions as indicated.

2.1 Cells and Media

1. 293FT cells (ATCC).
2. Dulbecco's minimal essential media (DMEM), high glucose, with 4 mM l-glutamine, 4500 mg/L glucose, 110 mg/L sodium pyruvate. Store at 4 °C.
3. 1× OptiMEM with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2.4 g/L sodium bicarbonate, 2 mM l-glutamine. Store at 4 °C.
4. 100× Antibiotic/antimycotic (penicillin/streptomycin/fungizone; Gibco). Store at 4 °C.
5. G418 sulfate: Resuspend 2 g with 40 mL ddH₂O to a concentration of 50 mg/mL, filter sterilize using 0.45 µm cellulose acetate membrane. Store in 1 mL aliquots at -20 °C.

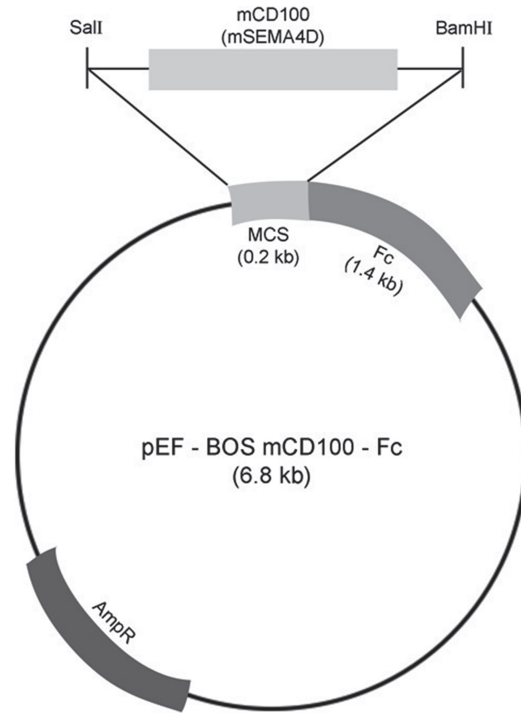


Fig. 1 pEF-BOS-mCD100-Fc vector map. The extracellular region of mouse CD100 (mCD100, also known as mSEMA4D) was inserted into a pEF-BOS-Fc backbone at the multiple cloning site (MCS). The CD100 portion can be cleaved out via enzyme digestion using SalI and BamHI

6. Fetal bovine serum (FBS): Triple 0.1 μm filtered. Store at -20°C .
7. 100 mm \times 20 mm Round tissue culture-treated dishes. Store at room temperature.
8. Fc-tagged SEMA4D plasmid DNA (Fig. 1) was a generous gift from Atsushi Kumanogoh, Osaka University [4] (*see Note 1*).

2.2 Lipofection

1. Lipofectamine 2000 (Invitrogen). Store at 4°C .
2. 50 mL Conical centrifuge tubes, polypropylene with cap, RNase/DNase-free, nonpyrogenic. Store at room temperature.
3. Super Low IgG FBS (HyClone), <0.005 mg/mL IgG. Store at 4°C .

2.3 Protein Purification

1. Amicon Ultra-15 Centrifugal Filter Units (Millipore). Store at room temperature.
2. 15 mL Conical centrifuge tubes, polypropylene with cap, RNase/DNase-free, nonpyrogenic. Store at room temperature.

3. Pierce Protein A Agarose, settled resin (Thermo Scientific): 6% Beaded agarose, 50% slurry in dH_2O with 0.02% sodium azide. Store at 4 °C.
4. Binding buffer (20 mM sodium phosphate, pH 4): Weigh out 1.38 g of sodium phosphate and transfer it into a sterile glass flask. Add 500 mL of ddH_2O to the flask and mix it thoroughly. pH the solution to 4.0 and filter sterilize (500 mL polystyrene, nonpyrogenic, sterile 2 μm filters). Store solution at room temperature.
5. Protease Inhibitor Cocktail Set I (Calbiochem): Resuspend in 1 mL ddH_2O per vial for 100 \times solution. Store at -20 °C.
6. Phosphate-buffered saline (PBS): Stock PBS, with 0.9 mM calcium chloride, and 0.49 mM magnesium chloride, comes as a 10 \times sterile solution (Cellgro). Dilute 1:10 ratio with ddH_2O into a convenient container. Store at room temperature.
7. Elution buffer (0.15 M glycine-HCl, pH 2.6): Weigh out 5.63 g of glycine and transfer it into a sterile glass flask. Add 500 mL of ddH_2O to the flask and mix it thoroughly. Then, pH the solution to 2.6 and filter sterilize (500 mL polystyrene, nonpyrogenic, sterile 2 μm filters). Store at 4 °C.
8. 0.6 mL Graduated microcentrifuge tubes, RNase/DNase free. Store at room temperature.
9. 1 M Tris-HCl, pH 9: Weigh out 60.57 g of Tris and transfer into a sterile glass flask. Add 500 mL of ddH_2O to the flask and mix it thoroughly. Then, pH the solution to 9.0 and filter sterilize (500 mL polystyrene, nonpyrogenic, sterile 2 μm filters). Store at room temperature.

2.4 SDS-PAGE Gel Electrophoresis

1. 30% Acrylamide/Bis solution. Store at 4 °C.
2. 1.5 M Tris-HCl pH 8.8 and pH 6.8: Dissolve 181.65 g Tris base into 1 L of ddH_2O , pH to 8.8 or 6.8. Store at 4 °C.
3. 10% Ammonium persulfate (APS): 1 g of APS in 10 mL ddH_2O , aliquot and store at -20 °C.
4. Tetramethylethylenediamine (TEMED). Store at room temperature.
5. 100% Ethanol. Store at room temperature.
6. Albumin, bovine serum, fraction V, low heavy metals (BSA). Store at 4 °C.
7. Radio-immunoprecipitation assay (RIPA) buffer: RIPA buffer concentrate (Cayman Chemicals, Ann Arbor, Michigan, USA), resuspend vial with 40 mL ddH_2O to yield 50 mM Tris-HCl, pH 7.6, 150 mM sodium chloride, 1% Tergitol (NP-40), 0.5% sodium deoxycholate, and 0.1% SDS. Store in aliquots at -20 °C.

8. 4× Sample buffer: 40 % Glycerol, 240 mM Tris-HCl pH 6.8, 8 % SDS, 0.04 % bromophenol blue, 5 % β-mercaptoethanol. Store at −20 °C.
9. Running buffer: Add 890 mL of ddH₂O into a convenient container, add 100 mL 10× Tris-glycine pH 8.3 (30.275 g Tris base and 144.134 g glycine, add 1 L ddH₂O, pH to 8.3), and 10 mL 10 % SDS (20 g sodium dodecyl sulfate in 200 mL ddH₂O), mix thoroughly. Store at 4 °C.
10. Amersham Full-Range Rainbow Molecular Weight Marker (GE Healthcare). Store at −20 °C.
11. Purified Mouse CD100 primary antibody (BD Transduction Laboratories). Store at −20 °C.

2.5 Silver Stain

1. Square glass Coplin jar with dimensions: 14.3 cm × 11 cm × 8 cm.
2. ProteoSilver[®] Silver Stain Kit (Sigma-Aldrich). Store at room temperature.
3. Table-top light box.

3 Methods

All procedures were performed at room temperature, unless otherwise stated. All procedures were done in a sterile environment, except for gel electrophoresis. Fc-tagged SEMA4D plasmid DNA (Fig. 1) was a generous gift from Atsushi Kumanogoh, Osaka University [4] (*see* **Note 1**).

3.1 Transfecting 293FT Cells with Plasmid SEMA4D DNA

1. Plate at least 20, 100 mm tissue culture-treated dishes (flasks are not recommended) with 1×10^6 293FT cells in each dish. Use DMEM + 10 % FBS + 500 µg/mL G418 sulfate + antibiotics/antimycotics.
2. Wait at least 2 days, or until the cells are about 70–80 % confluent (*see* **Note 2**). Prepare for transfection as follows: 60 µL of Lipofectamine per 100 mm dish, 24 µg of plasmid DNA (*see* **Note 3**) per 100 mm dish, 1.5 mL OptiMEM (no antibiotics added) per 100 mm dish. For example, for 20 100 mm dishes add 1.2 mL of Lipofectamine to 30 mL of OptiMEM (no antibiotics added) and 480 µg of plasmid DNA to a separate 30 mL of OptiMEM (no antibiotics added). Combine the two mixtures together (do not vortex) and incubate at room temperature for 15 min (Fig. 2).
3. During the above incubation, collect the media from the plates in 50 mL conical tubes. Centrifuge tubes at a minimum setting of $1000 \times g$ for at least 15 min. Collect the supernatant and discard any cellular debris at the bottom of the tubes. Aliquot the supernatant into fresh 50 mL conical tubes and store them in the −80 °C until protein purification. This will be used as a control SEMA4D.

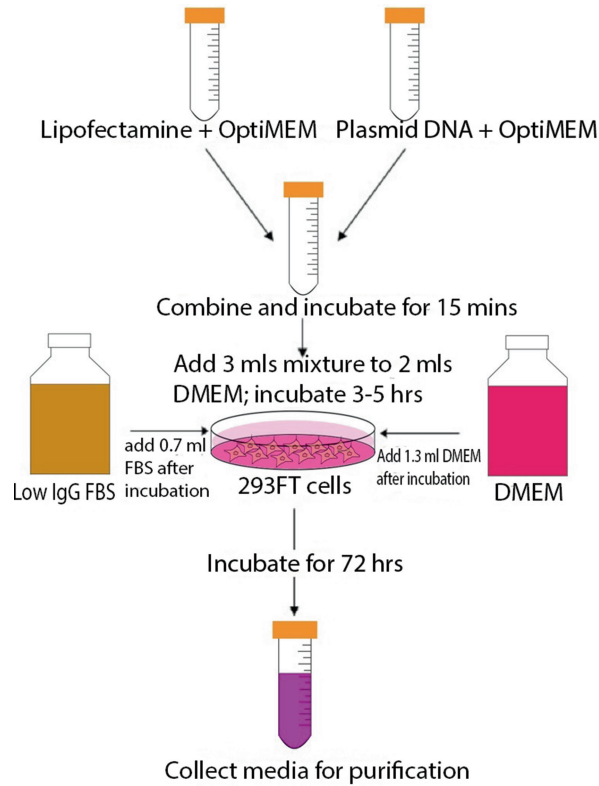


Fig. 2 Flowchart of transfection procedure, using Lipofectamine. Lipofectamine and plasmid DNA are added into antibiotic-free OptiMEM separately and then combined for a short incubation period. The mixture is then added onto 293FT cells with antibiotic-free DMEM and incubated again. Afterwards, low IgG FBS and additional antibiotic-free DMEM are added onto the cells and incubated for at least 72 h. Media from the cells is collected after incubation for the next purification step

4. Replace the media on the cells with 3 mL of the Lipofectamine+DNA mixture made in **step 2**. Add an additional 2 mL of DMEM (no antibiotics added) into each 100 mm dish.
5. Incubate cells in a standard cell incubator (37 °C+5% CO₂) for 3–5 h.
6. After incubation, add 1.3 mL of DMEM (no antibiotics) and 700 µl of low IgG FBS to each 100 mm dish. Replace dishes back into incubator for at least 72 h.

3.2 Purified SEMA4D Recombinant Protein

1. After the 72 h incubation, collect the media in 50 mL conical tubes and centrifuge at a minimum setting of 1000×*g* for 15 min to pellet any cellular debris. In the mean time, defrost the control supernatant from Subheading 3.1, **step 3**.
2. Transfer both the SEMA4D and the control supernatant to Amicon Ultra-15 tubes (each tube holds about 13 mL per

tube) for concentration. Typically these are split into two Amicon Ultra-15 tubes each, for a total of four tubes.

3. Centrifuge the tubes for at least 20 min at a minimum setting of $1000\times g$. Discard the flow through. Add additional supernatant into the same tubes, respectively, and continue to centrifuge until all of the supernatants have been concentrated. A total of about 1–2 mL of concentrated supernatant is ideal.
4. While the supernatants are concentrating, aliquot 1 mL of the 50% Protein A bead slurry into a 15 mL conical tube (*see* **Notes 4** and **5**) and centrifuge it so that the beads and solution are separated (speed and time are not important; however, we typically centrifuge for about 1–2 min at $2500\times g$). Using a pipette (with normal, uncut tips), carefully remove the supernatant from the beads and discard supernatant.
5. Wash the beads with 2 mL of binding buffer. Mix well (*see* **Note 6**), spin down the beads, and remove the binding buffer (carefully pipette it out, do not aspirate).
6. Transfer the concentrated supernatant (both SEM4D and control) into 15 mL conical tubes. Add the appropriate amount of protease inhibitor so that the final solution is $1\times$ (stock concentration is typically $100\times$).
7. Add 25% volume of binding buffer into the concentrated supernatant mixture. Add the entire mixture (now containing the concentrated supernatant, protease inhibitor, and binding buffer) into the 15 mL conical tube with the washed Protein A beads.
8. Rock the tubes on a plate rocker, at a medium speed setting, overnight at 4 °C.
9. The next day, centrifuge the tubes for 15 min at a minimum setting of $1000\times g$ to pellet the beads. Discard the supernatant.
10. Wash the beads with 1 mL of ice-cold $1\times$ PBS, three times. Do not pipette up and down; use the tapping method (*see* **Note 6**). Spin down beads and remove the supernatant for each wash.
11. Elute the proteins from the beads with 100 μ L of cold elution buffer, use tapping method to mix, and centrifuge to pellet down the beads. Aliquot the supernatant into convenient 0.6 or 1.5 mL microcentrifuge tubes. Elute beads a second time (elute beads at least twice).
12. Add 1 M Tris–HCl, pH 9 at a 1:50 ratio to the eluted SEMA4D and control SEMA4D. For example, if 100 μ L of protein was eluted, add 2 μ L of 1 M Tris–HCl, pH 9. This mixture is the purified SEM4D recombinant protein.
13. Store aliquots in the –80 °C freezer until use. Leave out one tube of purified SEM4D protein (for each elution) and control SEMA4D for quantitation via silver staining.

3.3 10% SDS-PAGE Gel Electrophoresis and Silver Stain

1. Prepare a 10% SDS-PAGE: Mix 6.25 mL of ddH₂O, 5 mL acrylamide, and 3.75 mL of Tris-HCl pH 8.3 into a 50 mL conical tube. Add 50 µl ammonium persulfate (APS) and 10 µl of TEMED. Cast gel within an 8.2 cm×10 cm x 1 mm glass cassette with 1 mm spacers; allow about 2 mm of space for the stacking gel. Add at least 1 mL of 100% ethanol on top of the gel mixture in the cassette to allow for even distribution. Allow gel to solidify before adding stacker gel. Once solidified, prepare the stacker gel mixture as follows: Add 3.05 mL of ddH₂O, 1.25 mL of Tris-HCl pH 6, 0.65 mL of acrylamide, 25 µl ammonium persulfate (APS), and 5 µl of TEMED into a 15 mL conical tube. Decant the ethanol on top of the gel and rinse the gel with ddH₂O at least twice. Do not open the cassette when rinsing. Decant any remaining ddH₂O and add the stacker solution to the brim of the glass cassette. Immediately insert a 10-well gel comb into the cassette and do not introduce any air bubbles. Allow stacker to solidify before running the gel.
2. Set up BSA protein standards: Prepare a 10 µg/mL, 5 µg/mL, and 1 µg/mL solution of BSA dissolved in RIPA buffer. Aliquot 10 µl of each standard into 0.6 mL microcentrifuge tubes. Add 5 µl of sample buffer and vortex gently.
3. Prepare SEMA4D protein and control protein: Aliquot 1, 5, and 10 µl of the purified SEMA4D protein into 0.6 mL microcentrifuge tubes for each elute. Add enough RIPA buffer into each tube so that the final volume for each tube is 10 µl. For the control protein, aliquot 10 µl into a separate microcentrifuge tube. Add 5 µl of sample buffer to each of the tubes and vortex gently.
4. Heat BSA standards, SEMA4D, and control protein on a hot plate at 95 °C to 100 °C for 5 min. Make sure that caps do not pop open during this time.
5. After heating, place samples on ice until cool and centrifuge them briefly to collect any samples that may have evaporated on the sides of the tubes.
6. Prepare gel apparatus with newly made SDS-PAGE and add running buffer to the brim of the container.
7. Load a reliable molecular weight marker (i.e., Full Range Rainbow Marker), BSA protein standards, SEMA4D protein, and control protein onto the 10% SDS-PAGE. Run the gel, immersed in ice or in a cold room, at 60 V and 500 Amp for 30 min or until the dye front has passed the stacking gel. Afterwards, switch the setting to 100 V and 500 Amp and run the gel until the dye front reaches the bottom of the gel.
8. Following electrophoresis, carefully separate the glass plates with a spatula so that the gel remains on one of the glass plates. Transfer the gel into a suitable container, containing enough ddH₂O to fully immerse the gel, and carefully remove the remaining glass plate.

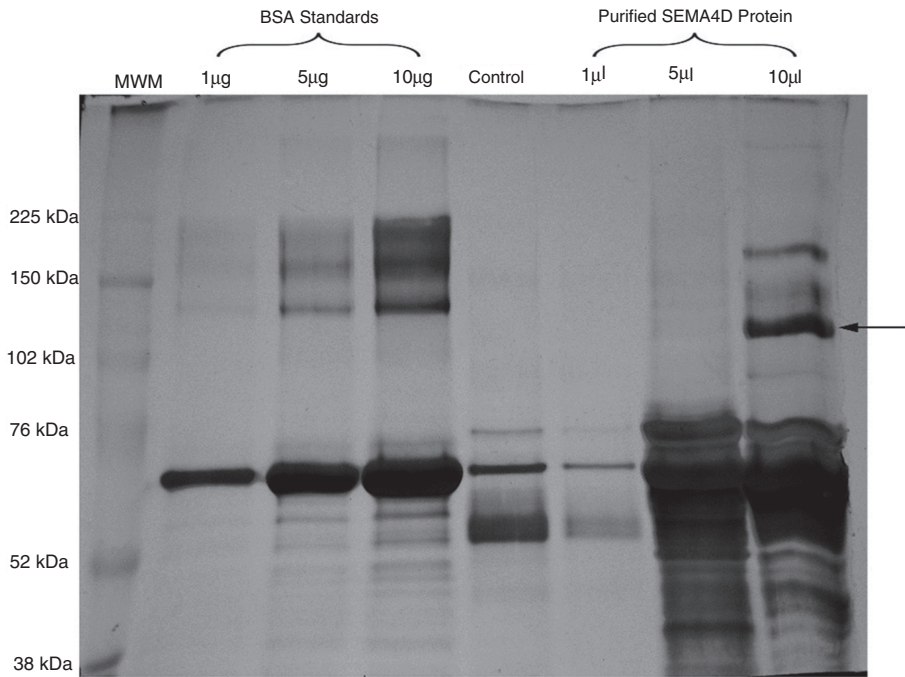


Fig. 3 Representative picture of a silver-stained polyacrylamide gel of purified SEMA4D using ProteoSilver® Silver Stain Kit from Sigma-Aldrich resolved on 10 % SDS-PAGE. The arrow is pointing to the band for SEMA4D at around 100 kDa. Based on BSA standard comparison, the estimated concentration of the purified SEMA4D is 2 µg/µl (*MWM* = molecular weight marker)

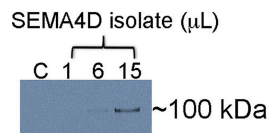


Fig. 4 Representative Western blot showing confirmation of the identity of purified protein as SEMA4D. Purified Fc-tagged-SEMA4D (1, 6, and 15 µl), and control ("C"; cell supernatant of untransfected 293FT cells) were resolved on 10 % SDS-PAGE and blotted using monoclonal antibodies against SEMA4D (*see Note 9*). Blot shows a band for SEMA4D at around 100 kDa

9. Proceed with silver staining protocol as per the manufacturer's instructions (*see Note 7*).
10. Once silver stain procedure is finished (Fig. 3), an estimate of the concentration of SEMA4D protein can be determined using the BSA standards (*see Note 8*; Fig. 3). The size of SEMA4D is about 100 kDa.
11. In addition, a Western blot (*see Note 9*) can be performed using the purified SEMA4D to confirm the identity of the protein (Fig. 4). Negative controls to include are culture supernatant from 293FT cells that were not transfected with plasmid DNA.

4 Notes

1. The extracellular region of mouse SEMA4D was inserted into the pEF BOS-Fc vector backbone. It can be enzymatically cut with SalI and BamHI and the vector contains an ampicillin resistance cassette for propagation in *E. coli*. For more detail on how the construct was made, please see Ref. [4].
2. 293FT cells tend to lift off very readily when they are above 80 % confluent. During the first section of the procedure, there will be several steps that require replacing the media on these cells at different intervals. Take care not to pipette media too quickly or forcefully into the cell culture dish. We recommend tilting the cell culture dish and pipetting the media onto one side of the dish, very close to the dish's brim, and then slowly placing the dish down flat so that the media distributes evenly.
3. Preparation of plasmid DNA from transformed *E. coli* was made using Qiagen EndoFree Plasmid Maxi Kit (Venlo, Limburg).
4. We found that Protein A agarose beads work the best. However, Fc tags are able to bind to both Protein G and A, so Protein G beads can be used instead of Protein A if necessary.
5. Use blunted or cut tips if using a pipette or serological pipette to aliquot the beads. This allows for maximum bead intake and minimal amount of beads stuck on the tip wall.
6. For all procedures that require mixing a solution that contains the Protein A beads, tap the side of the tube to mix. Do not pipette the mixture up and down because the beads adhere to the walls of the tips. It is also not recommended to vortex the solution because the solution may move toward the cap of the tube and get stuck onto the tube wall or cap.
7. The silver stain procedure was carried out exactly as per the instructions in the silver stain kit. However, there are a few tips we found useful when dealing with the silver-stained gel. The container that the SDS-PAGE is held in during the silver stain procedure should be glass, or at least clear. A large square glass Coplin jar is ideal. When the silver stain procedure is finished, placing the jar containing the gel onto a table top light box is best for visualization of the silver bands. If no light box is available, place the jar on top of a light-colored surface. In addition, when taking a picture of the silver-stained gel, gently remove the gel out from the jar and place it on a glass plate (i.e., the ones used for making the SDS-PAGE), as there tends to be a lot of bubbles that form in the resulting solution.
8. An estimate of the SEMA4D protein concentration can be determined by using BSA as a standard. Once the silver stain procedure is finished, one can visualize the SEMA4D band at

around 100 kDa. Compare the intensity of the SEMA4D band to the bands of the known BSA standard concentrations. Different volumes of SEMA4D isolate are loaded onto the gel so that one can easily determine the concentration of the protein if it is too concentrated or not concentrated enough. The BSA standards will not have a band at 100 kDa, but use the other bands (there should be a strong band between 76 and 52 kDa) to estimate the concentration of the protein.

9. Western blotting is a technique in which vertical gels made from polyacrylamide are used to resolve protein lysates based on the mass of the protein, with the aid of electric current (for a full discussion on the technique of Western blotting, please *see* Ref. [13]). Separated proteins are then transferred to a solid support, such as a nylon membrane or nitrocellulose, and then incubated with an antibody that detects the protein of interest. For Sema4D, we use a monoclonal antibody directed against Sema4D from BD Transduction Laboratories. We use the primary antibody at a concentration of 1:1000 in a 3% bovine serum albumen PBS solution overnight at 4 °C, followed by rinsing and then detection with a secondary horseradish-conjugated anti-mouse antibody (1:2000, Sigma) for 1 h at room temperature.

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