

Video Article

Expression, Isolation, and Purification of Soluble and Insoluble Biotinylated Proteins for Nerve Tissue Regeneration

Aleesha M. McCormick¹, Natalie A. Jarmusik¹, Elizabeth J. Endrizzi¹, Nic D. Leipzig¹

¹Department of Chemical and Biomolecular Engineering, University of Akron

Correspondence to: Nic D. Leipzig at nl21@uakron.edu

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Abstract

Recombinant protein engineering has utilized *Escherichia coli* (*E. coli*) expression systems for nearly 4 decades, and today *E. coli* is still the most widely used host organism. The flexibility of the system allows for the addition of moieties such as a biotin tag (for streptavidin interactions) and larger functional proteins like green fluorescent protein or cherry red protein. Also, the integration of unnatural amino acids like metal ion chelators, uniquely reactive functional groups, spectroscopic probes, and molecules imparting post-translational modifications has enabled better manipulation of protein properties and functionalities. As a result this technique creates customizable fusion proteins that offer significant utility for various fields of research. More specifically, the biotinylatable protein sequence has been incorporated into many target proteins because of the high affinity interaction between biotin with avidin and streptavidin. This addition has aided in enhancing detection and purification of tagged proteins as well as opening the way for secondary applications such as cell sorting. Thus, biotin-labeled molecules show an increasing and widespread influence in bioindustrial and biomedical fields. For the purpose of our research we have engineered recombinant biotinylated fusion proteins containing nerve growth factor (NGF) and semaphorin3A (Sema3A) functional regions. We have reported previously how these biotinylated fusion proteins, along with other active protein sequences, can be tethered to biomaterials for tissue engineering and regenerative purposes. This protocol outlines the basics of engineering biotinylatable proteins at the milligram scale, utilizing a T7 *lac* inducible vector and *E. coli* expression hosts, starting from transformation to scale-up and purification.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51295/>

Introduction

Proteins cover a wide-range of biomolecules that are responsible for many biological functions, ultimately leading to proper tissue formation and organization. These molecules initiate thousands of signaling pathways that control up-regulation and/or down-regulation of genes and other proteins, maintaining equilibrium within the human body. Disruption of a single protein affects this entire web of signals, which can lead to the onset of devastating disorders or diseases. Engineering individual proteins in the lab offers one solution for combating these adverse effects and offers an alternative to small molecule drugs. In 1977, a gene encoding the 14 amino acid somatostatin sequence was one of the first engineered polypeptides created using *E. coli*¹. Soon after in 1979, insulin was cloned in plasmid pBR322, transformed, expressed, and purified². Since then, recombinant proteins have expanded their influence to multiple fields of research such as biomaterials, drug delivery, tissue engineering, biopharmaceuticals, farming, industrial enzymes, biofuels, etc. (for reviews see references³⁻⁸). This is largely due to the versatility that the technique offers via the addition of application specific chemical moieties or protein sequences for purposes including, but not limited to, target protein identification, stabilization and purification.

Via recombinant DNA technology, recombinant proteins can be expressed in a variety of eukaryotic and prokaryotic host systems including mammalian, plant, insect, yeast, fungus, or bacteria. Each host offers different advantages and typically the best system is determined based on protein function, yield, stability, overall cost, and scalability. Bacteria cells often lack the post-translational modification mechanisms that eukaryotic hosts provide (*i.e.* glycosylation, disulfide bridging, etc.)⁵. As a result, mammalian and insect systems usually result in better compatibility and expression of eukaryotic proteins; however these hosts are typically more expensive and time-consuming⁹. Therefore, *E. coli* is the favored host for our expression system because cells expand rapidly in inexpensive growth conditions and the genetic expression mechanisms are well understood^{5,9}. Additionally, this system is easy to scale-up for production purposes and results in functional proteins despite the lack of post-translational modifications¹⁰. The *E. coli* K12 strain is chosen in this protocol for cloning because this strain offers excellent plasmid yields based on high transformation efficiencies. Additionally, an *E. coli* BL21 strain is utilized for expression because this host strain contains the T7 RNA polymerase gene which provides controlled protein expression and stability¹¹.

After host selection, further care must be taken in selecting the ideal expression vector to facilitate selected and controlled protein expression. Synthesizing recombinant proteins begins with a target DNA sequence that is cloned under the direction of bacteriophage T7 transcription

and translation signals, and expression is induced in host cells containing chromosomal copies of the T7 RNA polymerase gene¹². These vectors, derived from plasmid vector pBR322 (for review see reference¹³), are tightly controlled by the T7 promoter initially developed by Studier and colleagues¹⁴ and provide additional control through inclusion of the *lac* operator and *lac* repressor (*lacI*)^{15,16}. For recombinant protein engineering, this expression system offers the ability to tailor a specific amino acid sequence of a desired protein by inserting different target DNA sequences or to create fusion proteins made up of combined domains from single proteins. Additionally, some vector series include peptide tag modifications to be placed on the N or C terminus. For our design purposes, a histidine (His) tag was added to the DNA target sequence for purification and a 15 amino acid biotinylatable sequence was included for biotinylation^{17,18}. In this protocol a plasmid containing an ampicillin resistance gene, was chosen to carry our biotinylatable fusion protein sequences. Expression is controlled in this vector via the T7 *lac* promoter and is easily induced with isopropyl β -D-1-thiogalactopyranoside (IPTG).

Test expressions (small-scale cultures) are used to determine the presence and solubility of the target protein, which can be expressed in either a soluble or insoluble form to formulate purification procedures. A soluble protein expressed within the bacteria cell will undergo spontaneous folding to maintain its native structure¹⁹. Typically the native structure is thermodynamically favorable. In many cases the metabolic activity of the host is not conducive to the target protein, placing stress on the system that leads to insoluble protein production and the formation of inclusion bodies composed of insoluble protein aggregates. Thus the target protein denatures, rendering them generally biologically inactive²⁰. Both test expressions are scaled-up, and isolation procedures are determined by the solubility of the target protein. An additional renaturation or refolding step is required for insoluble proteins. The resulting recombinant proteins can be further purified using size exclusion chromatography.

In house recombinant protein production offers cost advantages over commercial products since milligrams of target protein can be isolated per liter of main culture. Most of the required equipment is available in a typical biological or chemical laboratory. Protein engineering allows for creation of custom fusion proteins with added functionalities which are not always commercially available. **Figure 1** depicts the main procedures involved in engineering recombinant proteins. With this expression system we have created many biotinylatable proteins, such as interferon-gamma, platelet-derived growth factor, and bone-morphogenetic protein²¹⁻²³, but we will focus on two proteins that we designed for axon guidance, NGF (29 kDa) and Sema3A (91 kDa)¹⁰ (for review see reference²⁴). Biotinylation is a common technique for identification, immobilization and isolation of labeled proteins utilizing the well-known biotin-streptavidin interaction²⁵⁻²⁷. Biophysical probes^{28,29}, biosensors³⁰, and quantum dots³¹ are some examples of systems that utilize the high affinity of biotin-streptavidin conjugation with a K_d on the order of 10^{-15} M²⁷. The *E. coli* biotin ligase, BirA, aids in the covalent attachment of biotin to the lysine side chain found within the biotin tagged sequence^{18,32}. Tethering biotin to materials and biomolecules has produced sustained delivery of growth factors to cell for multiple tissue engineering applications^{21,33-35}. Therefore, engineering these custom-designed biotinylatable proteins is a powerful tool that can transcend multiple research interests.

Protocol

1. Designing of Target Protein

- Using the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>) obtain an amino sequence for the target protein taking into account the species of interest and any splice variants. Select the amino acid sequence that corresponds to the active region of interest of the protein.
Note: For Sema3A, amino acids 21-747 were chosen. A fusion protein was designed with NGF where the sequence of barstar, amino acid 1-90, was added to NGF amino acids 122-241.
- To the N-terminus add the follow sequences: 6X-His tag for purification (HHHHHH), Tobacco Etch Virus (TEV) protease cut site for removal of His tag (ENLYFQG), biotin tagged sequence for biotinylation of protein at K (GLNDIFEAQKIEWHE) and flexible hinge with gaps that will allow room for proper protein refolding (EFPKPSTPPGSSGGAP).
Note: These sequences can vary depending on the desired fusion protein.
- Send complete amino acid sequence to a company for gene design/optimization for desired host species and synthesis. Subclone into a vector of choice using the BamHI-NotI site using a kit or by utilizing commercial services.

2. Making Agar Plates

Note: It is very important that all stocks of antibiotics, plates, buffers, etc. are stored properly (temperature and duration) and remain free of proteases (sterilized).

- Weigh out agar at 1.5 wt% and lysogeny broth (LB) at 20 g/L and place in a glass media bottle. A 500 ml bottle makes about 15 plates.
- Volumetrically add ultrapure water, mix well, and autoclave.
- Let bottle cool to the touch and add the appropriate antibiotics. Avoid premature solidification of agar; however, if bottle cools too much and solution begins to congeal, reheat in microwave.
Note: Our plasmid contains an ampicillin resistance gene. Therefore, all steps must contain ampicillin at 100 μ g/ml. The *E. coli* cloning K12 strain requires tetracycline (12.5 μ g/ml) antibiotic. BL21 bacteria cells do not require any additional antibiotic.
- Pour 25-30 ml of solution into 90 mm Petri dishes and allow time to dry.
- Label dish with appropriate antibiotics and store upside-down in 4 °C, with Parafilm or in a resealable bag.
Note: Plates are good for about one month.

3. Cloning of the Biotin Tagged Plasmid

Note: Conditions are done aseptically.

- Spin down plasmid vector at 6,000 x g for 3 min in order to pellet, and add 10 μ l of sterilized ultrapure water.
- Remove 50 μ l of chemically competent *E. coli* high transformation efficiency cells from -80 °C and immediately place on ice for 5-10 min.

3. Add 1 μ l of vector solution at 0.5-1 ng/50 μ l cells to the 50 μ l *E. coli* cells and place the remaining plasmid in -80 °C.
4. Place the bacteria cells containing the vector on ice for 5 min.
5. Heat shock the cell and vector mixture for 30-45 sec in 42 °C water bath and place cells back on ice for 2 min.
6. Add 250 μ l of super optimal broth with catabolite repression (SOC) medium (2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄, 20 mM glucose, ultrapure water; pH= 7.0) and shake at 250 rpm at 37 °C for 30 min.
Note: SOC should not be autoclaved but be filter sterilized through a 0.22 μ m filter.
7. Dry plates 10-15 min prior to plating cells.
8. Pipette 25, 50, and 100 μ l of cell solution onto separate agar plates containing the ampicillin (100 μ g/ml) and tetracycline (12.5 μ g/ml) antibiotics. Use glass beads to distribute solution evenly over agar plates.
9. Incubate plates upside-down at 37 °C for 15-18 hr.
10. Check for small individual bacteria colonies on plates and store upside-down at 4 °C until further use (**Figure 2A**).
 1. If no colonies are present:
 1. Check the freshness and sterility of buffers and supplies.
 2. Increase the amount of plasmid added to *E. coli* K12 strain.
 2. If large colonies are present or there is an overgrowth of colonies (**Figure 2B and 2C**), restreak a new agar plate using a sterile inoculation loop or plate cells at lower volume.
11. Inoculate 5 ml LB containing ampicillin (100 μ g/ml) and tetracycline (12.5 μ g/ml) antibiotics with a single colony of transformed *E. coli* cells. Grow-up cells overnight at 37 °C at 250-300 rpm.
12. The following day, use a plasmid isolation kit in order to isolate the target vector from the overnight grow-up and store purified plasmid at -80 °C until further use.
 1. **Optional:** Check purity and concentration of plasmid using absorbance reading obtained at 260 and 280 nm.

4. Transformation of Plasmid into Expression Host

Note: Conditions are done aseptically.

1. Repeat steps 3.2-3.10 for *E. coli* BL21 cells with the following changes:
Note: BL21 cells do not require any additional antibiotics, therefore only ampicillin is added to the agar plates for transformation.
 1. Add 2-5 μ l of isolated plasmid at 0.5-2 ng/50 μ l cells from step 3.12 to 50 μ l *E. coli* expression host cells.
 2. Heat shock cells for 60-90 sec.
 3. Shake solution containing SOC medium at 250 rpm for 60 min instead of 30 min.
2. Pluck a single colony of transformed bacteria cells from the Petri dish with a sterile applicator stick and place in a test tube containing 5 ml LB broth with the appropriate concentration of ampicillin (100 μ g/ml).
3. Place test tubes in shaker overnight at 37 °C at 250 rpm.
4. The following morning, take 200 μ l of the overnight grow-up and add it to 5 ml LB containing ampicillin at 100 μ g/ml.
5. Freeze down the remaining cells with 250 μ l 50% glycerol (sterile) to 750 μ l culture and keep in -80 °C.
Note: New test expression and scale-up procedures can be done by using a sterile applicator stick to obtain a scraping of frozen cells and inoculating LB with the appropriate antibiotics.
6. Place test tube in shaker at 250-300 rpm at 37 °C until optical density (OD) is measured between 0.7-0.8 at an absorbance of 600 nm.
7. Induce cells with IPTG at a final concentration of 1 mM.
8. Shake for an additional 4 hr at 37 °C at 300 rpm. Alternatively cells can also be shaken overnight at 18 °C at 250-300 rpm. This can produce a higher yield of plasmid depending on the target protein.
9. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Test Expression
 1. Take 500 μ l of culture and place in a 1.5 ml tube. Spin down at 13-15 x g for 5 min. Pour off supernatant liquid and label "Soluble." Resuspend pellet with vortexing in 200 μ l of loading dye (50 μ l 2-mercaptoethanol, 950 μ l Laemmli sample buffer).
Note: Soluble bacteria pellets will be used to determine if the recombinant protein is in the cytoplasmic regions of the bacteria cells. Pellets can be stored in -80 °C without loading dye until needed. A control bacteria culture that has not been transformed or induced can be treated as soluble as well for comparison.
 2. Take 1,000 μ l of culture and place in a 1.5 ml tube. Spin down at 13-15 x g for 5 min. Pour off supernatant liquid and label "Insoluble."
Note: Insoluble pellets will undergo denaturation procedures below in order to release proteins found in inclusion bodies of bacteria cells. Pellets can be stored in -80 °C until needed. A control bacteria culture that has not been transformed or induced can be treated as insoluble as well for comparison.
 1. Resuspend the insoluble pellet in 200 μ l Bugbuster and leave at room temperature for 30 min.
 2. Spin down sample again at 13-15 x g for 5 min and take 50 μ l of supernatant and add it to new "Insoluble" 1.5 ml tube.
 3. Then add 50 μ l of loading dye to this new sample.
 3. Boil both soluble and insoluble samples for 5 min to denature proteins for SDS-PAGE analysis.
 4. Load each sample into electrophoresis gel with standard ladder.
 5. In order to visualize the protein samples use a staining reagent and follow the company's protocol.
 6. Determine whether the protein expresses in the soluble and/or insoluble fractions by comparing these two lanes as well as comparing the lanes to the soluble and insoluble control lanes (see Note 4.9.1 and 4.9.2) on the SDS-PAGE gel (**Figure 2**). A dark band should appear around the molecular weight of the protein in the soluble or insoluble lanes. This will determine which isolation procedure to use in Protocol 6.
Note: If there is a band in both of these regions than either isolation used in Protocol 6 below can be performed, or the protein can be isolated both ways in order to determine which isolation method produces a higher yield of recombinant protein (Figure 3).

7. If a 4 hr and overnight induction was conducted, determine which induction method has the highest protein production for the scale-up process (**Figure 2**).

5. Scale-up Procedure and Main Culture

1. Prepare growth media with 85.7 g of Terrific Broth and 28.8 ml of 50% glycerol in 1.8 L of ultrapure water and autoclave on liquid cycle for 1 hr.
2. Start an overnight culture from frozen cell stock created in step 4.5.
Note: Conditions are done aseptically.
 1. Mix 20 ml of LB and with a final concentration of ampicillin at 100 µg/ml in a sterile 125 ml Erlenmeyer flask.
 2. Using a sterile applicator stick take a scrapping of transformed *E. coli* cells and drop applicator into the flask. Remove applicator stick and plug flask with foam stopper or cotton and cover with aluminum foil to maintain sterility.
 3. Shake overnight at 250 rpm at 37 °C.
3. Main Culture
Note: Conditions are done aseptically.
 1. Pour overnight culture into 1.8 L of sterile growth media and add ampicillin at 100 µg/ml and 6 to 8 drops of sterile antifoam 204 using a Pasteur pipette.
 2. Place cultures in a 37 °C water bath with 0.2 mm filtered compressed air bubbling into cultures through aeration stones.
 3. Once the OD_{600nm} reaches 0.7-0.8, induce with IPTG (1 mM) and allow induction to occur for either 4 hr at 37 °C or overnight at 18 °C depending on soluble/insoluble results from SDS-PAGE in step 4.9.
4. Collecting *E. coli* Cells
 1. Spin down the cell culture in 1 L centrifuge bottles (2 bottles/culture) for 15 min at 14,000 x g at 4 °C.
 2. Pour off supernatant and using a thin spatula, scoop the bacteria pellet into two 50 ml tubes. The cells can be pelleted again by centrifugation for a few minutes.
Note: Each 1.8 L culture will result in two bacteria pellets, one in each 50 ml centrifuge tube.
 3. Store pellets in -80 °C until protein isolation.

6. Isolation and Purification of Recombinant Protein

Note: If the protein is located in the soluble region based of SDS-PAGE Analysis from step 4.9 then "Native Isolation" will be used, but for insoluble proteins "Nonnative Isolation" procedures will be performed.

1. Cell Lysis
 1. Nonnative
 1. Remove transformed *E. coli* pellet from -80 °C freezer, and add 20 ml Lysis/Wash Buffer (**Table 1**) to each centrifuge tube.
 2. Vortex and shake the frozen pellet until it thaws and solubilizes into the buffer solution without any large chunks. Incubate on nutator overnight. The following morning, the pellet should now be a viscous slurry due to the denaturing of the protein from the buffer.
 3. Centrifuge the slurry at 20,500 x g at room temperature for 30 min. Transfer the supernatant to a fresh tube for isolation and discard the pellet.
 2. Native
 1. Obtain transformed *E. coli* pellet from -80 °C freezer.
 2. Add Lysis Buffer (**Table 1**) to centrifuge tube to reach a final volume of 30 ml, and dislodge frozen pellet by vortexing and shaking rigorously. Place pellet on ice.
 3. Wash sonicator probe with 70% ethanol at 100% amplitude for 1-2 min. Then repeat with ultrapure water.
 4. Sonicate bacteria pellet while on ice for 5 min (total elapsed time of 10 min).
 1. Set sonicator at 30% amplitude with pulse of 30 sec on/30 sec off.
 2. Bob centrifuge tube up and down during sonication interval in order to completely break up the cell pellet. At the end of the total elapsed time there should be no visible bacteria pellet leaving a stringy, viscous slurry.
 3. Clean the sonicator probe between different protein isolations as well as for storage using 70% ethanol and ultrapure water as described in step 6.1.2.3.
 5. Centrifuge the slurry at 20,500 x g at 4 °C for 30 min. Transfer the supernatant to a fresh tube for isolation and discard the pellet.
2. Ni-NTA Affinity Chromatography
 1. Nonnative
 1. Add 1 ml Ni²⁺-NTA resin solution to each centrifuge tube (2 ml resin total for a 1.8 L culture) and incubate at RT for at least 1 hr on nutator.
 2. Pour slurry into affinity column and allow solution to drip through stopcock valve completely into waste beaker.
 3. Perform 10 washes with 10 ml of Lysis/Wash Buffer for each wash.
 1. For the first two washes, add the 10 ml to the centrifuge tube to remove additional resin and then pour into column. The additional washes can be added directly to column.

2. After each wash, stir the resin with a glass stirring rod. Once the wash drips into the waste beaker, the next 10 ml can be added.
 4. After wash completely drips through, close stopcock valve, remove waste beaker and replace with 50 ml centrifuge tube for protein collection.
 5. Add 15 ml of Elution Buffer (**Table 1**) and stir-up resin, allowing solution to sit for 5 min. Open stopcock valve and collect elution. Repeat again with an additional 15 ml.
2. Native
1. Follow the Nonnative Affinity Chromatography above (Steps 6.2.1.1-6.2.1.4) except adjust the following parameters:
 1. Incubate Ni^{2+} -NTA resin solution at 4 °C for at least 1 hr on nutator.
 2. Use the appropriate Wash Buffer (**Table 1**).
 2. Add 5 ml of Elution Buffer (**Table 1**) and incubate with resin for 5 min.
 1. Open stopcock valve and collect elution until most of the solution has dripped through.
 2. Add 90 μl /well of Bradford reagent to clear 96-well plate. After each elution allow 10 μl of solution to drip from column into well containing 90 μl Bradford reagent.
 3. Continue adding 5 ml Elution Buffer at a time until Bradford assay no longer detects protein (color goes from dark blue to light blue to clear). This usually takes 4-5 elution volumes.
3. Dialysis/Renaturation
1. Make nonnative (renaturation) and native dialysis buffers (**Table 1**) and store in 4 °C.
Note: The pH of the dialysis buffers are determined by the target protein's isoelectric point (pI). As a rule of thumb proteins should be buffered at least one full point above or below their pI values.
 2. Transfer native and nonnative elutions to dialysis tubing with appropriate molecular weight cutoff (25,000 MWCO for NGF and 50,000 MWCO for Sema3A). Place each protein sample in respective dialysis buffer 1 for 4 hr at 4 °C and then replace with the respective buffer 2 overnight at 4 °C.
Note: Protein needs to be dialyzed in each buffer for at least 4 hr for proper refolding and buffer exchange to take place.
 3. Concentrate the dialyzed protein elutions to less than 5 ml using centrifuge spin concentrators.
Note: Proteins can further be purified by using fast protein liquid chromatography (FPLC) and concentrated again.
4. Determine protein concentration (mg/ml) by freeze drying a 10-50 ml sample in a preweighed microcentrifuge tube.
1. **Optional:** determine the extinction coefficient, ϵ , so that concentration of protein can be determined in future isolations by measuring the absorbance of a sample at 280 nm using Beer-Lambert's Law: $c = A_{280\text{nm}} / \epsilon l$, where l is the path length.

7. Biotinylation of Purified Protein

1. Dialyze proteins in 10,000 MWCO dialysis cassettes against 10 mM Tris (pH=8.0), changing the buffer every 4 hr with a total of 6 changes.
2. Transfer the recombinant proteins (now in 10 mM Tris buffer) to 2 ml micro-centrifuge tubes for biotinylation.
3. Biotinylate proteins using a biotin protein ligase kit.
4. Dialyze proteins against PBS (pH=7.4) using 10,000 MWCO dialysis cassettes with 3 buffer changes a day for 2 days.
5. Determine the percent biotinylation of proteins using a quantitation kit.
6. Determine the concentration again as described in 6.4 and store at 4 °C or aliquot and store at -20 °C for long term storage.

Representative Results

Cloning and Test Expression

When plating is performed properly, single isolated colonies should form to increase the chances of plucking clonal transformed bacteria cells (**Figure 2A**). However, if too many cells are plated, plates are incubated too long at 37 °C or transformation is questionable, colonies may cover the agar plate or form bigger aggregates of cells (**Figures 2B and 2C**). During test expression, NGF and Sema3A were first induced for 4 hr at 37 °C and SDS-PAGE analysis determined both proteins were located in the soluble fraction (**Figure 2D**). Protein expression seemed fair and therefore another test expression with overnight induction at 18 °C was examined. NGF resulted in better expression in the soluble fraction, whereas there was no noticeable difference with Sema3A (**Figure 2E**).

Isolation, Purification and Biotinylation

Both NGF and Sema3A were isolated using native isolation techniques as described in the above protocol and run through the FPLC column for purification. Additionally these recombinant proteins were isolated and purified nonnatively. Even though NGF was in the soluble fraction during test expression, native isolation procedures produced a low yield of NGF after both 37 °C (4.57 mg/main culture of 2 L) and 18 °C (6.11 mg/main culture of 2 L; **Figure 3A**, solid lines) inductions. However, a higher yield of NGF was obtained through nonnative isolation with renaturation (10.72 $\pm$ 1.8 mg/main culture of 2 L; **Figure 3A**, dashed line) after 18 °C, overnight induction. On the other hand, nonnative isolation resulted in no Sema3A production (**Figure 3B**, dashed line) with 8.61 $\pm$ 3.1 mg/main culture of 2 L for native isolation (**Figure 3B**, solid line). As a result, NGF was isolated under nonnative conditions after overnight induction at 18 °C, whereas, Sema3A underwent native isolation after 4 hr of induction at 37 °C. FPLC peaks were collected, and NGF and Sema3A samples were analyzed with SDS-PAGE (**Figure 3**). Additional protein peaks found in the FPLC output for Sema3A (**Figure 3B**) were collected and analyzed with SDS-PAGE and were not located in the Sema3A molecular weight region. These fractions are most likely degradation products because they did not behave functionally in cell based assays as did the Sema3A

peak indicated in **Figure 3B**. Proteins were biotinylated using a BirA enzyme and dialyzed to remove any unreacted species. Biotinylation was confirmed with a biotin quantification kit and fluorescent microplate reader.

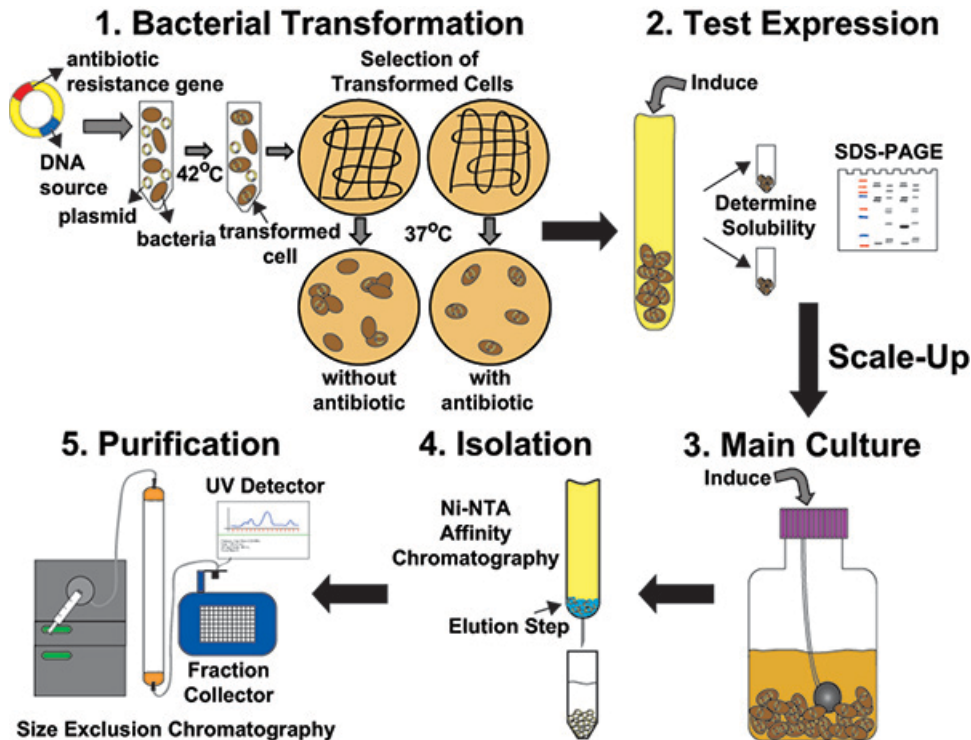


Figure 1. Protein engineering utilizing an *E. coli* expression system. The basic process of recombinant protein engineering involves designing of a plasmid for cloning and expression in *E. coli*. Transformed colonies are selected with the use of antibiotics. Small test expressions are used to optimize and identify the production and solubility of the target protein. This procedure is scaled-up to large batch cultivations (liter scale). Chromatography methods are used to isolate and purify the desired engineered protein. Modifications such as biotinylation can occur during batch cultivations or after purification.

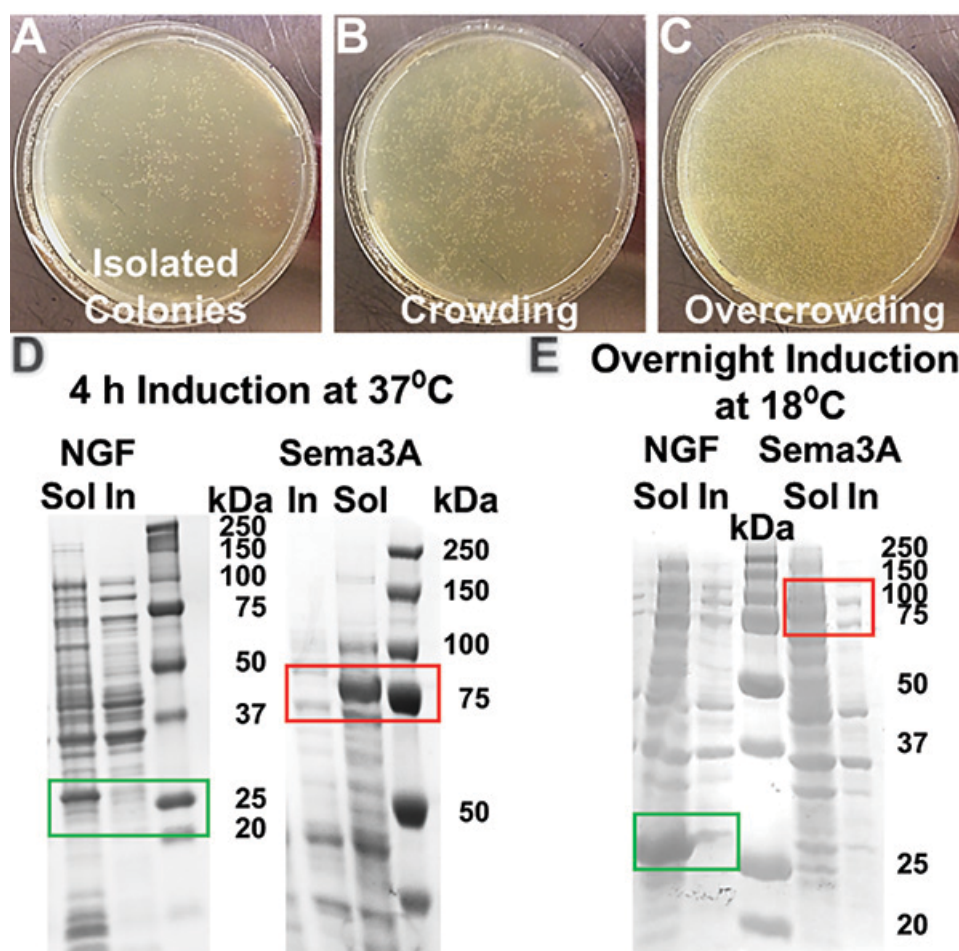


Figure 2. Optimizing test expression of NGF and Sema3A. (A) Plating 25 μ l of transformed *E. coli* K12 cells resulted in small isolated colonies. An individual colony should be selected for test expression. For BL21 transformed cells similar colony distribution should occur. (B-C) Plating 50 μ l and 100 μ l of K12 cells, respectively, resulted in overpopulation of bacterial colonies. Plucking from these plates could result in a lower probability of selecting a colony derived from a single transformed cell. (D) Test expressions of transformed cells were induced for 4 hr at 37 °C with IPTG, and SDS-PAGE shows that NGF fusion protein (29 kDa) and Sema3A fusion protein (91 kDa) are expressed in the soluble fraction. (E) Overnight induction at 18 °C shows NGF expression still in the soluble fraction; however, expression for Sema3A is not enhanced.

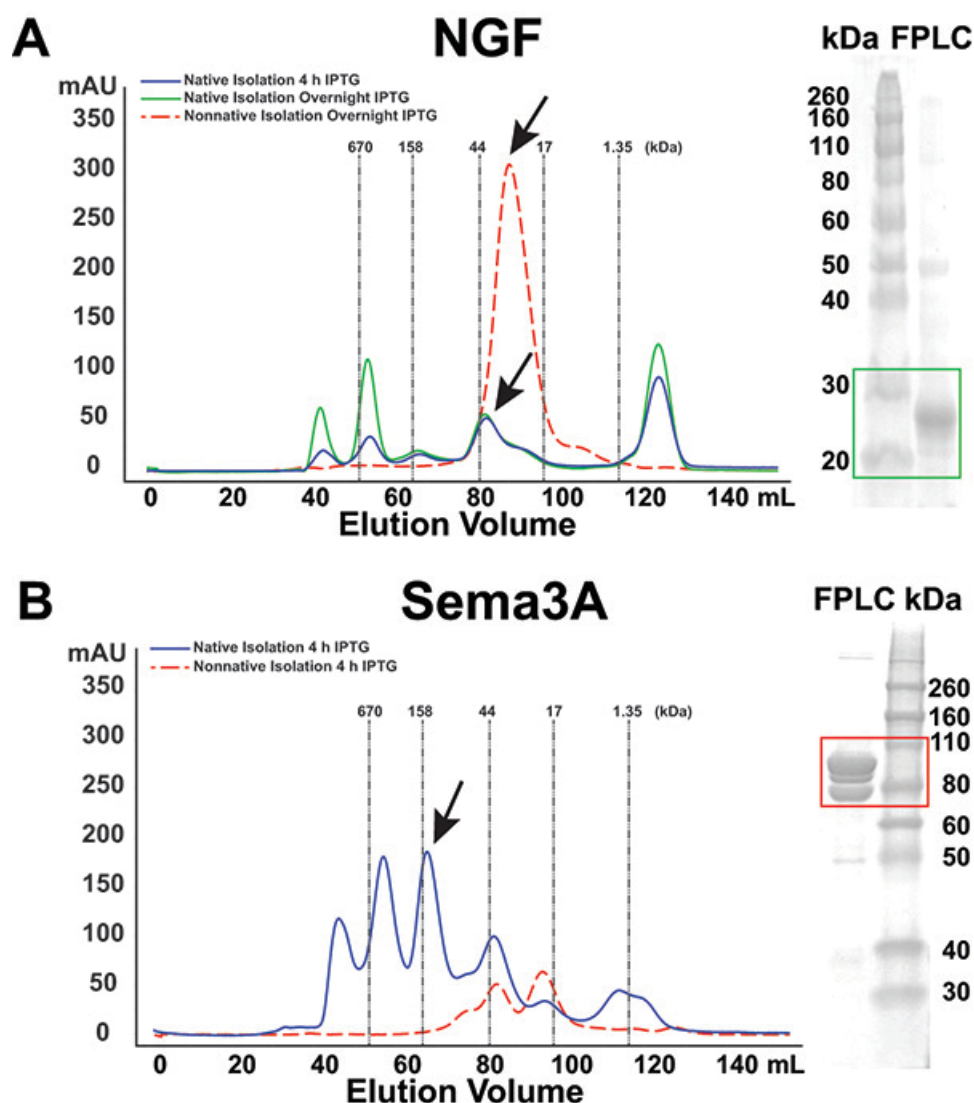


Figure 3. Purification of NGF and Sema3A using FPLC. (A) Nonnative isolation after 18 °C overnight induction (dashed line) resulted in better NGF yields compared to native isolation at both 4 hr, 37 °C and overnight, 18 °C inductions (solid lines). (B) For Sema3A, induction at 37 °C for 4 hr and native isolation condition produced better yields compared to nonnative isolation at the same cultivation parameters. SDS-PAGE shows FPLC peak collections (arrows) for both (A) NGF and (B) Sema3A. A gel filtration protein standard was run to confirm the molecular weight distribution of the peaks and is indicated in the background of the FPLC plots in kDa.

Isolation	Buffer	Components
Nonnative	Lysis/Wash	6 M GuHCl, 100 mM H ₂ NaPO ₄ , 10 mM Tris Base, 10 mM imidazole; pH=8.0
Elution	6 M GuHCl, 200 mM Glacial Acetic Acid (17.4 M)	
Dialysis (Renaturation)	Phosphate buffer saline (PBS): 21.7 mM NaH ₂ PO ₄ , 15.3 mM Na ₂ HPO ₄ , 149 mM NaCl	
Buffer 1: PBS, 0.2 M GuHCl, 1.99 mM dithiothreitol (DTT) in 4 L ultrapure water; pH=7.4		
Buffer 2: PBS in 4 L ultrapure water; pH=7.4		
Native	Lysis	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 10 mM imidazole; pH=8.0
Wash	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 20 mM imidazole; pH=8.0	
Elution	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 250 mM imidazole; pH=8.0	
Dialysis	Phosphate buffer saline (PBS): 21.7 mM NaH ₂ PO ₄ , 15.3 mM Na ₂ HPO ₄ , 149 mM NaCl	
Buffer 1: PBS, 1.99 mM DTT in 4 L ultrapure water; pH=7.4		
Buffer 2: PBS in 4 L ultrapure water; pH=7.4		

Table 1. Recombinant Protein Isolation Buffers.

Discussion

Recombinant protein engineering is a very powerful technique that spans many disciplines. It is cost-effective, tunable and a relatively simple procedure, allowing the production of high yields of custom-designed proteins. It is important to note that designing and expressing target proteins is not always straightforward. Basal expression and recombinant protein stability depend on specific choices of vector, *E. coli* cell strains, peptide tag additions and cultivation parameters. Our specific design criterion utilizes well established *E. coli* strains for protein engineering. Additionally, the plasmid vector contains a T7 *lac* promoter and ampicillin resistance gene for stable recombinant protein expression.

After obtaining highly purified subcloned plasmid, the first major procedure is to successfully transform the target protein into the host cell and determine the solubility of the protein. Test expressions are the best time to optimize the synthesis of the target protein. It is important to pluck small, isolated colonies (**Figure 2A**) to ensure better selection of bacteria cells containing the plasmid. Troubleshooting strategies such as restreaking to redistribute the colonies or incubating agar plates for shorter time periods could aid in better colony formations. It is no surprise that recombinant protein production induces stress on the host strain³⁶. During this stage if expression is poor, factors such as antibiotic and IPTG concentration as well as induction time and temperature can be adjusted to achieve the optimum target protein (for reviews see³⁷⁻³⁸). This was seen for NGF where the best expression resulted from overnight induction at 18 °C (**Figure 2E**).

Sema3A resulted in protein production for native conditions but nonnative isolation showed no protein production (**Figure 3B**). Main cultures of NGF were induced for 4 hr at 37 °C as well as overnight at 18 °C and isolated under native conditions. FPLC purification produced a lower yield of NGF compared to main cultures that were isolated nonnatively after overnight induction at 18 °C (**Figure 3A**). The formation of proteins in inclusion bodies is generally thought to be undesirable since renaturation is sometimes difficult and not always successful. This has led to the development of techniques to enhance protein solubility including the addition of soluble protein sequences³⁹⁻⁴¹. However, proteins have been shown to possess forms of conformational states while isolated in inclusion bodies, and parameters such as lower induction temperatures help to maintain these active structures⁴²⁻⁴⁶. When a protein can only be expressed in the insoluble form it is usually possible to renature the protein after isolation using simple techniques with very little yield loss as we have reported previously²¹.

We have shown how to successfully engineer and produce biotinylatable proteins using an *E. coli* cloning and expression system as the host yielding about 8-10 mg/main culture of 2 L. It is important to note that the overall sequence of events remains the same when producing new recombinant proteins (**Figure 1**); however, each custom-made protein should be treated on a case by case basis to determine how to specifically produce the highest yield of purified product. We have shown how NGF and Sema3A behave differently in the same cultivation system and how to optimize their production. Additionally, we are currently using another *E. coli* B host strain that can biotinylate *in vivo*⁴⁷ for other target proteins, demonstrating the importance of staying well-informed on the ongoing advances and modifications regarding recombinant protein engineering.

Disclosures

The authors have nothing to disclose.

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