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Approaches for Purifying Recombinant Proteins from Gram Negative Bacteria

Susan Burran

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APPROACHES FOR PURIFYING RECOMBINANT PROTEINS FROM GRAM NEGATIVE BACTERIA

by

SUSAN ADAMS BURRAN

Under the Direction of George Pierce, PhD

ABSTRACT

The ideal vaccine can generate a strong immune reaction without adverse effects on the body. Thus, many vaccines are now created using recombinant technology to accomplish this goal. Purification of recombinant proteins produced in Gram negative bacteria (GNB) presents several challenges, including reducing the concentration of contaminating host cell molecules to nontoxic levels. The most prevalent host cell molecule is lipopolysaccharide (LPS), which is a major constituent of the outer membrane of GNB. Residual LPS in final product presents a major problem for proteins intended for pharmaceutical application, as it is toxic to mammalian cells. An orthogonal approach is an FDA requirement for preparation of proteins intended for use as pharmaceuticals. The ideal approach not only reduces contaminants to acceptable measures, but also results in a high yield of properly folded protein, while maintaining an expeditious time table and keeping costs low. Though a truly universal scheme for processing proteins from GNB is not possible, a comprehensive study of scalable and certifiable methods currently
used for protein purification will be performed on multiple constructs in order to outline general principles for the system in a helpful blueprint, evaluating the effectiveness of different methods of retrieval of protein from inclusion bodies in particular. This approach is based upon the hypothesis that production of fusion proteins in the insoluble fraction results in a greater yield of pure protein with fewer processing steps.

INDEX WORDS: Gram negative bacteria, Lipopolysaccharide, Endotoxin, Protein, Purification, Inclusion bodies
APPROACHES FOR PURIFYING RECOMBINANT PROTEINS FROM GRAM NEGATIVE BACTERIA

by

SUSAN ADAMS BURRAN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University 2019
APPROACHES FOR PURIFYING RECOMBINANT PROTEINS FROM GRAM NEGATIVE BACTERIA

by

SUSAN BURRAN

Committee Chair: George Pierce
Committee: Sidney Crow
Eric Gilbert

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2019
DEDICATION

This dissertation is dedicated to my fantastic family. Specifically, to my extremely patient and understanding husband, Andy, without whom I would have gone insane many times over; and my parents and sister, Kathryn, who never doubted me and who’s unconditional love I treasure. I also would like to thank my wonderful in-laws who have given me their fullest support and encouragement. Finally, I would like to dedicate this work to my children, who are my greatest inspiration.
ACKNOWLEDGEMENTS

I would like to thank the members of the Pierce lab who provided me with lab support throughout this process. Additionally, I would like to thank Dr. Mugdha Vasireddi from Dr. Hilliard’s lab for aid in tissue culture. I would further like to thank Dr. Shelby Jones, and Dr. Trudy Ann Tucker for not only providing lab support but also providing mental health support, friendship, and generosity. Lastly, I would like to thank my support team at Dalton State College including my department chair, Marina Smitherman, dean, Randall Griffus, and my friends, Annabelle and Travis McKie-Voerste for providing unfailing support and accountability.
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1. INTRODUCTION

Industry is increasingly leaning towards the use of bacteria to produce various pharmaceutical products, particularly proteins. This is primarily because cultivation of bacteria saves both money and time in comparison to eukaryotic cell culture systems, and in addition a large amount of desired protein may be produced by bacterial systems. Industry leaders and researchers most commonly use *Escherichia coli* as an organism-expression system, owing to the extensive knowledge base surrounding this organism (1). Because of the widespread use of *E. coli*, culturing techniques have been developed which enable the achievement of very high cell densities. Generally, the goal of bacterial protein production is to generate a high concentration of cells that then express the preferred protein efficiently and then ensuring it is of high quality. It is very difficult to realize all these goals within the same procedure, so there is a constant effort to increase the pace and simplicity of production while maintaining high quality standards.

A major concern of this system is the production of endotoxin by *E. coli*. As a Gram-negative bacterium (GNB), *E. coli* contains lipopolysaccharide (LPS) as a vital component of its outer cell membrane, comprising 75% of the outer surface of *E. coli* (2). LPS is generally considered to be the major type of endotoxin, so named because of its close associated with the cell, thus differentiating it from secreted exotoxins. Once released from the surface of *E. coli*, which occurs both during rapid growth and lysis, LPS acts as a pathogen-associated molecular pattern (PAMP) and is a strong aggravator of the innate immune response. The LPS associates with a circulating glycoprotein known as LPS-binding protein (LBP); when this complex interacts with CD-14, it is recognized by TLR4 (Figure 1.1). TLR4 response triggers the expression of several pro-inflammatory cytokines, which will in turn lead to inflammation and toxic shock. Because of the risk of endotoxemia in mammals, it is generally considered unacceptable for phar-
maceuticals to contain >5EU/kg body weight (1EU is approximately 100pg of endotoxin) (3). The presence of endotoxins in the bloodstream can lead to toxemia, with symptoms including high fever, shock, bleeding disorders, organ failure, and death (4).
Figure 1.1 Interactions between LPS, LBP, and CD14 (5)
Removal of endotoxins is paramount to the safety of the bacterially-produced pharmaceuticals. This can be a difficult task as endotoxins are highly stable and often associate with the recombinant proteins (6). The general structure of an LPS molecule consists of an O-antigen repeating chain and core oligosaccharide (often referred to as the heteropolysaccharide region) covalently bound to Lipid A, a phosphoglycolipid. Lipid A is considered to be the toxic center of LPS, as bacteria deficient in Lipid A do not elucidate toxic shock in vivo (7). There is a significant amount of structural variation in the components of LPS among different bacterial species, especially in Lipid A. The general structure of Lipid A includes a phosphorylated disaccharide made of hexosamine residues, with fatty acid chains connected to the dimer. Different species possess distinctions in the structure of Lipid A, including the type of hexosamines, the degree of phosphorylation, and the number, location, and length of fatty acid chains. For E. coli it is well established that six asymmetrically organized fatty acid chains, containing fourteen carbons atoms each, are attached to a phosphorylated glucosamine dimer (Figure 1.2). The fatty acid chains of Lipid A generate a hydrophobic characteristic that allows LPS to form aggregates or strongly associate with other hydrophobic proteins, shielding the hydrophobic region. The core oligosaccharide, or heteropolysaccharide region, however, is hydrophilic. Together, these two regions give LPS an amphipathic characteristic which makes them very difficult to remove from solution. Additionally, the phosphate groups of Lipid A provide a negative charge, which gives LPS an anionic characteristic as well (6). This feature suggests that positively-charged proteins may be preferentially associated with endotoxin.
Figure 1.2 Diagram of cell envelope of Gram negative bacteria, inset Lipid A region of LPS (8)
Several methods have been developed to address the issue of sufficiently reducing endotoxins from microbially-derived protein solutions without tremendous sacrifice in yield or activity of the recombinant protein. As previously mentioned, LPS behaves as an amphipathic chemical because it contains hydrophobic, hydrophilic, and anionic regions. These unique chemical characteristics of LPS make it exceptionally stable; using extremes in heat and pH would likely destroy target protein before endotoxin concentration is satisfactorily reduced (9). More commonly, chemicals or affinity sorbents that exploit one of the areas with a distinctive chemical trait (i.e. the hydrophobic region) are utilized to segregate the endotoxin from the target protein and then the two may be safely separated. Detergents or chelating agents such as Triton-X or EDTA are commonly used to dissociate endotoxin from the desired protein, but remaining chemicals can alter cell membrane fluidity characteristics and therefore be potentially harmful to living cells (10). Additionally, residual detergents could be detrimental to the bioactivity of the target protein. Therefore, it is imperative that measures be taken to ensure there is no residual detergent or chelating agent in the preparation. Affinity chromatography with Polymyxin B has been shown to significantly reduce endotoxin (11), but if the target protein is strongly associated with endotoxin there is a risk of losing a significant amount of protein in this process (12). Size exclusion and filtration techniques for endotoxin removal are less effective, as there needs to be a significant difference in size between target protein and endotoxin. Combining a variety of approaches is widely accepted to be the most effective way of removing endotoxin efficiently without sacrificing the yield of the target protein (13).

Given all that is known about the structure and behavior of endotoxin, and considering the lack of any purification scheme that is considered universally effective at removing endotoxin from recombinant protein produced in GNB, designing recombinant proteins in silico may be a useful tool for future research efforts. Computational modeling has the potential to streamline the process of discovering an appropriate purification method, as researchers could design a recombinant protein that is unlikely to
associate strongly with endotoxin. Thus, screening potential constructs \textit{in silico} has the potential to vastly streamline the purification scheme while assuring highest level of activity of putative fusion proteins (unpublished work, Pierce lab).
1.1 Purpose of the Study

The intention of this project is to use an orthogonal multistep approach to deduce methods by which contaminating endotoxin can be effectively removed from microbially-produced recombinant fusion proteins, and which purification conditions maintain the highest level of bioactivity. This is based on the development of a relatively new platform to generate fusion proteins produced in Gram negative bacteria for use as vaccines.

Transformed *E. coli* is grown in a large bioreactor and plasmid expression is induced once the bacteria have reached a desired concentration. The fed-batch fermentation procedure has been optimized to increase cell yield and maximize the expression of the plasmid containing the recombinant protein. After fermentation, the cells are harvested and dewatered using a continuous flow 2-phase separation device, generating a thick cell paste. Once the cell paste is collected, the cells are disrupted and clarified via a multi-step process. Each step of this process is specifically geared to reduce cellular contaminants to within an acceptable range, yielding the largest possible amount of highly effective, purified protein (Figure 1.3).

Protein purification from microbial systems traditionally focuses on purification from the soluble fraction, due to the perceived difficulty of retrieval of native protein from within insoluble fraction. Overexpression of recombinant proteins in bacterial systems, especially *E. coli*, tends to result in the formation of aggregates of misfolded or partially folded intermediates (14). These aggregates, or inclusion bodies (IB), have many characteristics that make them readily segregate from other cellular components and fermentation media, a property that may be exploited in order to reduce the number of purification steps required to retrieve target protein. It is generally considered preferable to utilize mainly physical means for purification: this approach reduces the number of downstream steps required to remove chemical additives introduced in earlier steps (11). Additionally, there tends to be a high amount of protein within IBs and this protein is less likely than cytosolic protein to associate closely with other cellular
debris (15). Thus, there are advantages to exploring and optimizing purification schemes which focus specifically on the recovery of active protein produced within inclusion bodies.

One of the aims of this study is to evaluate each step of the protein purification procedure, assessing the effectiveness of different techniques on endotoxin reduction and activity of target protein. This assessment will be weighed against the time and resources required for each step or phase of the purification procedure, with the intent of establishing an optimized and efficient purification scheme.

**Figure 1.3 Protein purification scheme**
A future concern for protein purification from GNB is taking measures to reduce the iterative nature of the process. Initial purification steps are consistent regardless of target protein conformation, but little is known about how to predict which purification scheme is ideal for different proteins. In fact, there is no consensus about which properties of a protein make it more likely to be produced within the insoluble or the soluble fraction (16). Recombinant proteins overexpressed in *E. coli* tend to form cytoplasmic inclusions, especially when an inducer is used. It is widely accepted that this phenomenon is due to the amount of protein expressed overwhelming the chaperone system, and the likelihood of protein to form intermediates under the reducing conditions of the cytoplasm (17). Putative fusion proteins could be evaluated for structural and chemical similarity to other recombinant proteins using *in silico* modeling, and purification procedures adopted which best fit the characteristics of the fusion protein. While it may not be possible to completely prepare a purification scheme *a priori*, this approach could certainly reduce the time and cost associated with determining an ideal scheme for protein preparation.
1.2 Description of Constructs

The fusion proteins generated for this project include flagellin protein (from *Salmonella typhi-murium*), the major component of bacterial flagellar filament, because it acts as an agonist for toll-like receptor 5 (TLR5). TLRs recognize structurally conserved ligands/antigens present on microorganisms and play a fundamental role in the induction of the adaptive immune response (18, 19). There are 11 known TLRs, each with different specificity, and TLR5 is specific for flagellin. Unlike the other TLRs, TLR5 is the only TLR that recognizes a protein, making it an ideal innate immune system activator. Linkage of TLR5 ligands to vaccine antigens has been shown to increase immunopotency of the linked antigen (20, 21). Flagellin alone has been shown to act as an adjuvant to vaccines (22). Structurally, flagellin contains 5 domains, 4 of which directly interact with TLR5 (D0-D3). Of these, D0 and D1 are highly conserved and correspond to the C and N termini and are required for activation of the innate immune response via TLR5. The central regions, D2 and D3, are considered hypervariable and not required for activation of TLR5 (23). However, a portion of the D3 region is required for correct folding of flagellin, thus care must be taken if excising portions of the hypervariable region.

A construct was created in which full-length flagellin (FliC) from *S. typhimurium* was cloned into a plasmid vector and expressed in *E. coli* and grown in a bioreactor. The fermentation product (known as FA-4), was then purified following a purification scheme which evaluated the effectiveness of multiple purification conditions. This construct was generated for the purpose of evaluating the purification processes necessary for the full-length flagellin protein, under the presumption that fusion proteins based upon TLR5 stimulation by flagellin could be similarly purified. As previously mentioned, flagellin is sufficient to initiate a pro-inflammatory innate immune response. Use of flagellin as an adjuvant has been shown to increase effectiveness of vaccines, thus the development reliable method for rapid producing large amounts of highly pure, biologically active flagellin from a microbial system would be highly beneficial.
| D0 | D1 | D2-D3 | D1 | D0 |

Figure 1.4 Domains of Full-length flagellin (23)

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Figure 1.5 Structure of flagellin monomer and filament (24)
To construct the two fusion proteins used, fliC (S. typhimurium flagellin protein) was fused to immunologically significant regions of two different viruses in order to function as recombinant vaccines. Common production of vaccines is accomplished by disabling or inactivating the whole pathogen which may result in occasional adverse reactions. Subunit or recombinant vaccines can be advantageous because only the antigenic portion of the pathogen is introduced into the body, thus the safety of the vaccine is ensured. A challenge commonly associated with subunit and recombinant vaccines is the tendency of the resulting immune response to be somewhat weak. By fusing antigenic portions of viruses with flagellin, the effectiveness and safety of vaccines can be ensured. Once the recombinant constructs were created, they were then cloned into plasmid vectors and expressed in E. coli, grown in a bioreactor, and purified according to the schematic above (Figure 1.6). This imparts another advantage over traditional vaccine preparation: growing recombinant vaccines in a bioreactor may greatly increase yield (the average yield for prokaryotic systems is 3-4 x10^5 doses/L). The two viruses chosen for this analysis were influenza virus and Marburg virus.

The first recombinant protein (designated STF2:HA1-2, Solomon Island (SI)) linked full-length flagellin (FlIc) to a globular head subunit of Influenza A hemagglutinin (Figure 1.6). The morbidity of seasonal influenza virus is very high, causing well over 200,000 hospitalizations annually; additionally, it is indicated in an average of 30,000 deaths per year in the United States alone (25). Therefore, the availability of effective vaccines is of utmost importance to public health. The current vaccine is, at last report, indicated to be 47% effective against influenza A virus (26). Additionally, all of the current seasonal flu vaccine formulations are derived from virus grown in fertilized bird embryos; a new exception is the recently-approved Flucevlax, which is derived from virus grown in animal cell culture (27). Growth of viruses is notoriously difficult, as they are obligate intracellular parasites. Use of fertilized eggs as a means for viral growth is effective but is unfortunately time consuming. A significant problem with manufacture
of the seasonal influenza vaccine is that production of the world’s supply is a timely process, and diminishes the time available to effectively manufacture a vaccine against a pandemic strain (e.g. H5N1 or H7N9) should the need arise (28). An ideal vaccine would have a higher efficacy against the circulating strain of seasonal influenza A, could be manufactured in a timely manner, and would be allergen (ovalbumin) free. Early results indicate that purified STF2.HA1-2 (SI) also elicits a strong immune response in mice (20).

The purification procedure for STF2.HA1-2 (SI) was evaluated previously for protein produced within the soluble fraction under a specific set of denaturing conditions. This project expanded on the methodology used to extract the fusion protein in order to provide feedback following evaluation of different approaches.

![Figure 1.6 Structure of the recombinant protein STF2:HA1-2](20)
The final construct tested was a fusion protein in which the C-terminal region of flagellin was linked to a short epitope region of Marburg virus (GP132), and given the designation GP132:FliC (Figure 1.7). Marburg virus (MARV) is an agent of hemorrhagic fever. Prevalence of MARV is low, even in the populations in which it is considered endemic. However, MARV is considered an extremely dangerous human pathogen. In recent outbreaks, the mortality rates have reached 90% (29). Within as short as 5 days, infected individuals exhibit high fever, diarrhea, severe headaches, and malaise, and the possibility of hemorrhage and progressive organ damage. This symptom set can be very debilitating and due to the excessive fluid loss experienced by infected individuals, MARV is highly transmissible person-to-person. Additionally, there is no treatment beyond supportive care, which may be precluded in an outbreak situation: during an outbreak it might be difficult to secure sufficient necessary medical facilities, supplies, and staff to treat affected individuals. Thus, MARV is considered a potential bioterrorism agent (30).

Because this fusion protein was completely novel, the preferred method for extraction from the cell and subsequent purification was not established. Again, multiple purification methods were implemented and evaluated, building a purification scheme that met both the criteria of low contamination and high activity.

![Figure 1.7 Structure of fusion protein designated FliC:GP132](image)
1.3 Expected Results

Variation in the purification process is conditional upon whether the desired protein is produced in the soluble or insoluble fraction, or in combinations thereof. Production in the soluble fraction traditionally has been considered advantageous because the expressed protein is readily accessible for purification. However, in the soluble fraction, recombinant protein is more exposed to contaminating host protein, including destructive proteases, and must thusly be dissociated from contaminants. Additionally, proteins present in the soluble fraction preferentially associate with endotoxin. This implies that more purification steps be involved, which not only typically sacrifice the integrity of the native structure of the protein, but also increase the time, cost, and potential for user error due to the increased number of steps during the purification procedure (31).

If the recombinant protein is produced in inclusion bodies within the insoluble fraction, the apparent hurdle is to resolubilize the protein. The standard purification approach has been to focus on the soluble fraction because of the necessity to renature proteins recovered by the resolubilization step, which has historically been accomplished using strong denaturing conditions. This renaturation step has been characterized to be both the most expensive and time-consuming (32). However, the environment within inclusion bodies normally contains only the target protein (33). The higher level of protein expression in inclusion bodies combined with the natural shielding from degradation by proteases make purification from inclusion bodies desirable, if not preferable to, the standard approach. Therefore, a practical approach that accomplishes renaturation efficiently should have a more efficient purification procedure, thus making production of target protein within the insoluble fraction advantageous (34). The proposed approach explores a range of the concentrations of the denaturants during the resolubilization step.

Studies have shown that using a low concentration of denaturing agent at this stage of purification may result in fewer aggregates forming. It has been indicated that the use of urea as a denaturant may enhance the formation of protein aggregates in IBs due to hydrogen bonding interactions that affect the
formation of protein native structure (35). It is therefore hypothesized that using a lower concentration of denaturant may reduce misfolded protein aggregates and that the use of Guanidine-HCl as a denaturant, rather than urea, may yield high quality fusion protein from IBs. The hypothesized approach uses less stringent denaturing chemicals in order to reduce the likelihood of misfolded fusion protein or in formation of aggregates upon resolubilization from inclusion bodies (Figure 1.8).
Aspects of the purification process beyond whether the protein is produced in the soluble or insoluble fraction and the conditions involved in resolubilization of target protein will be considered. While it is a well-accepted generalization that protein overproduced in *E. coli* forms inclusions, purification of protein that may have been secreted from cells during fermentation will also be attempted. Additionally, conditions for the final refold step are evaluated, including analyzing the effect that varying redox pair systems and the amount of time refolding allowed may have on the integrity of purified protein. During this stage, the denaturing conditions are reversed either by dilution or dialysis in order to completely renature resolubilized protein while minimizing the formation of misfolded protein. Redox pairs (oxido-shuffling systems) are typically included in refolding buffers to achieve maximum yield and peak renaturation of target protein, as protein is more likely to fold into native conformation under oxidizing conditions (36).
2. EXPERIMENT

2.1 Preparation of Constructs

Purification procedural experiments were conducted on proteins constructed using recombinant DNA technology with the intention of using them as vaccines. Three such constructs were studied: a full-length flagellin and two fusion proteins built by linking flagellin with a truncated monomer of an antigenic portion of a virus. The full-length flagellin construct was prepared at Georgia State. The purpose of this construct was to evaluate systems for the purification of flagellin, since this protein itself has immunogenic properties, as well as to act as a point of comparison for the fusion proteins. To generate this construct, the full-length sequence of the flagellin protein from *S. typhimurium* (FliC) was ligated into the pETBlue™ plasmid (Merck, Whitehouse Station, NJ) using complementary restriction endonuclease cut sites. It was then transformed into the expression host: competent DE3 *E. coli*. A successful clone demonstrated induction of the plasmid and was designated FA4.

The first fusion protein, STF2:HA1-2 (SI), was acquired from and prepared by a group within private industry using similar protocols. Briefly, the gene for the globular head domain of hemagglutinin A was generated synthetically and fused to the C-terminus end of a full-length flagellin monomer (FljB) from *S. typhimurium*. The resulting fusion protein was inserted into the pET24 plasmid to yield the construct (20). Next, the plasmid was transformed into host in preparation for growth in a bioreactor.

To generate the FliC:GP132 fusion protein, GP132, oligonucleotide containing restriction enzyme cut sites was generated synthetically (Integrated DNA Technology, Coralville, Iowa). GP132 is a 9-mer peptide (15-mer amino acid sequence: GILLLSIAVLIALS) derived from the glycoprotein region of Marburgvirus and was found to act as an effective vaccine in mice: stimulating cytotoxic lymphocytes, resulting in the production of interferon γ, and offering complete protection upon challenge with Marburgvirus (37). The flagellin sequence was inserted into the pETBlue plasmid and the GP132 gene was ligated to the C-terminal end of the FliC flagellin gene. The plasmid containing the fusion protein was
then transformed into competent DE3 *E. coli* as above. A successful clone (one that expressed the fusion protein upon induction) was entitled 5088.

### 2.2 Fermentation and Initial Purification

The two constructs developed at GSU (FA4 and 5088) were expressed after induction with iso-propyl β-D-1-thiogalactopyranoside (IPTG) during a batch-fed 20L fermentation. IPTG induces expression of genes regulated by the *lac* operon by disabling the *lac* repressor (38). This results in high-density expression of recombinant proteins in *E. coli*. During fermentation, the temperature was maintained at 30°C, dissolved oxygen was maintained at 35%, and glucose was provided in a constant feed once the initial glucose concentration was depleted. The STF2:HA1-2 (SI) fusion protein was expressed by a similar protocol.

Overexpression of fusion proteins in *E. coli* does not always yield easily purified soluble proteins. Often, recombinant proteins are produced in dense insoluble aggregates within the cytoplasmic or periplasmic space known as inclusion bodies (IBs). Until recently it was believed that inclusion bodies contained solely misfolded non-functioning proteins. As of late, the advantages of overproducing target protein within a defined environment inside *E. coli* have been noted (33), and several procedures have been modified to exploit this feature (39, 40).

After the completion of fermentation, excess water was removed from the harvest using the Powerfuge Pilot (Carr, Clearwater, FL). This platform separates solid from liquid using simultaneous centrifugation and vacuuming, and processes at a rate of up to 60L/hour. Solid product obtained from the Powerfuge was designated “cell paste” and was immediately processed further or was stored at -80°C. If cell paste was stored, prior to processing it was thawed overnight at 4°C. Once thawed, the cell paste was evaluated for solidity. Depending on the firmness of the cell paste, it was diluted to either 7.5% or 15% solids using a 4% sucrose lysis buffer (P1) and stirred to mix completely. The diluted cell paste was then homogenized without pressure using an APV homogenizer (APV-1000, Delavan, WI) to generate a
fully resuspended cell paste. Resuspended cell paste was homogenized under pressure (690 bars, equivalent to 10,000 psi) in triplicate to completely lyse cells. Between homogenization passes, the resuspended cell paste was passed through a cooling coil to lower the temperature to no more than 5°C. This step was included as a control measure to reduce the risk of early denaturation of the target protein by heat. The resulting lysate was partially clarified by an initial centrifugation step, yielding both an insoluble fraction (pellet) and a soluble fraction (supernatant), both of which were analyzed (via SDS PAGE gel electrophoresis) for the presence of the target protein. Both fractions were processed further to determine which method emerged as more advantageous.

Cellular material was removed from the insoluble fraction by washing twice with ethylenediaminetetraacetic acid (EDTA) and Triton X-100, and then washing with a 0.3M urea solution. To extract the protein from inclusion bodies, the washed pellet was resuspended in varying concentrations of denaturant: either urea or guanidine-HCl. The concentrations of denaturant used ranged from 1M to 8M (Table 2.1). Following introduction of the denaturant, the resuspended pellet was centrifuged, yielding a refined lysate (RL). Because the RL was obtained from within inclusion bodies, it required fewer purification steps than RL from the soluble fraction.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Urea concentrations tested</th>
<th>Guanidine-HCl concentrations tested</th>
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<tr>
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<tr>
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<td>1M, 2M, 4M, 6M, 8M</td>
<td>1M, 4M, 6M</td>
</tr>
<tr>
<td>5088</td>
<td></td>
<td>1M, 6M</td>
</tr>
</tbody>
</table>

### 2.3 Additional Purification of the Soluble Fraction

Proteins present in the soluble fraction (the supernatant after initial centrifugation) were precipitated with polyethylene glycol (PEG) 3350 and centrifuged to separate precipitated proteins. As with
the insoluble fraction, the PEG pellet was resuspended in varying concentrations (see Table 2.1) of either Guanidine-HCl or urea to resolubilize desired protein. Insoluble solids were removed by a final centrifugation step to yield RL. This RL was then further purified to reduce contamination by endotoxins tightly associated with the desired protein.

2.3.1 **Tangential Flow Filtration (TFF)**

Refined Lysate obtained from the soluble fraction underwent a minimum of five passes through a Centramate (Pall, Port Washington, NY) tangential flow filtration membrane (1ft², 30kD molecular weight cut-off) to exchange denaturant-containing buffer for 50mM Tris buffer. Once the conductivity of the waste measured within a 10% range of the conductivity of the 50mM Tris buffer, the buffer exchange was satisfactorily completed. In addition to reducing the endotoxin, this filtration method facilitated the reduction of the nucleic acid concentration as reported by the Nanodrop 2000 (Thermo Fischer Scientific, Wilmington, DE), a micro-volume spectrophotometer. High concentrations of nucleic acids are known to interfere with anion exchange chromatography.

2.3.2 **Two-Phase Separation**

The detergent Triton X-114 was added to the TFF product, along with PEG. This technique utilized the hydrophobic nature of endotoxins, which associate preferentially with the surfactant to form a micelle. Centrifugation yielded a clarified aqueous phase and a disposable detergent phase, which contained the micelles. Thus endotoxins were discarded with the detergent phase (41). The aqueous phase was filtered in preparation for anion exchange chromatography.

2.4 **Anion Exchange Chromatography (AEX)**

AEX was used to remove charged impurities, including remaining host cell protein, endotoxin, incorrectly folded proteins, and buffer components from product derived from both the soluble and insoluble fractions. The recombinant protein was denatured in varying concentrations of either urea or
Guanidine-HCl, then refolded by diluting denatured protein into a nine-fold excess of refolding buffer (27, 42). Samples were allowed to refold for a period of two hours or overnight and quality of refolded protein was evaluated. The activity of protein refolded using two different oxido-shuffle systems (glutathione and cysteine) was assessed. Refolded protein was then loaded onto a pre-packed ToyoScreen column with SuperQ-650 resin, a known strong anion exchanger (Tosoh Bioscience, Stuttgart, Germany). The column was run on the ÄKTAexplorer® automated liquid chromatography system (GE Healthcare Biosciences, Uppsala, Sweden), and eluted as a single peak at a low salt concentration. Endotoxin remained bound to the column until it was washed with solution with a significantly higher salt concentration, thus further removing it from the product. This step also removed any remaining surfactant and nucleic acid (43).

2.5 Purification of Full-length Flagellin

2.5.1 Purification of the Insoluble Fraction

Cell paste generated via fermentation was thawed at room temperature and centrifuged to remove fluid. The pellet was resuspended in sucrose buffer using sonication. Resuspended cell paste was homogenized under pressure to lyse cells and then was centrifuged to separate the soluble proteins in suspension from the insoluble fraction contained within the pellet. The pellet was washed and resuspended in varying concentrations of urea or guanidine-HCl to generate clarified harvest. Clarified harvest was allowed to refold overnight in 9x refolding buffer. Refolded proteins were loaded onto a SuperQ column and eluted via AEX.

2.5.2 Purification of the Soluble Fraction

Following initial centrifugation, PEG 3350 was added to the supernatant (soluble fraction) to precipitate proteins. The soluble fraction was centrifuged again; this time the pellet was collected and
resuspended in varying concentrations of urea or guanidine-HCl. Tangential flow filtration was performed to exchange the denaturing buffer for Tris buffer. Triton X 114 was added to associate with the insoluble region of endotoxin and form micelles. These impurities were removed by centrifugation, and the aqueous phase was collected and filtered. The filtered aqueous phase was then added to 9x refolding buffer and allowed to refold overnight. The refolded protein was finally purified after anion exchange, which was accomplished using automated HPLC.

2.6 Purification of STF2:HA1-2 (SI)

2.6.1 Purification of Insoluble Fraction

Frozen cell paste obtained from fermentation was thawed at room temperature and resuspended in sucrose buffer. After homogenization, the clarified harvest was centrifuged to separate the soluble and insoluble fractions. The insoluble fraction (the pellet) was resuspended in buffer prepared with varying concentrations of urea or guanidine-HCl (Table 2.1). The resulting clarified harvests were diluted to a concentration of 2mg/mL and refolded overnight in preparation for anion exchange chromatography. Refolded protein was then loaded onto a SuperQ column and eluted either via a stepwise or gradient method. Eluate was collected and stored at 4°C. Process samples from each stage of purification were analyzed for quality by SDS-PAGE, BCA protein assay, and ET assay.

2.6.2 Purification of Soluble Fraction

After initial centrifugation, the soluble fraction present in the solution was treated with PEG to precipitate proteins. Precipitated proteins were collected after additional centrifugation as the pellet. This pellet was resuspended with varying concentrations of urea or guanidine-HCl and centrifuged again to remove any insoluble solids, yielding refined lysate. Buffer exchange was achieved using tangential flow filtration and the TFF product was further clarified by two-phase separation followed by filtration. Refined lysate was diluted to 2mg/mL and allowed to refold overnight. Refolded protein was then
loaded onto a SuperQ column and eluted either via a stepwise or gradient method. Eluate was collected and stored at 4°C. Again, samples taken during each stage of the procedure were analyzed for quality by multiple validation measures.

2.7 Purification of FliC:GP132

2.7.1 Purification of Insoluble Fraction

Cell paste from fermentation was allowed to thaw and then was resuspended in a sucrose buffer. Homogenization under pressure ensured that the cells were lysed sufficiently. An initial centrifugation step separated the insoluble fraction from the soluble fraction. The insoluble pellet was rinsed and then resuspended in varying concentration of guanidine-HCl. Anion exchange was then performed to reduce impurities. Process samples were analyzed by several methods to assure quality and to determine optimal conditions for purification.

2.7.2 Purification of Soluble Fraction

PEG 3500 was added to the supernatant from the initial purification step in order to solidify any proteins present. This solution was centrifuged, and the pellet was collected and resuspended with buffer containing varying concentrations of guanidine-HCl in order to resolubilize target protein. Another centrifugation removed any insoluble impurities, resulting in refined lysate. The refined lysate was then passed through tangential flow filtration to exchange the denaturing buffer for a Tris buffer. Following buffer exchange, Triton X-114 was added in order to form close associations with the nonpolar regions of LPS. Centrifugation forced these impurities into the detergent phase, which was discarded. The aqueous phase was filtered and allowed to refold overnight using varying redox species in the refolding buffer: cysteine/cystine or glutathione redox systems. Finally, anion exchange was performed to remove any remaining impurities.
2.7.3 Purification of Secreted Protein

Process samples from fermentation indicated that target protein was present in the conditioned media (CM), rather than solely within cell paste (Figure 2.1). Thus, a platform to purify protein which may have been secreted during fermentation was also tested. Spent media from fermentation was collected and initially clarified by centrifugation (8000rpm, 45min). Both the supernatant and pellet were collected and processed by secondary purification methods in order to determine the efficacy of purification of secreted protein.

![Image of C-terminal GP132 fusion 5088 fermentation](image)

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<td>Pre-Induction soluble</td>
</tr>
<tr>
<td>4</td>
<td>Pre-Induction insoluble</td>
</tr>
<tr>
<td>5</td>
<td>Post-induction 1hr CM</td>
</tr>
<tr>
<td>6</td>
<td>Post-induction 1hr soluble</td>
</tr>
<tr>
<td>7</td>
<td>Post-induction 1hr insoluble</td>
</tr>
<tr>
<td>8</td>
<td>Post-induction 2hr CM</td>
</tr>
<tr>
<td>9</td>
<td>Post-induction 2hr soluble</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>

Figure 2.1 Pre and Post-Induction Fermentation Samples (FliC:GP132)
2.8 Validation

2.8.1 Western Blotting

Western blotting was done in order to provide additional confirmation of the presence of fusion protein in samples from various stages of the purification process. The preparations that were carried through to AEX were analyzed by Western blotting, performed using primary antibody specific for FliC flagellin from *Salmonella typhimurium*. Protein was loaded onto a polyacrylamide gel and separated on the basis of size via gel electrophoresis. Blotting of the gel was accomplished by sandwicking the gel and a pre-cut cellulose membrane between soaked blotting pads. Protein was then electrophoretically transferred to the cellulose membrane using the Xcell II® Blot Module (Invitrogen, Grand Island, NY) at 360mA for 1 hour. The membrane was then blocked with 0.5% milk solution to reduce nonspecific binding of antibodies. After blocking, the transfer membrane was washed in triplicate and incubated with the primary antibody: anti-FliC. Secondary antibody (rat anti-Mouse IgG horseradish peroxidase (HRP)) was applied in order to be detected by chemiluminescence. Finally, the image was created using Western Lightning-Plus-ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer, Walthan, MA) and the ImageQuant® LAS 4000 (GE Lifesciences, Piscataway, NJ).
Figure 2.2 Western Blot Procedure (from MilliporeSigma)
2.8.2 TLR5 Bioassay

The TLR5 bioassay was performed in order to provide verification of the presence of active FliC in purified samples, thus indicating proper folding of the inserted protein. The preparations that were carried through to AEX were analyzed by a TLR5 ligand stimulation assay (adapted from InvivoGen, San Diego, CA). In this assay, HEK-Blue hTLR5® cells were co-transfected with an hTLR5 gene and secreted embryonic alkaline phosphatase (SEAP) gene under the control of NF-κB promoter. Stimulation of hTLR5 by flagellin activated NF-κB, inducing production of SEAP. A detector, Quanti-Blue® was used to indirectly detect the amount of alkaline phosphatase present due to the stimulation of hTLR5 via changes in optical density. Increases in optical density were considered directly proportional to increases in TLR5 activity. Samples were compared with a flagellin standard (positive control) and ET-free water (negative control) in order to determine activity of purified protein. Additionally, HEK-Blue Null® cells were used in this assay to correct for endogenous TLR5 in HEK cells.
Figure 2.3 Process of TLR5 Bioassay for Validation of Innate Immune System Activation

Plate preparation
• Add test samples, controls to bottom of wells
• Add 180μL of cell suspension (~25,000 cells) per well.
• Incubate the plate at 37°C in a CO₂ incubator for 20-24 h.

Absorption reading
• Add 180μL of QUANTI-Blue™ per well of a flat-bottom 96-well plate.
• Add 20μL of induced HEK-Blue™-hTLR5 Cells supernatant OR induced HEK-Blue™-Null1 Cells supernatant
• Incubate the plate in a 37°C incubator for 1-3 h.
• Determine SEAP levels using a spectrophotometer at 620-655 nm.

Calculate Corrected Activity
• ΔOD between TLR5 and null
2.8.3 Endotoxin Assay

All preparations that were carried through to AEX were tested to determine the amount of endotoxin remaining after each step of the purification process. This is a necessity in order to maintain compliance with current Good Manufacturing Practices (cGMP). The current FDA standard for measurement of endotoxin is the Limulus amebocyte lysate (LAL) kinetic chromogenic assay (Kinetic-QCL®, Lonza, Walkersville, MD). The basis of the assay is the response of horseshoe crabs to GNB. Presence of LPS leads to degranulation of amebocytes, releasing the protease zymogens and pre-clotting enzyme which are involved in a cascade resulting in coagulation. Factor C is a LPS-binding protein, that, once activated by LPS, will in turn activate Factor B. Activated Factor B is then able to convert the pre-clotting enzyme into its active form, which leads to the formation of a gel clot. This pathway traps the bacteria in the clot, thus helping the horseshoe crab evade infection (14). The commercial LAL assays contain one of the proenzymes, which will split a chromogenic substance from a colorless substrate, causing a color change (Figure 1.3). This color change is measured continuously throughout the course of the reaction, and the reaction time is inversely related to the endotoxin concentration in the measured sample. Diluted samples were combined with the LAL reagent and substrate mixture and placed inside an incubating plate reader. Each assay was run along with an endotoxin standard, diluted serially, to generate a standard curve to determine the concentration of endotoxin in each sample.
Figure 2.4 LAL Kinetic Assay for Endotoxin Detection (44)
2.8.4  **BCA Protein Quantification**

All samples were subjected to a BCA (bicinchoninic acid) chromogenic protein quantification (Pierce™ BCA Protein Assay, ThermoFischer Scientific, Waltham, MA) in which the optical density of each sample combined with the BCA reagent was measured at a wavelength of 562nm. The relationship between optical density and protein concentration is directly proportional, thus this method provides an accurate measure of the amount of target protein produced.

---

**Figure 2.5 BCA Protein Quantification Assay**
2.8.5 SDS-PAGE assay

All samples were run using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to visualize the production and purification of the target protein. Samples were diluted and added to buffer prior to being loaded into the gel. Electrophoresis was allowed to continue for an hour and then the gel was stained with Coomassie blue. The gel was destained with water and then imaged on a light box.

Figure 2.6 SDS PAGE Assay (Wangler, 2017)
3. RESULTS

3.1 Results for FliC

During initial purification of the full-length flagellin construct, both insoluble and soluble fractions were processed using varying concentrations of Urea (1M-8M). Process samples were taken and protein quantified using a BCA protein assay. Additionally, these samples were run using SDS-PAGE to detect target protein (55kDa) (Figures 3.1, 3.2). Based on early sampling, the relationship between denaturant concentration and yield of target protein appears to be directly proportional. There was a reduced yield of protein in the insoluble fraction compared to soluble: insoluble refined lysate protein yield ranged from 542 – 1733μg/ml, while the range for soluble refined lysate ranged from 987μg/ml to 9684μg/ml. However, protein produced in the soluble fraction required additional processing, so these results were anticipated. Following initial purification of the insoluble fraction, the refined lysate was refolded for at least 2 and up to 24 hours using varying redox systems in the refolding buffer and samples were filtered prior to anion exchange chromatography.
Figure 3.1 SDS PAGE 1 of Initial Purification of FliC from 20 Liter Fermentation Run.

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<td>1</td>
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<tr>
<td>2</td>
<td>Recrystallized Cell pellet</td>
<td>13750 µg/mL</td>
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<td>5</td>
<td>Lysate</td>
<td>14769 µg/mL</td>
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<tr>
<td>6</td>
<td>Insoluble pellet interphase</td>
<td>22771 µg/mL</td>
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<td>7</td>
<td>Pooling Supernatant</td>
<td>17851 µg/mL</td>
</tr>
<tr>
<td>8</td>
<td>Insoluble Pellet wash step</td>
<td>None detected</td>
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<tr>
<td>9</td>
<td>Insoluble refined lysate spin pellet (5M Gdn-HCl)</td>
<td>1333 µg/mL</td>
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<tr>
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<td>Insoluble refined lysate spin pellet (2M Urea)</td>
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**Figure 3.2 SDS PAGE 2 of Initial Purification of FliC from 20 Liter Fermentation Run.**

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<td>Soluble refined lysate spin pellet (4M Urea)</td>
<td>12022 µg/mL</td>
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<td>9556 µg/mL</td>
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<td>11291 µg/mL</td>
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<td>6</td>
<td>Soluble refined lysate (5M Gdn-HCl)</td>
<td>9684 µg/mL</td>
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<td>Soluble refined lysate (4M Urea)</td>
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<td>Soluble refined lysate (6M Urea)</td>
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<td>Insoluble refined lysate (2M Urea)</td>
<td>542 µg/mL</td>
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<td>12</td>
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<td>Insoluble refined lysate (6M Urea)</td>
<td>1733 µg/mL</td>
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</table>
The initial processing of the full-length flagellin construct was repeated using varying concentrations of Guanidine-HCl (Gdn-HCl) (1M-6M) as the denaturant (Figures 3.3, 3.4). Again, based on early sampling, there is a directly proportional relationship between denaturant concentration and protein yield. Notable exceptions to this trend were samples processed by the insoluble procedure using 1M Gdn-HCl: these samples had similar protein yield (1264μg/mL) as samples processed using a much higher concentration of denaturant. Because these samples had high protein concentration and used a lower concentration of Gdn-HCl, these samples were selected further processing along with samples processed using 6M Gdn-HCl. Following initial purification of the insoluble fraction, the refined lysate was refolded for at least 2 and up to 24 hours using varying redox systems in the refolding buffer. Samples were then filtered in preparation for column loading. The protein yield was again slightly higher in the soluble fraction, though the difference was not as great compared to the results when urea was used as the denaturant. This trend was expected, as the soluble fraction requires additional downstream steps before anion exchange chromatography.
Figure 3.3 SDS PAGE 3 of Initial Purification of FliC from 20 Liter Fermentation Run.
Figure 3.4 SDS PAGE 4 of Initial Purification of FliC from 20 Liter Fermentation Run
Refined lysates resulting from the soluble purification procedure required further clarification using TFF and 2-phase separation procedures. Only samples which were initially purified using 6M Urea had adequate protein remaining (892 μg/ml) after secondary purification to be further processed (Figure 3.5). These samples were filtered following the 2-phase centrifugation step in order to be processed by anion exchange chromatography.

![Figure 3.5 SDS PAGE of TFF and 2 Phase Separation of FliC from 20 Liter Fermentation Run.](image)

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Samples prepared by the insoluble procedure using either 1M or 6M Gdn-HCl, and samples prepared by the soluble procedure using 6M Urea had adequate protein yield to be processed through to anion exchange chromatography. Western blotting revealed similar hybridization patterns for protein processed in both the insoluble and soluble fractions (Figure 3.6). The presence of the 55kDa band corresponding with FliC is clearly present in all samples prior to AEX; the band is not visible in the samples which follow AEX and this is likely due a low concentration of the sample being loaded onto the gel. During anion exchange elution, a large peak was seen that corresponded to purified FliC. This is depicted on the chromatogram (Figure 3.7: this sample was processed via the insoluble procedure using Gdn-HCl (1M) as the denaturant); protein concentration of this peak was quantified using a BCA protein assay (377µg/mL). Following anion exchange, endotoxin assays indicated that both procedures yielded a low concentration of endotoxin (Table 3.1). Results from the endotoxin assay show that refined lysate from insoluble and soluble clarification procedures had similar levels of contamination due to host cell lipopolysaccharide. The insoluble preparations that underwent AEX had much lower ET levels than soluble preparations, however the 6M Gdn-HCl preparation contained no detectable protein. The soluble preparations had detectable protein at all stages but had a significantly higher level of endotoxin after AEX than insoluble preparations (Figure 3.8). This finding indicates that protein within the soluble fraction may be more difficult to decontaminate than protein within the insoluble fraction. A theoretical yield of 979mg purified protein was determined to be obtainable from a 20L fermentation run, using the optimized purification scheme (insoluble procedure using 1M Gdn-HCl as the denaturant). This corresponds to nearly 500,000 doses per run, assuming the effective dose is 5μg. Endotoxin contamination was reduced to 1.85EU/μg, which is within the acceptable range of exposure for a vaccine.
**Figure 3.6 Western Blot of FliC samples, insoluble and soluble fractions**
Figure 3.7 Anion Exchange Chromatogram FliC.
Table 3.1 Endotoxin levels detected using LAL assay, FliC

<table>
<thead>
<tr>
<th>Sample</th>
<th>EU/mL</th>
<th>[protein] µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Gdn RL (ins)</td>
<td>3.64E+07</td>
<td>1183</td>
</tr>
<tr>
<td>AEX Peak 1</td>
<td>7.26E+02</td>
<td>392</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>EU/mL</th>
<th>[protein] µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>6M Gdn RL (ins)</td>
<td>2.63E+07</td>
<td>1166</td>
</tr>
<tr>
<td>AEX Peak 1</td>
<td>3.96E+02</td>
<td>None detected</td>
</tr>
<tr>
<td>AEX Peak 2</td>
<td>7.40E+01</td>
<td>None detected</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>EU/mL</th>
<th>[protein] µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>6M Gdn RL (sol)</td>
<td>1.38E+07</td>
<td>1902</td>
</tr>
<tr>
<td>CAP</td>
<td>5.55E+05</td>
<td>892</td>
</tr>
<tr>
<td>AEX Peak 1</td>
<td>5.55E+04</td>
<td>13</td>
</tr>
</tbody>
</table>

Figure 3.8 Endotoxin Levels during secondary purification, FliC
The purified protein must be properly refolded in order to stimulate TLR5. The TLR5 activity was quantified by measuring the optical density of samples which should increase as the activity increases. The percent activity was calculated by comparing the optical density of samples against the flagellin standard (Figure 3.9). Samples processed by both the insoluble and soluble procedures had high percent activity compared to the positive standard (86.75% and 91.41%, respectively). Additionally, protein purified by the insoluble procedure was positive for TLR5 activity in all preparations following protein refolding, while only one of the peaks from AEX from the soluble procedure was positive for TLR5 activity. These results, together with the protein quantification results indicate that the insoluble procedure seemed to generate a higher level of properly folded flagellin.

![TLR-5 Activity of FliC Purified from Insoluble and Soluble Fraction](image)

**Figure 3.9 TLR5 Percent Activity, insoluble vs soluble (FliC)**
Finally, the conditions for renaturation of solubilized protein were evaluated. There were no discernable differences in activity or quantity of protein based upon the time permitted for refolding (2 hours or 24 hours, data not shown). However, altering the redox species did impart an effect on the ability to stimulate TLR-5. When the cysteine/cystine (cys/(cys)2) system was used, the percent activity was greater than the flagellin standard (111.74%). Changing the oxido-shuffle to glutathione (GSH-GSSG) resulted in a reduction of activity (90.34%). The effect of the redox species used in refolding buffer may be minor, however when considering all the steps required for protein purification, even these slight differences may be impactful and worthy of consideration (Figure 3.10).

![Figure 3.10 TLR5 activity, varying redox species (Flc)](image_url)
3.2 Results for STF2.HA1-2 (SI)

Soluble and insoluble fractions of the second construct, STF2.HA1-2 (SI), were initially purified using varying concentrations of urea (1M, 2M, 4M, 6M) as the denaturing agent. By analysis of SDS-PAGE and BCA assay data, use of a urea at high concentrations was found to deliver a high yield of protein (between 1337μg/ml and 8910μg/ml), but with residual contamination from host cell proteins (Figures 3.11, 3.12). The bands seen at around 37kDa represent contaminant, and the standard denoted “SI standard” (Lane 14) corresponds to the size of the target protein (76kDa).

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>[Protein]</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>2</td>
<td>Resuspended Cell paste</td>
<td>20533 μg/mL</td>
</tr>
<tr>
<td>3</td>
<td>Lysate</td>
<td>24269 μg/mL</td>
</tr>
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<td>Lysate prespin sup</td>
<td>22424 μg/mL</td>
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<tr>
<td>5</td>
<td>Insoluble refined lysate spin pellet (1M Urea)</td>
<td>2612 μg/mL</td>
</tr>
<tr>
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<td>Insoluble refined lysate spin pellet (2M Urea)</td>
<td>6021 μg/mL</td>
</tr>
<tr>
<td>7</td>
<td>Insoluble refined lysate spin pellet (4M Urea)</td>
<td>1908 μg/mL</td>
</tr>
<tr>
<td>8</td>
<td>Insoluble refined lysate spin pellet (6M Urea)</td>
<td>1239 μg/mL</td>
</tr>
<tr>
<td>9</td>
<td>Insoluble refined lysate (1M Urea)</td>
<td>None detected</td>
</tr>
<tr>
<td>10</td>
<td>Insoluble refined lysate (2M Urea)</td>
<td>330 μg/mL</td>
</tr>
<tr>
<td>11</td>
<td>Insoluble refined lysate (4M Urea)</td>
<td>2164 μg/mL</td>
</tr>
<tr>
<td>12</td>
<td>Insoluble refined lysate (6M Urea)</td>
<td>1337 μg/mL</td>
</tr>
<tr>
<td>13</td>
<td>PEG pooled supernatant</td>
<td>10094 μg/mL</td>
</tr>
<tr>
<td>14</td>
<td>SI Standard</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>MW Marker</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.11 SDS PAGE 1 of Initial Purification of STF2.HA1-2(SI) from 20 Liter Fermentation Run.
<table>
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<tr>
<th>Lane</th>
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<th>Protein (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>MW marker</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Resuspension (6M Urea)</td>
<td>13933</td>
</tr>
<tr>
<td>3</td>
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<td>12717</td>
</tr>
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<td>4</td>
<td>Resuspension (4M Urea)</td>
<td>7389</td>
</tr>
<tr>
<td>5</td>
<td>Resuspension (1M Urea)</td>
<td>7421</td>
</tr>
<tr>
<td>6</td>
<td>Soluble refined lysate spin pellet (1M Urea)</td>
<td>15021</td>
</tr>
<tr>
<td>7</td>
<td>Soluble refined lysate spin pellet (4M Urea)</td>
<td>16610</td>
</tr>
<tr>
<td>8</td>
<td>Soluble refined lysate spin pellet (6M Urea)</td>
<td>13731</td>
</tr>
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<td>9</td>
<td>Soluble refined lysate spin pellet (8M Urea)</td>
<td>9983</td>
</tr>
<tr>
<td>10</td>
<td>Soluble refined lysate (1M Urea)</td>
<td>None detected</td>
</tr>
<tr>
<td>11</td>
<td>Soluble refined lysate (4M Urea)</td>
<td>790</td>
</tr>
<tr>
<td>12</td>
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<td>6262</td>
</tr>
<tr>
<td>13</td>
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<td>8910</td>
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<td>14</td>
<td>SI Standard</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>MW Marker</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.12 SDS PAGE 2 of Initial Purification of STF2.HA1-2(SI) from 20 Liter Fermentation Run.
Further experiments were performed in which varying concentrations (1M, 4M, 6M) of Guanidine Hydrochloride (Gdn-HCl) were used as the denaturant. The SDS-PAGE and BCA data from these preparations indicated that the protein yield when using Gdn-HCl as a denaturant was higher than the yield associated with urea in both insoluble and soluble fractions (3924μg/ml – 16914μg/ml), regardless of the concentration used (Figures 3.13, 14). Again, the relationship between denaturant concentration and protein yield appears to be directly proportional but using Gdn-HCl even at low concentration still returned a relatively high concentration of target protein with less residual contamination. Using Gdn-HCl as a denaturant appeared to resolve many of the contaminating host protein issues that were seen in preparations using urea.

Figure 3.13 SDS PAGE 3 of Initial Purification of STF2.HA1-2(SI) from 20 Liter Fermentation Run.
Figure 3.14 SDS PAGE 4 of Initial Purification of STF2.HA1-2(SI) from 20 Liter Fermentation Run.

<table>
<thead>
<tr>
<th>Lane</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>2</td>
<td>PEG Pooled Sup</td>
<td>16878 µg/mL</td>
</tr>
<tr>
<td>3</td>
<td>Soluble refined lysate spin pellet (1M Gdn-HCl)</td>
<td>9628 µg/mL</td>
</tr>
<tr>
<td>4</td>
<td>Soluble refined lysate spin pellet (4M Gdn-HCl)</td>
<td>2566 µg/mL</td>
</tr>
<tr>
<td>5</td>
<td>Soluble refined lysate spin pellet (4.6M Gdn-HCl)</td>
<td>4013 µg/mL</td>
</tr>
<tr>
<td>6</td>
<td>Soluble refined lysate (1M Gdn-HCl)</td>
<td>5049 µg/mL</td>
</tr>
<tr>
<td>7</td>
<td>Soluble refined lysate (4M Gdn-HCl)</td>
<td>169134 µg/mL</td>
</tr>
<tr>
<td>8</td>
<td>Soluble refined lysate (4.6M Gdn-HCl)</td>
<td>15000 µg/mL</td>
</tr>
<tr>
<td>9</td>
<td>Empty</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>15</td>
<td>MW Marker</td>
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</tr>
</tbody>
</table>
Following initial purification of the insoluble fraction, refined lysate generated using Gdn-HCl as a denaturant was further clarified by AEX chromatography (Figure 3.15). The purpose of AEX was to reduce contamination by endotoxin. All concentrations of Gdn-HCl resulted in greatly reduced ET (Figure 3.16), but a higher concentration of Gdn-HCl (6M) resulted in better removal of endotoxin from preparations. Additionally, the protein yield was far greater (1302μg/mL) when using a high concentration of denaturant. These results indicate that using a 6M concentration of Gdn-HCl as the denaturant is best suited for the purification of this construct and further demonstrate the value of harvesting the protein within the insoluble fraction. This process generates a theoretical yield of purified protein from a 20L fermentation run is 3.2g, corresponding to approximately 650,000 doses. An effective dose was assumed to be 5μg, however, significantly lower doses have been shown to be effective for this fusion protein (20). The residual endotoxin contamination is minimal: 1.5EU per dose.
**Figure 3.15 SDS-PAGE of AEX Fractions (SI)**

<table>
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<th>[Protein]</th>
</tr>
</thead>
<tbody>
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<td>MW</td>
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</tr>
<tr>
<td>2</td>
<td>SI Standard</td>
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</tr>
<tr>
<td>3</td>
<td>Load Flow Thru</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>A1 Fraction</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>A2 Fraction</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A3 Fraction</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>A4 Fraction</td>
<td>1302 μg/mL</td>
</tr>
<tr>
<td>8</td>
<td>A5 Fraction</td>
<td>2787 μg/mL</td>
</tr>
<tr>
<td>9</td>
<td>A6 Fraction</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>A7 Fraction</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>A8 Fraction</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>A9 Fraction</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Empty</td>
<td></td>
</tr>
<tr>
<td>14</td>
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<td></td>
</tr>
<tr>
<td>15</td>
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</tr>
</tbody>
</table>

**Figure 3.16 Endotoxin Levels during Secondary Purification (SI)**
3.3 Results for FliC:GP132

Early sampling during fermentation indicated that a large quantity of the fusion protein designated FliC:GP132 was found in the spent media (Figure 3.16). Thus, initial experimentation focused on an approach designed to maximize potentially secreted target protein. Spent media was collected centrifuged to initially clarify desired protein from cell contaminants. The resulting insoluble and soluble fractions were processed further. The insoluble pellet was rinsed and then resuspended in resolubilizing buffer using varying concentrations of Gdn-HCl (1M, 6M). PEG was added to the soluble fraction (supernatant) to precipitate the target protein. A second centrifugation step was performed to collect precipitated protein. The pellet was recovered, rinsed, and resuspended in buffer containing either 1M or 6M Gdn-HCl. The yield of protein was very low for this procedure: below 400µg/mL for all conditions. Thus, no further processing of these samples was attempted.

Cell paste was processed by both the insoluble and the soluble procedure, using either 1M or 6M Gdn-HCl as the denaturant. Purification of the previous constructs (full-length flagellin and the SI fusion protein) indicated that Gdn-HCl was a more efficacious denaturant than urea. Process samples were analyzed by SDS-PAGE to detect presence of target protein (~50kDa) and by BCA protein assay to quantify protein. Samples processed by the insoluble procedure resulted in low protein yield (590-12634µg/mL); additionally, host cell contaminants were present. Thus, only samples from the soluble procedure were processed further. Yields following preliminary processing using the soluble procedure ranged from 6016µg/mL when using 6M Gdn-HCl to 10664µg/mL when using 1M Gdn-HCl. These initial results indicate that this construct is optimally processed using the soluble fraction at a low concentration of Gdn-HCl (1M) (Figure 3.17).
Figure 3.17 SDS PAGE 1 of Initial Purification of FliC:GP132 from 20 Liter Fermentation Run.

<table>
<thead>
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<th>Lane</th>
<th>Sample</th>
<th>[Protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MW</td>
<td>10,023 µg/mL</td>
</tr>
<tr>
<td>2</td>
<td>Resuspended Cell Extract</td>
<td>10,023 µg/mL</td>
</tr>
<tr>
<td>3</td>
<td>Pass 1 with Pressure</td>
<td>15,417 µg/mL</td>
</tr>
<tr>
<td>4</td>
<td>Pass 2 with Pressure</td>
<td>15,967 µg/mL</td>
</tr>
<tr>
<td>5</td>
<td>Lysate</td>
<td>19,730 µg/mL</td>
</tr>
<tr>
<td>6</td>
<td>PEG Pooled Supernatant</td>
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</tr>
<tr>
<td>7</td>
<td>RL Spin Pellet (ins) 6M Gdn</td>
<td>4373 µg/mL</td>
</tr>
<tr>
<td>8</td>
<td>RL Spin Pellet (ins) 1M Gdn</td>
<td>2859 µg/mL</td>
</tr>
<tr>
<td>9</td>
<td>RL (ins) 1M Gdn</td>
<td>590 µg/mL</td>
</tr>
<tr>
<td>10</td>
<td>RL (ins) 6M Gdn</td>
<td>12634 µg/mL</td>
</tr>
<tr>
<td>11</td>
<td>RL Spin Pellet (sol) 1M Gdn</td>
<td>11,886 µg/mL</td>
</tr>
<tr>
<td>12</td>
<td>RL Spin Pellet (sol) 6M Gdn</td>
<td>21,090 µg/mL</td>
</tr>
<tr>
<td>13</td>
<td>RL (sol) 1M Gdn</td>
<td>10,664 µg/mL</td>
</tr>
<tr>
<td>14</td>
<td>RL (sol) 6M Gdn</td>
<td>6016 µg/mL</td>
</tr>
<tr>
<td>15</td>
<td>MW marker</td>
<td></td>
</tr>
</tbody>
</table>
Following initial purification, buffer exchange was performed using tangential flow filtration (TFF), followed by a 2-phase spin and filtration in order to reduce contaminating GNB components. Total protein yield for samples after secondary processing (called “post-TFF refined lysate”) was 3377µg/mL (Figure 3.18). The post-TFF refined lysate was then refolded for either 2 or 24 hours using varying redox species in preparation for column loading and final clarification by anion exchange chromatography.

The chromatogram generated by AEX showed a peak at ~15% gradient, indicating the target protein was eluted (Figure 3.19). Protein concentration of the first peak was quantified using a BCA protein assay (3238µg/mL). SDS-PAGE analysis also indicated the presence of target protein (Figure 3.20). Significant protein loss occurred during column-binding (4019µg/mL), which could be a result of over-loading protein onto the column, or poor binding of the protein due to the presence contaminants.
Figure 3.18 SDS PAGE 2 of TFF and 2 Phase Separation of FliC:GP132 from 20 Liter Fermentation Run.
Figure 3.19 Anion Exchange Chromatogram FliC:GP132.
Figure 3.20 SDS-PAGE of AEX Peaks (FliC:GP132)
Samples processed through AEX were validated using Western Blotting (Figure 3.21). Hybridization of the purified protein to the antibody indicated that the epitope portion of the fusion protein was present and detectable. Endotoxin levels were quantified following each stage of purification. The concentration of ET decreased after each process step, with the largest decrease in contamination occurred following 2-phase separation (Figure 3.22). Endotoxin levels were reduced from $10^9$EU/mL in the refined lysate to $10^4$EU/mL following AEX.

![Western Blot of purified FliC:GP132 samples from 30 Liter Fermentation Run.](image1)

**Figure 3.21 Western Blot of purified FliC:GP132 samples from 30 Liter Fermentation Run.**

![Endotoxin Levels during Secondary Purification (FliC:GP132).](image2)

**Figure 3.22 Endotoxin Levels during Secondary Purification (FliC:GP132)**
Finally, biological activity of purified FliC:GP132 was measured using a TLR-5 assay. The refolding efficiencies of different redox species used in the refolding buffer was compared. Refolded protein was highly active (101.65% activity – 131.67% activity) compared to the flagellin standard. Refolding protein in the presence of the glutathione redox system appeared to yield more active than protein refolded using the cysteine system (Figure 3.23).

![TLR-5 Activity of FliC:GP132 refolded with different redox species](image)

**Figure 3.23 TLR-5 Activity, varying redox species (FliC:GP132)**

Endotoxin contamination was 18EU/μg, which indicates that this preparation does not meet the criteria for pharmaceutical-grade protein. Processing the soluble fraction was more effective for this construct. Further, a low concentration (1M) of Gdn-HCl was considered to be optimal. If processed under the recommended conditions, the theoretical yield for FliC:GP132 is 8.1g protein per 20L fermentation run. Thus, there is a potential for obtaining 1.6 million doses of vaccine from a single 20L fermentation run (assuming a 5μg dose).
4. CONCLUSIONS

In this study, a multistep approach was taken to optimally purify recombinant proteins produced in an *E. coli* expression system: full-length flagellin, flagellin fused to an antigenic portion of influenza, and flagellin fused to an antigenic region of Marburgvirus. Purification schemes which recovered target protein from both the insoluble and soluble fractions were compared, as approaches which only focus on one fraction fail to capture valuable protein produced in the opposite fraction. It is unclear what characteristic about the constructs causes the difference in the expression in the insoluble or soluble fraction, as well as whether the protein can be best purified from the insoluble or soluble fraction. There is still no universal procedure for purification of proteins from GNB, and purification systems need to be developed which maximize retrieval of proteins from both insoluble and soluble fractions. The ideal purification structure should yield target protein produced in high quantity, be easily purifiable, and have similar activity to protein in native conformation. These targets were met for each of the constructs examined, though the methodology used varied. For full-length flagellin (FliC) and the flagellin-influenza fusion protein (STF2:HA1-2(SI)), processing protein from the insoluble fraction was shown to result in high yields: up to 1-3g of protein per 20L fermentation run. For the flagellin-Marburgvirus fusion protein (FliC:GP132), greater yield was obtained by processing the soluble fraction (up to 8g protein per 20L fermentation run).

The approach taken in this study was intended to create a blueprint for protein purification from GNB. Variables which were examined included the type of denaturant used, under which conditions protein was best resolubilized, as well as comparing the quantity and quality of protein from the insoluble and soluble fractions. It was hypothesized that purification in the insoluble fraction may reduce the number of purification steps required for highly active protein. A second hypothesis posited that altering the type and concentration of denaturant could reduce loss of target protein due to misfolding or the formation of aggregates. TLR5 activity for the FliC construct purified from the insoluble fraction was
comparable to the flagellin standard. Additionally, endotoxin levels were greatly reduced in samples processed by the insoluble procedure, compared to the endotoxin contamination in samples from the soluble fraction. Endotoxin contamination for the SI construct purified from the insoluble fraction using Gdn-HCl was similar to previously reported levels using urea as a denaturant and purifying solely from soluble fraction (data confidentially reported). Bioactivity of FliC:GP132 was like flagellin standard according to TLR5 assays, though endotoxin was levels were higher than desirable.

The use of Gdn-HCl as a denaturant was effective for all the constructs purified. It has been widely shown that Gdn-HCl can be used effectively to purify proteins from Gram negatives, but usually at higher concentrations than appeared necessary for the preparations SI, FliC, and FliC:GP132. Using a lower concentration of denaturant during the initial stages of the purification process is advantageous in that there should be fewer downstream steps required to remove it from the preparation. The concern with purification of proteins from the insoluble fraction has always been that protein will be misfolded or aggregated and not functional. These data show that active, properly folded protein was able to be obtained from the insoluble fraction using lower concentration of denaturant than has been previously demonstrated.

In order to take full advantage of the speed and low-cost nature of bacterial protein expression systems, more focus has been put on methods that extract protein produced within inclusion bodies. It was hypothesized that production of target protein within inclusion bodies could be exploited because of the lack of host cell contaminants within inclusion bodies as well as the tendency of *E. coli* to express a high concentration of recombinant protein therein. Use of this method yields active product at the high concentration with little endotoxin contamination. It appears that that is the case for certain proteins, but not for all. For instance, this approach was not suitable for the purification of the construct containing
Marburg virus, FliC:GP132. In the future, use of computational models to predict the solubility of constructs may be useful in predicting whether target protein is produced within the soluble or insoluble fraction.

Purification of proteins from inclusion bodies requires some distinct considerations. The most crucial step is refolding of solubilized protein, as misfolding often occurs within inclusion bodies. Refolding methods vary based on the technique used to refold protein, the type chemical that aids in refolding, whether there are chaperone molecules present, and length of the process. Guanidine-HCl acts as a chaotrophic agent: using a low concentration of this denaturant to resolubilize protein from IBs can maintain or recover native structure of target proteins, while urea has been shown to sometimes increase the likelihood of secondary structures or aggregates forming. The time allowed for refolding (2 hours or overnight) did not influence the quality of protein, which was expected. Rapid dilution was utilized to refold protein and allowing the protein to stir once refolded for a longer period of time should not have an effect on the activity of protein. The redox system used in refolding buffer (either oxidized and reduced cysteine or glutathione) were evaluated for effectiveness based on TLR5 activity: both systems were efficient for all constructs tested, though there were variations seen among different constructs. For FliC, higher activity was observed when using the cysteine/cystine (cys/(cys)2) redox system within the refolding buffer. Conversely, refolding FliC:GP132 in the presence of the glutathione redox system appeared to yield more active than protein refolded using the cysteine system. The effect of the redox species used in refolding buffer may be minor, however when considering the totality of variables involved in creating an ideal protein purification scheme, slight variances such as these cannot be ignored.

Anion exchange chromatography was used as the final purification step in all purification procedures studied. A significant amount of protein was lost to column flow-through; this was especially true for the FliC:GP132 fusion protein. This result, combined with the high level of residual endotoxin contamination indicate that it is likely that endotoxin contamination prevented proper binding of protein to
the column during the final chromatography step. This is an issue that may have been reduced by removing excess ET in prior stages of purification. Repeating the 2-phase spin or introducing this step earlier in the process may reduce ET prior to loading on the column. Adding this step does not greatly affect the amount of time or resources needed for protein purification, nor does it introduce any additives that would have to be removed by adding an additional step later in purification. AEX chromatography is a purification step ideally placed near the end of the purification scheme because the product eluted from the column should have very minimal contaminants and therefore will require few, if any, downstream steps.

Purification of proteins from Gram negative bacteria can be challenging, and unfortunately, there is still no one universal method to be used to achieve this goal. The iterative nature of the purification process may be reduced or optimized by performing a meta-analysis of the literature of proteins purified from *E. coli*, in order to build a database of protein characteristics and successful purification schemes. This, combined with *in silico* protein folding and characterization, may provide insight into which purification system is the most appropriate for the putative protein. This may be especially useful when the putative protein is truncated or a fusion protein. The expression and characteristics of such proteins may be difficult to predict, thus *in silico* modeling would greatly reduce troubleshooting. Moreover, it may be more advantageous to produce recombinant protein in newer cell-free systems, recombinant *E. coli* which does not express LPS at all (45), or within a eukaryotic or Gram-positive bacterial expression system. At any rate, a blueprint of all possible protein purification methodologies is a useful tool regarding expediting the process of purification and reduction of unpredictable variables. This study integrates valuable information into such a blueprint by demonstrating that a less stringent denaturant may be used to effectively purify biologically active recombinant protein from the insoluble fraction of an *E. coli* expression system.
REFERENCES


APPENDICES

Appendix A- Insoluble Procedure for Initial Purification of Recombinant Proteins

Procedure:

Preparing the Cell Extract

1. Thawing Cell Extract
   a. Record bioreactor batch information, construct ID, and amount (weight and quantity) of cell extract to thaw
   b. Remove frozen cell extract from storage (record storage temperature)
   c. Place frozen cell extract in 4°C to thaw overnight

2. Addition of Lysis Buffer
   a. Retrieve cell extract and Lysis Buffer (P1) from refrigerator
   b. Add a small amount of Lysis Buffer to volumetric container with the capacity to hold 1.5L
   c. Begin stirring Lysis Buffer with a stir bar
   d. Add cell extract to stirring Lysis Buffer
   e. Rinse cell extract bottle with Lysis Buffer
   f. Continue to add a total of 1L of Lysis Buffer to cell extract.
   g. Mix on stir plate for at least 15 minutes
   h. Place prepared cell extract in fridge to store or into ice bath to cool to <5°C
Homogenization

1. Prepare the Homogenizer
   a. Replace water in recirculating cooling loop with fresh ddH₂O, place into ice bath
   b. Begin pumping (set flow to 50-100mL/min)
   c. Clean APV
      1. Fill hopper with 2L chilled ddH₂O and run through
      2. Fill feed hopper with chilled lysis buffer and recirculate 3-5 minutes at 0psi (0bar)
      3. Discard lysis buffer

2. Resuspend Cell Extract
   a. Record start temperature of the prepared cell extract
   b. Fill feed hopper with prepared cell extract
   c. Run sample through the APV at 0psi (0bar)
   d. Take sample of resuspended cell extract (“Resuspended Cell Extract”)
   e. Cool resuspended cell extract to <5ºC

3. Homogenization
   a. Fill feed hopper with resuspended cell extract
   b. Run sample through the APV at 10,000psi (690bar)
   c. Cool lysate to <5ºC
   d. Repeat steps a-c until the lysate has been homogenized 3 times under pressure
   e. After the final pass, collect the lysate and cool to 14ºC
   f. Take a sample of the lysate (“Lysate”)
   g. Record final weight and volume of lysate
   h. If not processed immediately, store @ 4ºC
4. Clean the Homogenizer
   a. Fill feed hopper with ddH2O (1L)
   b. Run through and discard
   c. Fill feed hopper with fresh ddH2O (1L)
   d. Allow to recirculate at least 5min
   e. Drain hopper and discard
   f. Fill feed hopper with 0.1M NaOH
   g. Allow to recirculate 5-10min
   h. Drain hopper and discard

Clarification by centrifugation

1. Initial clarification
   a. Turn on centrifuge (Avanti J-20 XP) and set temp to 15ºC
   b. Aliquot equal volumes of the lysate into centrifuge bottles
   c. Load into centrifuge and spin for 45min @ 8,000rpm (decel to slow)
   d. After centrifugation, collect supernatant via pipetting, pool supernatants and measure total weight and volume
   e. Take sample of the pooled supernatant (“Pooled Supernatant”)
   f. Add Pellet Resuspension Buffer (P3, 1mL) to micro-centrifuge tube and add a loopful of pellet for sampling (“Initial Spin Pellet”)
   g. Discard the pellet
**PEG Precipitation**

1. Addition of PEG
   a. Determine the amount of PEG-3350 required:
      - PEG = (pooled supernatant volume) \* 140g/L
   b. Weigh out required amount of PEG
   c. Slowly add the PEG to the pooled supernatant
   d. Mix on stir plate for at least 15 minutes

2. PEG Precipitation Spin
   a. Turn on centrifuge (Avanti J-20 XP) and set temp to 15ºC
   b. Aliquot equal volumes of the prepared supernatant into centrifuge bottles
   c. Load into centrifuge and spin for 60min @ 10,000rpm (decel to zero)
   d. After centrifugation, decant and pool supernatants, measure total volume
   e. Take sample of pooled supernatant (“PEG Pooled Supernatant”)
   f. Discard supernatant

**Resuspension**

1. Resuspension of PEG pellet
   a. Add required volume of Resuspension Buffer (SOL1a or SOL1b) to pooled PEG pellets
      - Volume = volume of pooled supernatants from previous spin
   b. Disrupt mechanically using a disposable inoculation needle
   c. Mix with a stir bar until the pellet has been evenly resuspended
   d. Take sample of the resuspended pellet (“PEG Resuspension”)
2. Refined Lysate Spin
   a. Turn on centrifuge (Avanti J-20 XP) and set temp to 30ºC
   b. Aliquot equal volumes of the PEG resuspension into centrifuge bottles
   c. Load into centrifuge and spin for 60min @ 10,000rpm (decel to max)
   d. After centrifugation, collect and pool the supernatants
   e. Measure the total volume of the pooled supernatants, take sample ("Refined Lysate")
   f. Label Refined Lysate with batch number and date; store at 4ºC
   g. Add Pellet Resuspension Buffer (P3, 1mL) to micro-centrifuge tube and add a loopful of pellet for sampling ("RL spin Pellet")
   h. Discard the pellet

Solutions:

Lysis Buffer (P1)
50mM Tris, 125mM NaCl, 4% Sucrose, 10mM EDTA, 0.05% TritonX-100, pH 8

- Add 750ml ddH₂O to a 1L beaker with a stir bar
- Add 40g Sucrose
- Stir until completely dissolved
- Add 6.06g Tris
- Add 7.3g Nicol
- Add 3.72g EDTA
- Add 0.5g TritonX-100
- Stir for ~ 10 min
- Adjust pH using Acetic Acid
- Adjust volume to 1L with ddH₂O
- Filter sterilize, store at 4°C
Resuspension Buffer (SOL1a)

200 mM Acetic Acid, 1M Guanidine-HCl, pH 4

- Add 600ml ddH₂O to a 1L beaker with a stir bar
- Add 95.53g Guanidine-HCl
- Stir until completely dissolved
- Add 12mL of Glacial Acetic Acid
- Stir for ~ 10 min
- Adjust pH using NaOH.
- Adjust volume to 1L with ddH₂O
- Filter sterilize, store at RT

Resuspension Buffer (SOL1b)

200 mM Acetic Acid, 6M Guanidine-HCl, pH 4

- Add 600 ml ddH₂O to a 1L beaker with a stir bar
- Add 573.18g Guanidine-HCl
- Stir until completely dissolved
- Add 12mL of Glacial Acetic Acid
- Stir for ~ 10 min
- Adjust pH using NaOH.
- Adjust volume to 1L with ddH₂O
- Filter sterilize, store at RT
Pellet Resuspension Buffer (P3)

100 mM Tris, 8 M Urea, pH 8

- Add 500ml ddH2O to a 1L beaker with a stir bar
- Add 480.48g Urea
- Stir until completely dissolved
- Add 12.112g Tris
- Stir until completely dissolved
- Adjust volume to 1L with ddH2O
- Adjust pH using Acetic acid
- Filter sterilize, store at RT
Appendix B- Soluble Procedure for Initial Purification of Recombinant Protein

Preparing the Cell Extract

1. Thawing Cell Extract
   a. Record bioreactor batch information, construct ID, and amount (weight and quantity) of cell extract to thaw
   b. Remove frozen cell extract from storage (record storage temperature)
   c. Place frozen cell extract in 4°C to thaw overnight

2. Addition of Lysis Buffer
   a. Retrieve cell extract and Lysis Buffer (P1) from refrigerator
   b. Add a small amount of Lysis Buffer to volumetric container with the capacity to hold 1.5L
   c. Begin stirring Lysis Buffer with a stir bar
   d. Add cell extract to stirring Lysis Buffer
   e. Rinse cell extract bottle with Lysis Buffer
   f. Continue to add a total of 1L of Lysis Buffer to cell extract.
   g. Mix on stir plate for at least 15 minutes
   h. Place prepared cell extract in fridge to store or into ice bath to cool to <5°C

Homogenization

1. Prepare the Homogenizer
   a. Replace water in recirculating cooling loop with fresh ddH₂O, place into ice bath
   b. Begin pumping (set flow to 50-100mL/min)
   c. Clean APV
      1. Fill hopper with 2L chilled ddH₂O and run through
      2. Fill feed hopper with chilled lysis buffer and recirculate 3-5 minutes at 0psi (0bar)
      3. Discard lysis buffer
2. Resuspend Cell Extract
   a. Record start temperature of the prepared cell extract
   b. Fill feed hopper with prepared cell extract
   c. Run sample through the APV at 0psi (0bar)
   d. Take sample of resuspended cell extract ("Resuspended Cell Extract")
   e. Cool resuspended cell extract to <5ºC

3. Homogenization
   a. Fill feed hopper with resuspended cell extract
   b. Run sample through the APV at 10,000psi (690bar)
   c. Cool lysate to <5ºC
   d. Repeat steps a-c until the lysate has been homogenized 3 times under pressure
   e. After the final pass, collect the lysate and cool to 14ºC
   f. Take a sample of the lysate ("Lysate")
   g. Record final weight and volume of lysate
   h. If not processed immediately, store @ 4ºC

4. Clean the Homogenizer
   a. Fill feed hopper with ddH2O (1L)
   b. Run through and discard
   c. Fill feed hopper with fresh ddH2O (1L)
   d. Allow to recirculate at least 5min
   e. Drain hopper and discard
   f. Fill feed hopper with 0.1M NaOH
   g. Allow to recirculate 5-10min
   h. Drain hopper and discard
Clarification by centrifugation

1. Initial clarification
   a. Turn on centrifuge (Avanti J-20 XP) and set temp to 15ºC
   b. Aliquot equal volumes of the lysate into centrifuge bottles
   c. Load into centrifuge and spin for 45min @ 8,000rpm (decel to slow)
   d. After centrifugation, collect supernatant via pipetting, pool supernatants and measure total weight and volume
   e. Take sample of the pooled supernatant (“Pooled Supernatant”)
   f. Add Pellet Resuspension Buffer (P3, 1mL) to micro-centrifuge tube and add a loopful of pellet for sampling (“Initial Spin Pellet”)
   g. Discard the pellet

PEG Precipitation

1. Addition of PEG
   a. Determine the amount of PEG-3350 required:
      - PEG = (pooled supernatant volume) * 140g/L
   b. Weigh out required amount of PEG
   c. Slowly add the PEG to the pooled supernatant
   d. Mix on stir plate for at least 15 minutes
2. PEG Precipitation Spin
   a. Turn on centrifuge (Avanti J-20 XP) and set temp to 15°C
   b. Aliquot equal volumes of the prepared supernatant into centrifuge bottles
   c. Load into centrifuge and spin for 60min @ 10,000rpm (decel to zero)
   d. After centrifugation, decant and pool supernatants, measure total volume
   e. Take sample of pooled supernatant (“PEG Pooled Supernatant”)
   f. Discard supernatant

Resuspension

1. Resuspension of PEG pellet
   a. Add required volume of Resuspension Buffer (SOL1a or SOL1b) to pooled PEG pellets
      - Volume = volume of pooled supernatants from previous spin
   b. Disrupt mechanically using a disposable inoculation needle
   c. Mix with a stir bar until the pellet has been evenly resuspended
   d. Take sample of the resuspended pellet (“PEG Resuspension”)

2. Refined Lysate Spin
   a. Turn on centrifuge (Avanti J-20 XP) and set temp to 30°C
   b. Aliquot equal volumes of the PEG resuspension into centrifuge bottles
   c. Load into centrifuge and spin for 60min @ 10,000rpm (decel to max)
   d. After centrifugation, collect and pool the supernatants
   e. Measure the total volume of the pooled supernatants, take sample (“Refined Lysate”)
   f. Label Refined Lysate with batch number and date; store at 4°C
   g. Add Pellet Resuspension Buffer (P3, 1mL) to micro-centrifuge tube and add a loopful of pellet for sampling (“RL spin Pellet”)
   h. Discard the pellet
Solutions:

Lysis Buffer (P1)

50mM Tris, 125mM NaCl, 4% Sucrose, 10mM EDTA, 0.05% TritonX-100, pH 8

- Add 750ml ddH$_2$O to a 1L beaker with a stir bar
- Add 40g Sucrose
- Stir until completely dissolved
- Add 6.06g Tris
- Add 7.3g NaCl
- Add 3.72g EDTA
- Add 0.5g TritonX-100
- Stir for ~ 10 min
- Adjust pH using Acetic Acid
- Adjust volume to 1L with ddH$_2$O
- Filter sterilize, store at 4°C

Resuspension Buffer (SOL1a)

200 mM Acetic Acid, 1M Guanidine-HCl, pH 4

- Add 600ml ddH$_2$O to a 1L beaker with a stir bar
- Add 95.53g Guanidine-HCl
- Stir until completely dissolved
- Add 12mL of Glacial Acetic Acid
- Stir for ~ 10 min
- Adjust pH using NaOH.
- Adjust volume to 1L with ddH$_2$O
- Filter sterilize, store at RT
**Resuspension Buffer (SOL1b)**

200 mM Acetic Acid, 6M Guanidine-HCl, pH 4

- Add 600ml ddH$_2$O to a 1L beaker with a stir bar
- Add 573.18g Guanidine-HCl
- Stir until completely dissolved
- Add 12mL of Glacial Acetic Acid
- Stir for ~ 10 min
- Adjust pH using NaOH.
- Adjust volume to 1L with ddH$_2$O
- Filter sterilize, store at RT

**Pellet Resuspension Buffer (P3)**

100 mM Tris, 8 M Urea, pH 8

- Add 500ml ddH2O to a 1L beaker with a stir bar
- Add 480.48g Urea
- Stir until completely dissolved
- Add 12.112g Tris
- Stir until completely dissolved
- Adjust volume to 1L with ddH2O
- Adjust pH using Acetic acid
- Filter sterilize, store at RT
Appendix C - Tangential Flow Filtration and 2 Phase Separation

Procedure:

Preparing the TFF unit

1. Pressure Hold Test
   a. Drain the system
   b. Tighten backpressure valve and clamp permeate line
   c. Introduce at least 5psi pressure to the system
   d. After 2 minutes, if the pressure has not dropped by 1psi, continue to next step. If the pressure has dropped, tighten both clamps and repeat until pressure is maintained.

2. Retentate Flow Rate / Clean Water Flux
   a. Open backpressure valve and clamp permeate line
   b. Direct both permeate and retentate lines to waste.
   c. Direct feed line to reservoir of ddH2O
   d. Measure flow rate
   e. Direct retentate line to Feed Reservoir and unclamp permeate line
   f. Adjust backpressure to 5psi
   g. Establish permeate flow rate and calculate flux \([0.06 \times \text{flow rate}}/0.02\]. Target flux is 200LMH
   h. Drain system

3. Sanitize with Sanitization Buffer (P4)
   a. Make up fresh 0.1N NaOH (P4) for each use
   b. Open backpressure valve and direct feed and retentate lines to reservoir containing sanitization buffer
   c. Once pH of permeate is at least 10, direct permeate line to sanitization buffer reservoir
   d. Recirculate 15 minutes, and drain the system
4. Rinse w/ ddH2O
   a. Direct retentate and permeate lines to waste
   b. Direct feed line to a reservoir containing ddH2O
   c. Flush 400mL through (as measured by the volume of the retentate)
   d. Clamp permeate line and measure pH of retentate
   e. Once pH of the retentate is 8 or less, open permeate line and tighten backpressure valve to give 5psi of pressure
   f. Measure pH of permeate
   g. Once the pH of the permeate is 8 or less, drain the system

5. Equilibration
   a. Clamp permeate line and open backpressure valve
   b. Direct both feed and retentate lines to a reservoir containing diafiltration buffer (P2)
   c. Establish a consistent flow and unclamp the permeate line
   d. After 1 minute, check the pH of the permeate line. The pH of the permeate line should be at the same pH of the diafiltration buffer (+/- 0.5)
   e. Record pH and volume of permeate. Save 2x 1mL samples for endotoxin (ET) assay (“TFF EQ”).
TFF and 2-Phase

1. Sample Prep and Concentration
   
a. Retrieve refined lysate (RL) and record relevant sample information, including volume and appearance of sample
b. Decant RL into fresh bottle, leaving behind white precipitate
c. Record new volume (“original volume”) and pH of sample
d. Add 1M Tris (P5) to bring pH of RL to 8, record volume of 1M Tris added
e. Obtain 1x 1mL sample (“TFF start”)
f. Measure conductivity of sample
g. Direct feed and retentate lines to bottle containing sample
h. Open permeate line and adjust backpressure valve to 5psi
i. Concentrate RL to original volume (until volume in permeate reservoir is equivalent to volume of 1M Tris added)
j. Take 1x 1mL samples of permeate, RL, and retentate (“Post Concentration RL”)
2. Buffer exchange

   a. Measure and record conductivity of diafiltration buffer

   b. Add diafiltration buffer feed line into sample reservoir

   c. Adjust backpressure valve to 5psi

   d. Begin running the system, adding diafiltration buffer to sample at the same rate as permeate outflow (maintain the original volume of RL)

   e. Once one diavolume (equivalent to the original volume of RL) has collected in the permeate reservoir, take 1mL samples of the retentate and permeate ("Diavolume 1")

   f. Measure the conductivity of the permeate

   g. Repeat Steps d-f until 5 diavolumes have been run and the permeate conductivity is within 10% of the conductivity of the diafiltration buffer
3. Filter Wash

a. Drain TFF into retentate reservoir and record final volume

b. Add 100 mL diafiltration buffer to clean container, direct feed and retentate lines to this container

c. Direct permeate line to waste

d. Clamp the permeate line and recirculate for at least one minute to establish consistent flow

e. Unclamp the permeate line and adjust backpressure to 5psi

f. Continue to recirculate until volume remaining in feed reservoir is concentrated an amount equivalent to hold-up volume of the system (~30mL)

g. Record final volume of the wash

h. Pool wash and retentate from buffer exchange

i. Record the weight and volume of the pooled retentates

j. Take sample (“Post-TFF RL”)

k. If the Post-TFF RL is not processed immediately, store @ 4°C
4. 2-Phase Spin Procedure

   a. Add Triton X 114 (0.01 * weight of post-TFF RL)

   b. Stir for at least 15 minutes

   c. Add PEG 3350 (0.05 * weight of post-TFF RL)

   d. Stir until fully dissolved

   e. Aliquot prepared post-TFF RL into centrifuge bottles

   f. Begin 2-phase spin (10,000rpm at 24ºC for 3 hours, set decel to zero)

   g. Collect aqueous phase and detergent phase

   h. Take sample detergent phase ("Detergent Phase"), discard remainder

   i. Filter aqueous phase into clean container

   j. Weigh clarified aqueous phase and sample ("CAP")

   k. Store CAP @ 4ºC

Cleaning TFF unit

1. Rinse with ddH2O

   a. Direct retentate and permeate lines to waste

   b. Open permeate clamp and adjust backpressure valve so that permeate flow and retentate flow are equal

   c. Direct feed line to reservoir containing 800mL ddH2O

   d. Flush all the water through the system

   e. Drain the TFF
2. Sanitize with 0.1N NaOH
   a. Open backpressure valve
   b. Direct feed and retentate lines to reservoir containing 0.1N NaOH
   c. Direct permeate line to waste
   d. Recirculate for about 1 minute
   e. Measure the pH of the permeate
   f. Once the permeate is at least 10, direct the permeate line to the feed reservoir
   g. Recirculate for 15 minutes
   h. Drain the TFF

3. Rinse with ddH2O
   a. Direct retentate and permeate lines to waste
   b. Tighten backpressure valve so there is flow through the permeate line
   c. Flush 400mL ddH2O through the system
   d. Measure the pH of the permeate
   e. Once the pH of the permeate is 8 or less, open the backpressure valve and tighten the clamp on the permeate line
   f. Measure the pH of the retentate
   g. Once the pH of the retentate is 8 or less, drain the system
4. Store in 0.1N NaOH (P4)
   a. Open the backpressure valve
   b. Direct feed, retentate, and permeate lines to vessel containing 0.1N NaOH
   c. Start the pump
   d. Once the pH of the permeate is at least 10, stop the pump
   e. Clamp all tubing from the TFF unit to seal
   f. Store TFF unit at room temp

**Solutions:**

**Diafiltration Buffer (P2)**

50mM Tris, 125mM NaCl, 10mM EDTA, pH 8
- Add 750ml ddH2O to a 1L beaker with a stir bar.
- Add 6.06g of Tris
- Add 7.3g of NaCl
- Add 3.72g of EDTA
- Adjust pH using Acetic Acid
- Adjust volume to 1L with ddH2O
- Filter sterilize, Store at 4°C

**Sanitization Buffer (P4)**

0.1N NaOH
- Add 990mL ddH2O to clean bottle
- Add 10mL 10N NaOH, swirl to mix
- Store @ RT
**pH Adjustment Buffer (P5)**

**1M TRIS**

- Dissolve 121.1g tris(hydroxymethyl)aminomethane in 1L ddH2O
- Filter sterilize
- Store @ RT
Appendix D - Phase 2 Purification

Procedure:

Preparation

1. Add Guanidine-HCl to a 100mL aliquot of Refined Lysate (RL) sufficient to bring the concentration of Guanidine to 1M (9.53g).
   a. Mix on stir plate for at least 15 minutes
2. Determine protein concentration of Denatured RL using BCA protein assay
3. If required, dilute RL to 2mg/mL using Dilution Buffer (see Solutions); Final volume = at least 25mL

Refolding

1. Record pH, conductivity, and batch information of Refolding Buffer (A1)
2. Calculate volume of prepared RL containing at least 50mg protein (~25mL)
3. Add required volume of Refolding Buffer
   a. Volume Refolding Buffer = 9*Volume of Diluted RL
4. Slowly add Diluted RL (5mL/min) to Refolding Buffer and stir to refold for 2 hours
5. Measure and record pH and conductivity of Refolded AEX Load
6. Take sample (“Refolded AEX Load”)

ÄKTA prep, Sample Load and Run

1. Buffer prep set up for AEX
   a. Direct A11 inlet to 0.1M Tris solution
   b. Direct A2 inlet to 0.1M HCl
   c. Direct B1 inlet to sterile ddH2O
   d. Direct B2 inlet to 2M NaCl
2. Buffer line set up for additional methods (Clean/Strip/Store)
   a. Direct A16 inlet to A3 (Cleaning Solution)
   b. Direct A17 inlet to A2 (Stripping Solution)
   c. Direct A18 inlet to 20%EtOH

3. Remove pH meter from storage solution and put in line

4. Manually prime system pumps for ALL buffer lines
   a. Manual select Pump: Flow- 5mL/min (insert)
   c. Execute commands
   d. Loosen corresponding valve port
   e. Connect syringe
   f. Draw all air from buffer line
   g. End command
   h. Repeat for each buffer line used

5. Direct line from Refolded AEX Load (250mL sample to load) to sample valve (S1)

6. Prime sample line
   a. Manual select Pump: Sample Flow_960- 5mL/min (insert)
   b. Manual select Flowpath: Sample Valve S1 (insert)
   c. Execute commands
   d. Watch for flow through with no bubbles.
   e. End command
7. Begin method queue “R3 Complete” on AKTA computer
   
   a. Method “Strip and Clean”
      
      i. Click “ok” on all options
   
   b. Method “Gradient Super Q 40411”
      
      i. Frac 950 collection- set to “row by row”
      
      ii. Evaluation Procedure- set to integrate full report
      
      iii. Click “ok” on all other options
   
   c. Method “Clean and Strip”
      
      i. Click “ok” on all options
   
   d. Method “EtOH for storage”
      
      i. Click “ok” on all options
   
8. Collect post load wash, fractions, and load flow through, store at 4°C.
   
9. Replace pH meter into storage solution.
Solutions:

Refolding Buffer (A1)

20mM Tris, 0.1M Trehalose, 2mM CaCl₂, 3mM Cysteine, 0.3mM Cystine, 1mM EDTA, 0.1% PS-80, pH 8.0

- Add 1g of PS-80 into tared 1L beaker
- Add 750ml ddH₂O with a stir bar
- Add 2.42g Tris
- Add 34.23g Trehalose
- Add 0.294g CaCl₂
- Add 0.527g Cysteine
- Add 0.072g Cystine
- Add 0.372g EDTA
- Stir ~15min
- Adjust pH with Glacial Acetic Acid
- Adjust volume to 1L with ddH₂O
- Filter sterilize, Store at RT
- Place 2-week expiration date on container
**Stripping Buffer (A2)**

100mM Tris, 1M NaCl, pH 8 (Volume: 1L)

- Add 750ml to a 1L beaker with a stir bar
- Add 12.114g Tris
- Add 58.44g NaCl
- Stir ~10 min
- Adjust pH using Acetic Acid
- Adjust volume to 1L with ddH₂O
- Filter sterilize, store @ RT

**Q Cleaning Buffer (A3)**

0.5N NaOH, 1M NaCl

- Add 750mL ddH₂O to a beaker with a stir bar
- Add 58.44g NaCl
- Stir ~10min to mix thoroughly
- Slowly add 20.0g NaOH pellets (exothermic reaction)
- Stir ~10min to mix thoroughly
- Adjust volume to 1L
- Filter sterilize, store @ RT
**Dilution Buffer (P3)**

100 mM Tris, 1M Guanidine-HCl, pH 8

- Add 500ml ddH2O to a 1L beaker with a stir bar
- Add 95.53g Guanidine-HCl
- Stir until completely dissolved
- Add 12.112g Tris
- Stir until completely dissolved
- Adjust pH using Acetic acid
- Adjust volume to 1L with ddH2O
- Filter sterilize, store at RT

**20% EtOH**

- Add 800mL ddH2O to sterile beaker
- Add 200mL 100% EtOH (molecular grade)
- Store at RT

**Buffer Prep 0.1M Tris (BP1)**

- Add 500mL ddH2O to a 1L beaker with a stir bar
- Add 24.22g Tris
- Stir until completely dissolved
- Adjust volume to 2L with ddH2O
- Filter sterilize, Store at RT
**Buffer Prep 2M NaCl (BP2)**

- Add 500mL ddH$_2$O to a 1L beaker with a stir bar
- Add 233.8g NaCl
- Stir until completely dissolved
- Adjust volume to 2L with ddH$_2$O
- Filter sterilize, store at RT

**Buffer Prep 0.1M HCl (BP3)**

- Add 500mL ddH$_2$O to a 1L beaker with a stir bar
- Add 8.56g 36%HCl
- Stir to mix
- Adjust volume to 1L with ddH$_2$O
- Filter sterilize, store at RT

**Buffer Prep Sterile ddH$_2$O (BP4)**

- Add 1L ddH$_2$O to beaker
- Autoclave liquid cycle at least 20 min
- Store at RT
Method Queue Specifications:

1. Column information:

   Anion Exchange

   ToyoScreen SuperA-650M 5mL prepacked column
   (Tosoh part number 21363)

   Maximum pressure: 3 bar (0.3 MPa)

   Dimensions: 3 cm H x 1.46 cm D

2. Buffer Prep:

   Species: Tris

   pH: 8 – range = 7.5-8.5

3. Gradient:

   0-100% B (100% B = 1 M NaCl)

   10 Column Volumes

4. Clean and Strip/Strip and Clean:

   Clean with 3CV Stripping Solution (A2)

   Strip with 3CV Cleaning Solution (A3)
Appendix E- TLR5 Bioassay

Procedure

Ensure that both the HEK-Blue™-hTLR5 and HEK-Blue™-Null1 cells have been passaged with Test media at least once prior to performance of the assay (see HEK Cell Maintenance Procedure).

HEK-Blue™-hTLR5 Cells

Day 1:

- Add 20 μl of each sample per well of a flat-bottom 96-well plate.
- Add 20 μl of a positive control (flagellin from *S. typhimurium*, 100 ng/mL) in one well.
- Add 20 μl of a negative control (sterile, endotoxin-free water) in one well.
- Prepare a cell suspension of HEK-Blue™-hTLR5 Cells at ~140,000 cells per ml in Test Medium which contains 10% (v/v) heat inactivated FBS.
- Add 180 μl of cell suspension (~25,000 cells) per well.
- Incubate the plate at 37°C in a CO₂ incubator for 20-24 h.

Day 2:

- Prepare QUANTI-Blue™ following the instructions on the pouch.
- Add 180 μl of resuspended QUANTI-Blue™ per well of a flat-bottom 96-well plate.
- Add 20 μl of induced HEK-Blue™-hTLR5 Cells supernatant.
- Incubate the plate at 37°C incubator for 1-3 h.
- Determine SEAP levels using a spectrophotometer at 620-655 nm.
HEK-Blue™-Null1 Cells

Day 1:

- Add 20 μl of each sample per well of a flat-bottom 96-well plate.
- Add 20 μl of a positive control (TNFα, 100 ng/ml) in one well.
- Add 20 μl of a negative control (sterile, endotoxin-free water) in one well.
- Prepare a cell suspension of HEK-Blue™-Null1 Cells at ~280,000 cells per ml in Test Medium which contains 10% (v/v) heat inactivated FBS.
- Add 180 μl of cell suspension (~50,000 cells) per well.
- Incubate the plate at 37°C in a CO2 incubator for 16-20 h.

Day 2:

- Prepare QUANTI-Blue™ following the instructions on the pouch.
- Add 180 μl of resuspended QUANTI-Blue™ per well of a flat-bottom 96-well plate.
- Add 20 μl of induced HEK-Blue™-Null1 Cells supernatant.
- Incubate the plate at 37°C incubator for 1-3 h.
- Determine SEAP levels using a spectrophotometer at 620-655 nm.

Once SEAP levels have been determined for both TLR5 and Null cells, subtract the Null ODs from the TLR5 ODs to correct for endogenous TLR5 activity in the HEK cells. The OD is directly proportional to the amount of TLR5 stimulation.
Appendix F- HEK Cell Maintenance

HEK-Blue® hTLR5 and HEK-Blue® Null cell maintenance:

Frozen cells (3rd passage) are stored in cryogenic vials in liquid N₂ for long term storage.

Growth medium (for both cell types):

DMEM (500mL)—remove 58.5mL DMEM and discard
add 50 mL FBS
add 2.5 mL Pen/Strep
add 5 mL glutamine
add 1 mL Normocin

Procedure:

Add 5mL Growth media to T-25 flasks (1 per cell type).
Label flasks with date, passage #, dilution (1:6), and cell type.
Warm to 37°C.
Thaw cryovials and transfer 1mL cells to Growth media.
After 4 days, or once confluency = 70-80%, transfer to Test media.

Test medium (Null):

⇒ To 50mL Growth media, add 50μL Zeocin

Test medium (hTLR5):

⇒ To 50mL Growth media, add 50μL Zeocin and 150μL Blasticidin.

Procedure:

Add 11.5mL Test media to T-75 flasks (2 per cell type).
Label flasks with date, passage #, dilution (1:24), and cell type.
Warm to 37°C.
Obtain cultures from incubator and check for 70-80% confluency.
Draw off spent media and wash twice with PBS. Draw off PBS and add fresh Test media (2mL).

Pipet cells off flask, and tap gently to release.

Add 500µL cell suspension to warmed new media, incubate @ 37°C, 5%CO₂.

Transfer to fresh media every 4 days, or once confluence = 70-80%.

** For frozen cells (after 20 passages, dispose of cell line and start from frozen cells).
Appendix G- Endotoxin Assay

Procedure

Pre-Assay Steps:

All samples are tested for a pH of 8 using pH testing strips. Each sample is added to a pH strip until the tip of strip is wet, and then waits 30 seconds for color change. The strip is then compared to the test strip colors on the box to determine the pH of the sample. If samples are below 8 to 8.5, a buffer solution is added to increase the pH of the sample.

LAL-free water is used for all dilutions, and all pipet tips and plates are pyrogen-free. All steps are done according to the manufacturer’s procedure.

Preparation of the Standard:

The standard is *an E. coli* endotoxin which is rehydrated with 2.3 mL of LAL-free water prior to use (stable for one month). The concentration of this solution is 50 EU/mL. Diluent is LAL-free water.

Standard should be vortexed before each use for 10 – 15 minutes. The standard should be at room temperature for 15 minutes prior to use. Standard should be stored at 4°C.

<table>
<thead>
<tr>
<th>Standard Curve Dilutions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
</tr>
<tr>
<td>1:5</td>
</tr>
<tr>
<td>1:50</td>
</tr>
<tr>
<td>1:500</td>
</tr>
<tr>
<td>1:5000</td>
</tr>
</tbody>
</table>

Each dilution should be vortexed for ~ 1 minute between each serial dilution. Each sample should also be vortexed before it is added to microplate.
Sample Preparation:

Pre-dilution:

All samples from initial purification must be pre-diluted to $10^7$.

All samples from TFF/2Phase must be pre-diluted to $10^3$.

All samples from AEX need not be pre-diluted.

Assay:

Once samples have been pre-diluted (if necessary), prepare the dilutions for the assay as follows.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Sample Volume(µL)</th>
<th>Diluent (µL)</th>
<th>Total Volume(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>80</td>
<td>320</td>
<td>400</td>
</tr>
<tr>
<td>1:25</td>
<td>80 of 1:5</td>
<td>320</td>
<td>400</td>
</tr>
<tr>
<td>1:125</td>
<td>80 of 1:25</td>
<td>320</td>
<td>400</td>
</tr>
<tr>
<td>1:625</td>
<td>80 of 1:125</td>
<td>320</td>
<td>400</td>
</tr>
</tbody>
</table>

Each dilution should be vortexed for ~ 1 minute between each serial dilution. Each sample should also be vortexed before it is added to microplate.

Samples are incubated at 37°C for 5 minutes.

The LAL reagent is then rehydrated with 2.6 mL LAL-free water and shaken until fully dissolved. LAL reagent is then added to a reservoir.

100µL of the LAL reagent is then added to every well with a sample by a multichannel pipette. The plate is then run for 40 minutes at 37°C using a pre-set protocol using the SoftMax Pro software on a SpectraMax Plus apparatus. It is important that no bubbles are present in the plate wells.
Parameters for SpectraMax Plus apparatus:

Lonza Protocol

- 405 nm
- Onset OD of 0.2
- Interval of 2:30 min
- Reads: 40
- Constant Temperature of 37 degrees Celsius

The data is then analyzed and calculated via SoftMax pro software.

Standard Curve is generated with a log-log curve. A trend line is added to find the slope of the line and the $R^2$ value. The equation is then used to calculate the x value. The x value for each sample is then multiplied by the dilution factors for each to obtain the amount of endotoxin for each sample.
Appendix H- BCA Protein Quantification

Procedure

(Adapted for Thermo Scientific Pierce BCA Protein Assay Kit)

Prepare the working reagent- 1-part Reagent B : 50 parts Reagent A

- Calculate the amount needed:

  - (# of samples x # of replicates x # of dilutions) + standards (in duplicate)

Add 25µL of diluted standard to each well, in duplicate.

Serial dilution of standards:

  2000µg (neat)
  1000µg (1:2 dilution of previous)
  500µg (1:2 dilution of previous)
  250µg (1:2 dilution of previous)
  125µg (1:2 dilution of previous)
  62.5µg (1:2 dilution of previous)
  31.25µg (1:2 dilution of previous)
  15.625µg (1:2 dilution of previous)
  0µg (ddH2O)

Add 25µL of sample (diluted 1:10 and 1:20) to each well, in duplicate.

Add 200µL working reagent to each well

Incubate 30min @ 37°C (in the dark)

Read Abs (562nm) using Spectrophotometer

Generate chart plotting concentration of standards against absorbance.

  - Add trend line
  - Show R2 value and slope equation (y=mx+b)
Calculate protein concentration of samples:

- Average replicate ODs
- (Averaged value-b)/m
- Multiply by dilution factor
- Average dilutions of same samples
Appendix I- SDS-PAGE Assay

Procedure:

Preparing Samples

1. Retrieve samples from -20°C

2. Preparing non-reduced samples:
   a. Add sample, ddH2O, and 4x buffer to microcentrifuge tube according to the chart below.
   b. Vortex each tube for ~10s to mix thoroughly.
   c. Briefly centrifuge samples.
   d. Heat on 95°C heating block for 5 minutes.
   e. Allow to cool briefly.

<table>
<thead>
<tr>
<th>Step</th>
<th>Sample</th>
<th>ddH2O</th>
<th>4x sample buffer</th>
<th>Ratio</th>
<th>μg Protein/10μL loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Purification</td>
<td>4</td>
<td>26</td>
<td>10</td>
<td>1/10</td>
<td>1</td>
</tr>
<tr>
<td>TFF/2phase</td>
<td>12.5</td>
<td>17.5</td>
<td>10</td>
<td>1/2</td>
<td>5</td>
</tr>
<tr>
<td>Refold/ AEX</td>
<td>30</td>
<td>0</td>
<td>10</td>
<td>3/4</td>
<td>7.5</td>
</tr>
<tr>
<td>HIC</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>1/4</td>
<td>2.5</td>
</tr>
<tr>
<td>Bulk formulation</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>1/4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

3. Preparing reduced samples (for CDC samples):
   a. Add sample (4μL), ddH2O (20μL), 10X Reducing Agent (6μL), and 4X Buffer (10μL).
   b. Vortex each tube for ~10s to mix thoroughly.
   c. Briefly centrifuge samples.
   d. Heat on 95°C heating block for 5 minutes.
   e. Allow to cool briefly.
**Loading Gel(s)**

1. Open pre-cast gel(s) and carefully remove tape and comb.
2. Rinse gel(s) thoroughly with ddH2O.
3. Insert gel(s) and spare plate, if necessary, into gel running apparatus.
4. Lock securely into place.
5. Add sufficient MOPS buffer (cool) to the upper and lower chambers.
6. Add 4µL molecular weight marker to at least one well per gel using gel-loading tips.
7. Add 10µL prepared sample to the well using gel-loading tips.

**Running Gel(s)**

1. Place the top of the gel-running apparatus onto the bottom chamber.
2. Set the power supply to 200V, 1 hour.
3. Start the run by pushing the “run” button.

**Viewing**

1. Fixing
   a. Once the run is finished, turn off the power supply and remove the gel(s).
   b. Prepare fixing solution.
   c. Carefully pry open gel cassette(s) using the gel knife.
   d. Place gel(s) into basket.
   e. Add required amount of fixing solution and allow gel(s) to shake for 10min @ RT.
2. Staining
   a. Add prepared Coomassie Blue (10mL per gel) to gel(s) in fixing solution.
   b. Allow gel(s) to continue shaking for 3-16 hours.
3. De-staining
   a. Remove the staining solution by decanting.
   b. Add 200mL ddH2O.
   c. Shake the gel(s) for at least 3 hours or until stain is sufficiently reduced.

4. Imaging
   a. Turn on light box of Gel Imager,
   b. Gently place gel on light box (one at a time), smooth out any bubbles,
   c. Manually adjust focus and aperture closure of the camera,
   d. Acquire image of the gel,
   e. Adjust exposure time and contrast as needed,
   f. Save modified image on a jump drive as both a jpg and a tif file.

Solutions:

Running Buffer

- Add 950 mL of ddH2O to flask
- Add 50 mL 20X NuPAGE MOPS SDS Running Buffer
- Store @ 4ºC

   ✔ Running buffer may be recycled up to 3 times

Fixing Solution

<table>
<thead>
<tr>
<th>FIXING SOLUTION</th>
<th>1 GEL</th>
<th>2 GELS</th>
<th>3 GELS</th>
<th>4 GELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water (mL)</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>Methanol (mL)</td>
<td>30</td>
<td>60</td>
<td>90</td>
<td>120</td>
</tr>
<tr>
<td>Acetic Acid (mL)</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>