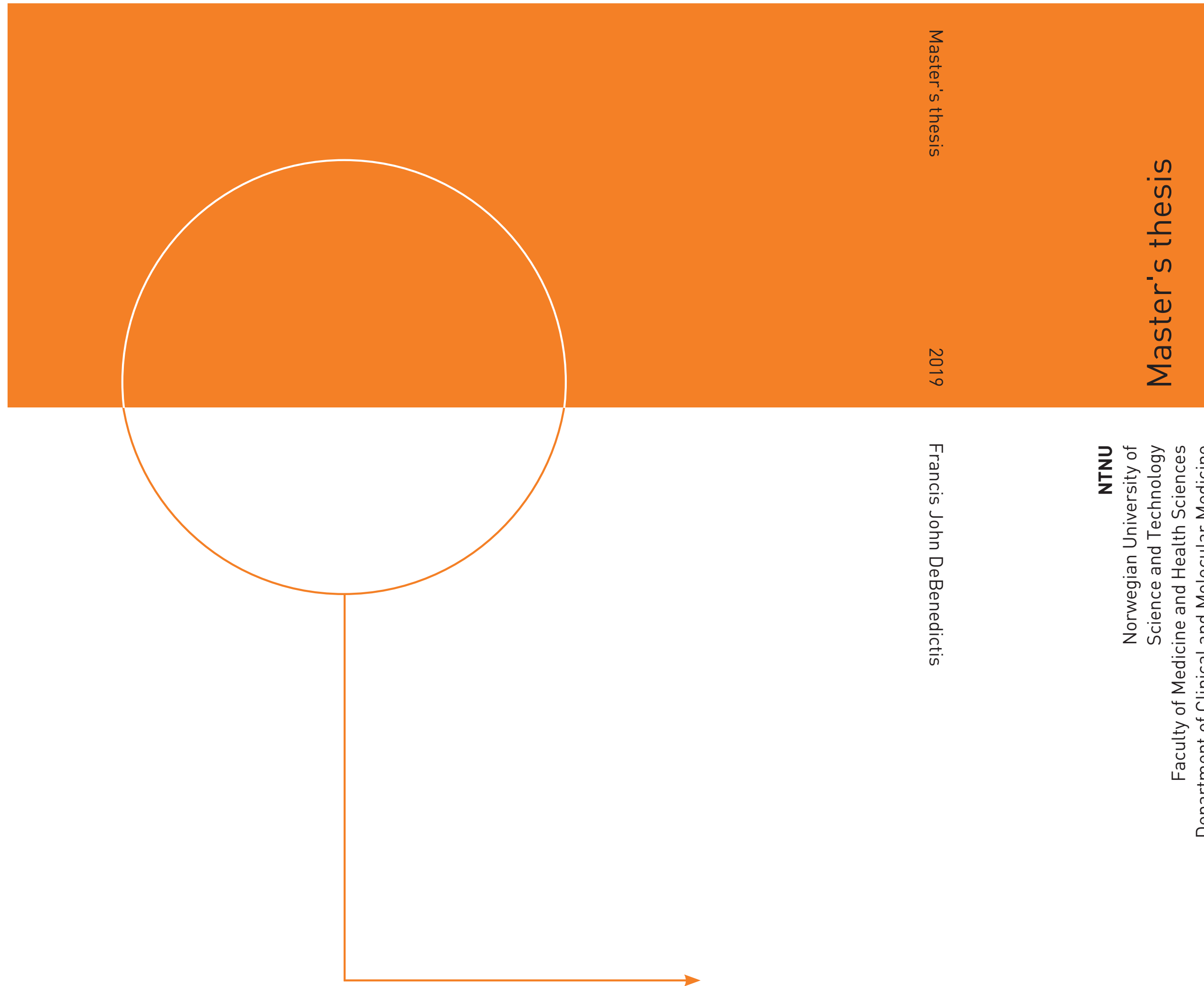

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An Agile Approach to Novel Recombinant Antithrombin Production for use in Biologics Development

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Molecular Medicine
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Abstract

Background

Sepsis is a deadly medical condition caused by a runaway inflammatory response to systemic infection in which the release of extracellular cytotoxic histones has been linked to disease progression (1-3). Heparin has been found to bind to and inhibit histone-mediated sepsis progression and cytotoxicity (4), but medical heparin is an anticoagulant and increases the risk of haemorrhaging in haemostatic factor depleted sepsis patients. Antithrombin III (AT) can be used to separate unfractionated heparin into an anticoagulant fraction and a non-anticoagulant fraction. Non-anticoagulant heparin could be greatly beneficial for neutralizing extracellular histones in sepsis patients without increasing haemorrhagic risk, thereby increasing the survival rate of septic patients without the potential for unintended lethal side effects (5).

Scope

The scope of this project is to develop a production method for recombinant human AT in a prokaryotic expression vector. The primary goal is to assess the viability of prokaryotic recombinant AT production for medical use in purifying non-anticoagulant heparin. The secondary goals include the assessment of a novel cell-free production method in contrast to the prokaryotic expression and establishing the identity of the purified protein through biochemical means such as mass spectrometry and plasmon resonance analysis. These secondary goals were established to be conducted once the primary goal had been satisfied.

Methods

A pET30a(+) plasmid carrying a human antithrombin III insert was amplified in DH5α cells and transformed into a BL21(DE3) pLysS expression host. Recombinant protein production was induced with IPTG, harvested, purified with either Ni-NTA HisTrap column and/or Heparin-Sepharose column. Purity was assessed with Coomassie Brilliant Blue staining and Western Blot, and functional activity was determined by anti-IIa activity assay. Results were analysed and the process was reviewed for sources of error or improvement. Recommended changes were introduced to the protocol and tested in a subsequent batch production cycle. Each batch cycle further refined the process and advanced the protocol towards the completion of the primary goal: production and purification of recombinant AT.

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INTRODUCTION

Sepsis is a life-threatening condition which arises from a dysregulated inflammatory immune response to infection or injury which leads to organ damage and impaired blood homeostasis (6). Mortality from sepsis ranges from 30% to as high as 80% depending on the severity and source, and there are approximately 19 million cases of sepsis each year worldwide (7). Early diagnosis and therefore rapid treatment are critical in improving patient outcomes. In murine models, extracellular histones have been found to be major mediators of sepsis progression and mortality (1, 3). Histones are normally sequestered in the nucleus but can be released into circulation during apoptosis, necrosis (8), and possibly most relevantly NETosis (9, 10). NETosis is a process by which, during infection, activated neutrophils can form neutrophil extracellular traps (NETs). These NETs are extracellular fibre matrices of DNA and globular proteins which bind pathogens and, with dying cells, release histones into circulation. These circulating histones are directly cytotoxic by forming pores in cell membranes and activating the NLRP3 inflammasome response (11-13). The NLRP3 response causes caspase-1-dependent cytokine secretion (IL-1B and IL-18) and begins the runaway inflammatory response central to sepsis progression (14, 15). Additionally, these histones can upregulate the NF-kB and MAPK pathways resulting in increased endothelial destruction, thrombin generation, clotting, and microvascular leakage leading to microthrombi formation and coagulation dysregulation. This dysregulation in haemostasis, and the subsequent depletion of haemostatic factors known as consumption coagulopathy, can directly cause tissue injury and hypoxia, progressing the severity of sepsis and organ damage. The consumption coagulopathy following systemic inflammatory response puts sepsis patients at increased risk of internal haemorrhaging following the increased systemic microthrombi formation. Histones, therefore, are a clear target for mediating sepsis progression and injury. Heparin has been identified as an agent for neutralizing circulating extracellular histones to slow the runaway feedback cascade (4, 5). Heparin, a highly negatively charged polysaccharide, can bind to positively charged circulating histones and can inhibit their cytotoxic and inflammatory generating effects in a non-anticoagulatory manner (16). Heparin can be separated into two forms by fractionation based on molecular weight. Low molecular weight heparin does not produce such significant anticoagulation effects (17). In addition, low molecular weight heparin has been shown to bind to and therefore mediate histone effects in sepsis. This shows non-anticoagulant inducing low-molecular weight heparin to be a clinically important biologic agent for sepsis treatment.

Heparin is a naturally occurring polysaccharide which binds to antithrombin III (AT) *in vivo* to increase ATs rate of thrombin inhibition over 1000-fold. AT is a 432 amino acid long plasma serine

protease inhibitor that primarily acts to inhibit the activities of the proteases thrombin and Factor Xa *in vivo* to prevent coagulation. It is a glycoprotein mainly produced in the liver with 4 available glycosylation sites. The two forms of AT, alpha and beta, differ in glycosylation levels, with the fully glycosylated alpha form comprising the majority in blood plasma. The beta form contains a single un-occupied glycosylation site which increases its affinity for the cofactor heparin. AT is an intrinsically unstable protein that can inhibit its target proteases by offering a pseudo-substrate to the protease as "bait." The proteases take the pseudo-substrate bait and are destroyed in what has been described as a "mousetrap mechanism." Through affinity-chromatography, it is possible to remove the high affinity AT-binding heparin, which has a relatively high anticoagulant activity, from the non-anticoagulant heparin (18). Thus, allowing recovered non-anticoagulant heparin to be used in treatment of sepsis without unduly increasing haemorrhagic risk to the patient.

The production of recombinant human proteins in different expression vectors has become a well-defined field in biotechnology. The available options for the production of these proteins ranges from baculoviral transformed immortalized human epithelial cells to the simple prokaryote *E. coli.* Each expression vector comes with a variety of benefits and drawbacks. For example, some enzymes require the addition of certain chemical groups post-translation to remain efficacious. Immortalized eukaryotic cell lines, such as the Chinese hamster ovary line, are able to provide these complex post translational modifications distinct to eukaryotes. However, eukaryotic cell lines have large nutrient, space, and physical requirements to maintain growth and protein production when compared to the relatively simple needs of bacteria like *E. coli*. The use of bacteria like *E. coli* for protein production is a simple procedure involving the transformation of the bacteria with the recombinant genetic material and growth in simple volume batches ranging from 100mL to over 4,000L in large scale corporate manufacturing plants. The drawback that comes along with this ease of use is that bacteria are unable to provide the post-translation modifications necessary for a large part of human enzymes to remain functional. AT however does not require the post-translational glycosylation found *in vivo* to remain effective, and therefore can be produced, theoretically, in a prokaryotic expression vector. Each recombinant protein expression requires tailoring of the production process to maximize protein of interest yields. No two strains of bacteria are alike, nor any two proteins. For this reason, the ability to successfully produce a new protein of interest in a given expression vector must be assessed, and the production method revised to overcome the unique challenges which present themselves in every new production.

To possibly create a more effective treatment for sepsis it is necessary to first develop a repeatable methodology for effectively generating human antithrombin to separate anticoagulant

heparin from non-anticoagulant heparin. A verifiable, repeatable methodology for producing recombinant AT will allow for the quality production of medicine grade non-anticoagulant heparin, which may be used to save lives.

METHODS

DH5A PLASMID AMPLIFICATION

Transformation of competent DH5α cells with customized pET30a+ vector containing recombinant human AT insert was performed by heat shock. The heat shock method utilizes rapidly increasing temperature to create pores in the plasma membrane while a calcium rich environment overcomes electrostatic repulsion of the plasmid from the membrane, allowing the plasmid to enter the cell through the temporary pores. DH5α cells were chosen for their *recA1* and *endA1* mutations improving insert stability and the ability to conduct blue/white screening for cells which contain the gene of interest. The vector, Fig. 1, carried the AT insert, with a C-terminal heptahistidine tag (6xHis), between a 5'NdeI and 3'XhoI restriction site and contained a kanamycin resistance control selector. The cloned plasmid was purified by Qiagen MidiPrep kit and verified by Sanger sequencing performed by the Clinical Genetics laboratory of Maastricht University. After purification the vector was cloned into competent Bl21(DE3) pLysS *E. coli* for expression. This work was performed by, and thanks is given to, undergraduate researcher Femke Witsel.

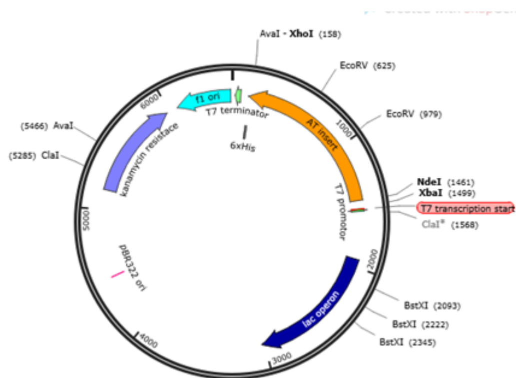

Figure 1. Vector map of pET30(+) plasmid with AT insert with C terminal 6xHistag.

EXPRESSION

Colonies of transformed BL21(DE3) pLysS cells were grown overnight on LB agar plates with kanamycin (34ug/mL) and chloramphenicol (50ug/mL). Kanamycin is used as a selective agent to remove bacteria which do not contain the vector containing plasmid and chloramphenicol is used to maintain the

pLysS plasmid containing the T7 lysozyme. Single colonies were selected and aseptically transferred, in successive quantities, to 1L LB broth cultures containing the same antibiotics and grown to an Optical Density at 600nm (OD_{600}) of 0.6 in 37°C with shaking; measured with Cary 50 Bio UV-Visible Spectrophotometer. This OD_{600}, measured with a 1mL sample, is approximately 4.8 x 10^8 cells. Once the culture reached the desired OD_{600} Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM to induce recombinant protein production. IPTG allosterically binds the tetrameric lac repressor and induces the lac operon T7 RNA polymerase (T7 RNAP) transcription. T7 RNAP concentration then increases and overcomes inhibition from the pLysS T7 lysozyme, allowing transcription of the gene of interest to proceed. Cultures were then incubated overnight at 28°C with shaking. This lower temperature was selected to decrease inclusion body formation during recombinant protein production. The expression host Bl21(DE3) pLysS was chosen for its easy protein induction ability with the T7 polymerase under control of the lac UV5 promoter but suppressed basal target transcription controlled by the T7 lysozyme (pLysS). Additionally, the strains lack of protease's lon and OmpT improves protein of interest (POI) yields.

Harvest

After overnight induction, 1L cultures were centrifuged at max speed (3428 rcf), 4°C, for 30 minutes using Eppendorf® 5804 R centrifuge in pre-massed centrifuge canisters. The supernatant was decanted, and the resulting pellet was massed and resuspended in appropriate chromatography binding buffer. The suspension was frozen and thawed to rupture bacterial cell walls and additionally sonicated five times at 20 kHz for 30 second intervals with 30 second cooling intervals in between. Afterwards, the suspension was centrifuged a second time at 3428 rcf for 30 minutes to further remove cellular debris.

Purification

Two different chromatography methods were chosen to purify the recombinant AT for ease of use and accessibility. First, an immobilized metal ion affinity chromatography (IMAC) HisTrap column from GE Life Sciences utilizing nickel-nitrilotriacetic acid (Ni-NTA) covalently bound to agarose beads as the capture agent for the 6xHis tag. Second, a HiTrap HP heparin-sepharose column, also from GE Life Sciences, was used which utilizes heparin as the solid phase affinity agent and eluted the bound protein through increased ion concentration. The HisTrap chromatography methods utilizes the 6xHis tags ability to chelate the nickel ions in the column, thereby separating out proteins which do not contain the 6xHis tag and cannot bind the Ni-NTA substrate as strongly. The heparin-sepharose affinity chromatography

capitalizes on ATs affinity for heparin as a cofactor. The recombinant AT binds to the immobilized heparin as other proteins wash through and is released from the column with increasing ion concentration.

Initial purification of cell lysate was done using a Ni-NTA column which was regenerated before use. Crude cell harvest dissolved in binding buffer containing urea and imidazole was run over the Ni-NTA agarose column, then washed with a low imidazole washing buffer to remove protein that had not bound to the Ni-NTA. Bound protein was then eluted from column by high imidazole elution buffer which overcomes the interaction between the 6xHis tag and the metal ions.

Chromatography was initially performed using the AKTA Prime Plus automatic chromatography system. However mechanical failures in the machine loading pumps and suspected POI loss in loading procedures prompted the switch from an automated system to a more manual chromatography protocol utilizing a peristaltic pump and hand sampling of flow through, wash, and elution fractions for protein content on a separate NanoDrop microvolume spectrophotometer.

IMMUNOBLOT

Immunoblot assay was utilized for rapid detection of AT in purification samples. A 2uL sample is applied to a polyvinylidene difluoride (PVDF) membrane and dried. The film is then blocked with 5% milk in tris-buffered saline with Tween20 solution (TBST), washed, and probed with primary and secondary antibody in the same manner as Western Blotting. Primary antibody was mouse monoclonal anti-AT from Cloud-Clone Corp. (A20180502518) and secondary antibody was polyclonal sheep anti-mouse HRP conjugate from Dako (P0447). The chemiluminescent HRP substrate was WesternBright ECL from Advansta (K-12045-D20).

PURITY AND IDENTITY

Purity of the recovered samples were assessed with Coomassie Brilliant Blue R-250 staining after 12% SDS-PAGE. As Coomassie Brilliant Blue binds non-specifically to all proteins suspended in the acrylamide gel it is easy to visualize non-protein of interest contaminants which might remain in the finished purification preparation. Purification samples were tested for AT content by Western Blot analysis after SDS-PAGE with the same antibodies used in immunoblot detection.

ANTI IIA ACTIVITY ANALYSIS

An assay was developed to test the purified ATs functionality. Using a chromogenic substrate, Chromogenix S-2238, which releases a chromophore when cleaved by thrombin as a reporter, AT activity was assessed by depression of signal generation when compared to a reference curve. Purification

fractions were incubated with heparin and factor IIa (thrombin) at 37°C before the chromogenic substrate was added. Signal generation was measured every 30 seconds over 15 minutes in a BIO-TEK Instruments Ultra Microplate Reader EL808. The S-2238 substrate contains a D-Phe-Pip-Arg-pNA sequence which is cleaved by thrombin to release the colour changing pNA. The reaction with antithrombin can be summarized as:

Heparin + AT -> [AT Hep.]

[AT Hep.] + [IIa (excess)] -> [FIIa-AT-Hep.] + [residual FIIa]

[FIIa (residual)] + S-2238 substrate peptide -> pNA (colour change)

An activity of ΔA/min = 0.05 (at 37°C) is obtained by using 0.1 mM substrate with an excess of available thrombin for activation. Changes in the rate of colour formation, specifically a depression of the rate, indicates inhibition of the thrombin activity.

PRODUCTION PROCESS DEVELOPMENT

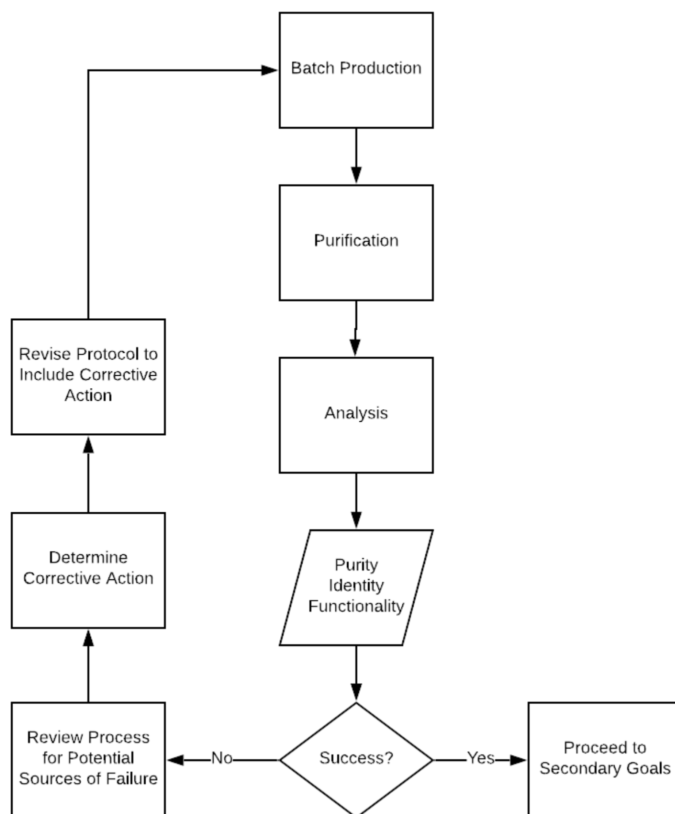

Figure 2. Process Map Diagram. This process map depicts the workflow for the batch production cycle and the incorporation of corrective actions.

This process map, Fig. 2, visualizes the process development approach used to develop and refine the production methodology of this project. The initial protocol was developed after review of current literature involving recombinant production techniques and training on tools which were selected, i.e. standard HisTrap protocol from General Electric Life Sciences. Each batch was an individual culture growth and induction. After each successive batch, success criteria (identity and purity) were analysed and presented to peer group (including supervisor and department PhD candidates) for discussion, and possible improvements to the protocol were identified. Each possible improvement was then incorporated in the next production round and results were analysed for outcome improvement defined by product yield and purity. In total 10 significant production batches were produced, and several intermediate revisions and tests occurred between batches.

The project progression can best be understood by visualizing the project process through its plan and schedule (Chart 1). These activities are major components of the development and translation cycle for a pharmaceutical product or component. Throughout the project lifecycle I utilised internationally recognized project management techniques to plan and schedule the work and measure project schedule performance. I started with the baseline Project Management Institute (19) project management model and tailored it to fit the specific needs of the project. These project management practices, combined with actual project accomplishments, provided schedule performance measurements and quality assurance measures to aid in creating a quality product by design through verifiable, repeatable, documented process'.

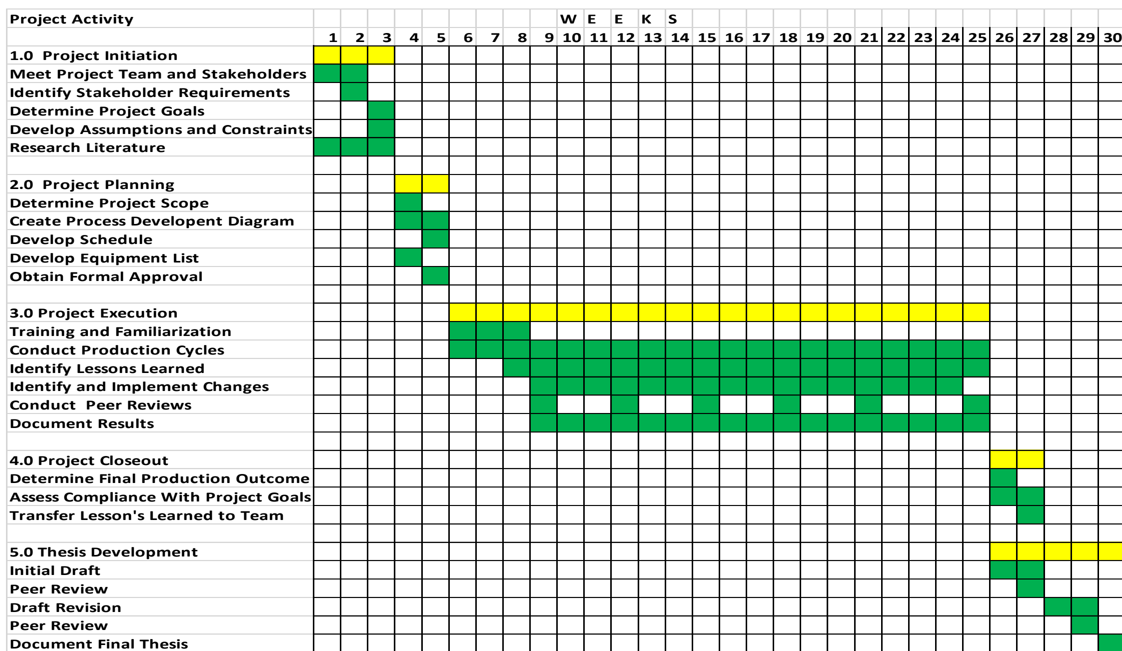

Chart 1: Project Plan and Schedule outlining project processes in their timeframe.

The project initiation phase develops the business case for why the project is useful and necessary to those who are impacted by its outcome-the stakeholders. The stakeholders then describe what is required for a successful project and those requirements are translated into goals for the project team to accomplish. The described potential project is evaluated for assumptions that were present in its initiation and the constraints that may limit the project, such as time, money, and resource availability. This transitions into the project planning phase where the description of how the project will be accomplished is created, known as a project management plan. This plan includes a scope, defining what is part of the project and what is not, the schedule to evaluate when work will be done, and includes final formal approval to begin work in accordance with the plan. Once the project is formally allowed to proceed work begins in the execution phase. The first step is training on relevant equipment and procedures. A few practice trials usually are involved so that relevant operating lessons can be learned hands-on. In this project the execution phase was iterative, meaning once one cycle of work was completed it was evaluated and a new cycle was planned with the same methodology incorporating lessons learned. These development cycles continue until either a limiting constraint is reached, such as the end of allowed time period, or until the primary goal is achieved as determined by the pre-set requirements. At the project closeout the team reviews and confirms the work is complete according to the requirements developed from the planning phase. This project process is able to create quality by design in the final product by creating a production methodology which can be verified through each repetition. Since the product of this project, antithrombin, is intended for the development of biologics for medical use quality by design is an essential aspect of the methodology. The produced at would not be useable in subsequent projects for biologics development if it was not possible to verify the product identity and purity. The best way for a business to ensure those two components is through developing a method which reliably produces the same result each time.

RESULTS

PROCESS OPTIMIZATION

Cycle	Change from previous Cycle	Failure	Corrective Action Identified	Notes
1	(Not applicable)	Failure to bind to Ni-NTA column	Reduce imidazole content in binding buffer from 200mM to 100mM.	Cycle repeated twice. Initially operator error was suspected.

2	Reduced imidazole in binding buffer.	Failure to bind to Ni-NTA column	Further reduce imidazole content to 2mM.	Immunoblotting revealed some AT adherence to column, but majority continued to flow through.
3	Reduced imidazole in binding buffer.	Failure to remove cellular contaminants	Increase washing step from 5 column volumes to 10 column volumes.	Histidine rich background protein contamination is a common occurrence in HisTrap purification. (20)
4	Washing step increased to remove weakly bound contaminates. Additionally, change to elution buffer done in 1% gradient increments instead of 100% switch.	Contaminants continue to co-elute with protein of interest over total elution gradient.	Removal of HisTrap chromatography column from protocol.	Background protein contaminants presenting problem for purification preparation. Additionally, high imidazole content in elution buffer requires dialysis removal before following steps.
5	Heparin-sepharose chromatography as primary purification step.	Failure to bind to heparin-sepharose	Sodium ion concentration too high in binding buffer.	Initial sodium concentration determined by manufacturer.
6	Sodium concentration reduced from 200mM to 20mM.	Protein Degradation	Not applicable	Tolerance of column for urea was unclear. Binding buffer contained no urea for protein solvation.
7	Not applicable	Inclusion bodies from harvest not properly dissolved. Low protein content in solution.	Use urea in binding buffer to dissolve inclusion bodies and dissolve protein into solution.	Additional clarification obtained from manufacturer indicating tolerance of column for up to 8M urea.
8	Addition of urea in binding buffer.	None	Not applicable	9.5mg yield

Chart 2: Table of Documented Changes

An initial protocol was crafted from relevant literature review and collaboration with peers to tailor the workflow to the application at hand. This protocol incorporated HisTrap Ni-NTA chromatography followed by HiTrap heparin-sepharose chromatography and involved dissolving the bacterial crude cell harvest in a high urea, medium imidazole buffer for use in the HisTrap. Initial production cycles did not produce positive purification results from HisTrap chromatography, though induction was successful as demonstrated by immunoblotting crude cell harvest extracts for POI. Two additional cycles were run, each time reducing the imidazole content first, from 200mM to 100mM and then from 100mM to 2mM. It was hypothesized that the 200mM imidazole, which was supposed to prevent nonspecific binding, was inhibiting 6xHis tag binding to the Ni-NTA substrate. The third cycle successfully accomplished AT binding

to the column but did not result in a purified POI as elution fractions contained large amounts of cellular contamination shown by Coomassie Brilliant Blue. The fourth cycle incorporated a longer washing step to remove weakly bound contaminants and a gradient buffer change to elution buffer as opposed to a single step switch to attempt to identify where most contaminates would elute vs POI. The resulting elution profile did not yield useful results as the protein appeared to elute non-specifically over the gradient. It was then decided to remove the HisTrap chromatography step from the protocol and attempt heparin-sepharose chromatography as the primary purification step. This decision was made due to the consistently poor purity obtained from the HisTrap chromatography and the need for intermediate dialysis between HisTrap and heparin-sepharose purification to remove the imidazole content, which required 48hours in which the AT began to degrade significantly.

The subsequent heparin-sepharose purification was also unsuccessful. The AT was unable to bind to the column and immediately flowed through. This was determined to be caused by high sodium content in the binding buffer inhibiting interaction between the AT and heparin substrate. Dialysis was performed to remove the salt content but due to the time delay, the AT appeared to degrade and was not easily detectable after re-purification. The final purification cycle incorporated all previous lessons learned in the finalized protocol and resulted in approximately 9.5mg of purified protein of interest measured by NanoDrop A280 reading (Fig. 3). Coomassie Brilliant Blue visualization displays little cellular contaminants and POI indicated at correct molecular weight (Fig 4). Western Blot analysis shows very little protein loss in the column flow through and the initial washing step. Two bands were detected in elution fractions compared to single band in the starting material, possibly a molecular weight change as a result of the addition of heparin from the sepharose column, which depending on sequence can be between 3 and 30kDa. Functionality assay with anti-IIa showed almost total functionality retained and supported A280 protein concentration report (Fig. 5). The final protocol developed for batch production of recombinant antithrombin is in the appendix.

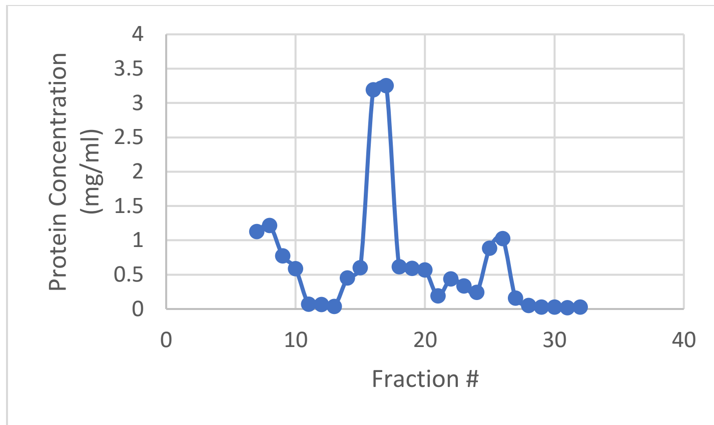

Figure 3. A280 Elution profile from NanoDrop microspectrophotometer. Each fraction is 1.5mL elution from heparin-sepharose column. Fractions 1 through 10 are from the wash step. Fractions 10 through 30 are elution fractions.

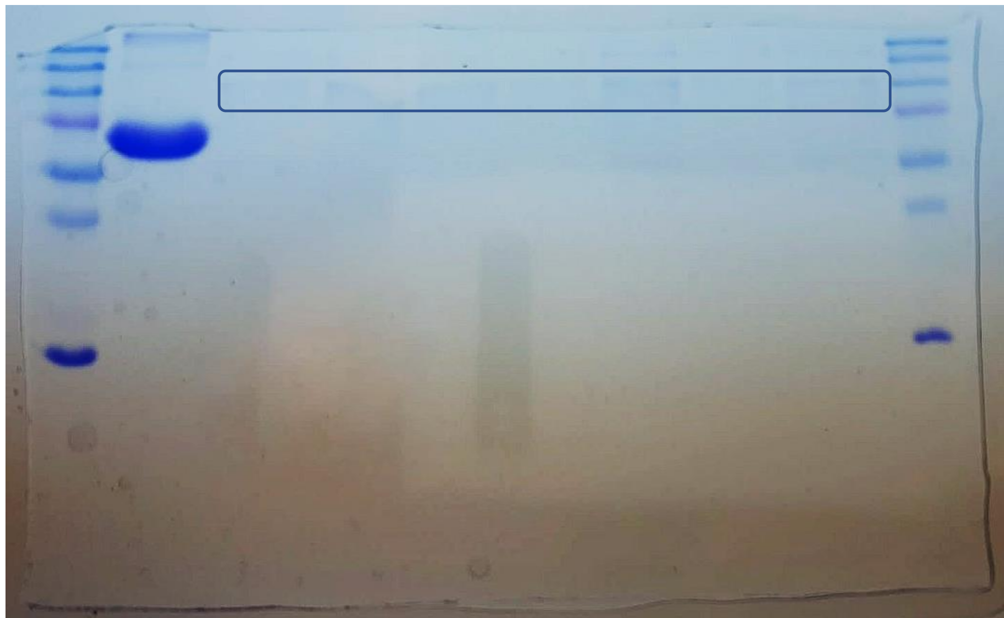

Figure 4. Coomassie Brilliant Blue staining after SDS-PAGE. Lane 1 is a positive bovine AT control. Successive lanes are purification fractions from heparin-sepharose column. The Coomassie Brilliant Blue shows relatively little bacterial contaminants and a band appears at the expected length which is slightly larger than the bovine control.

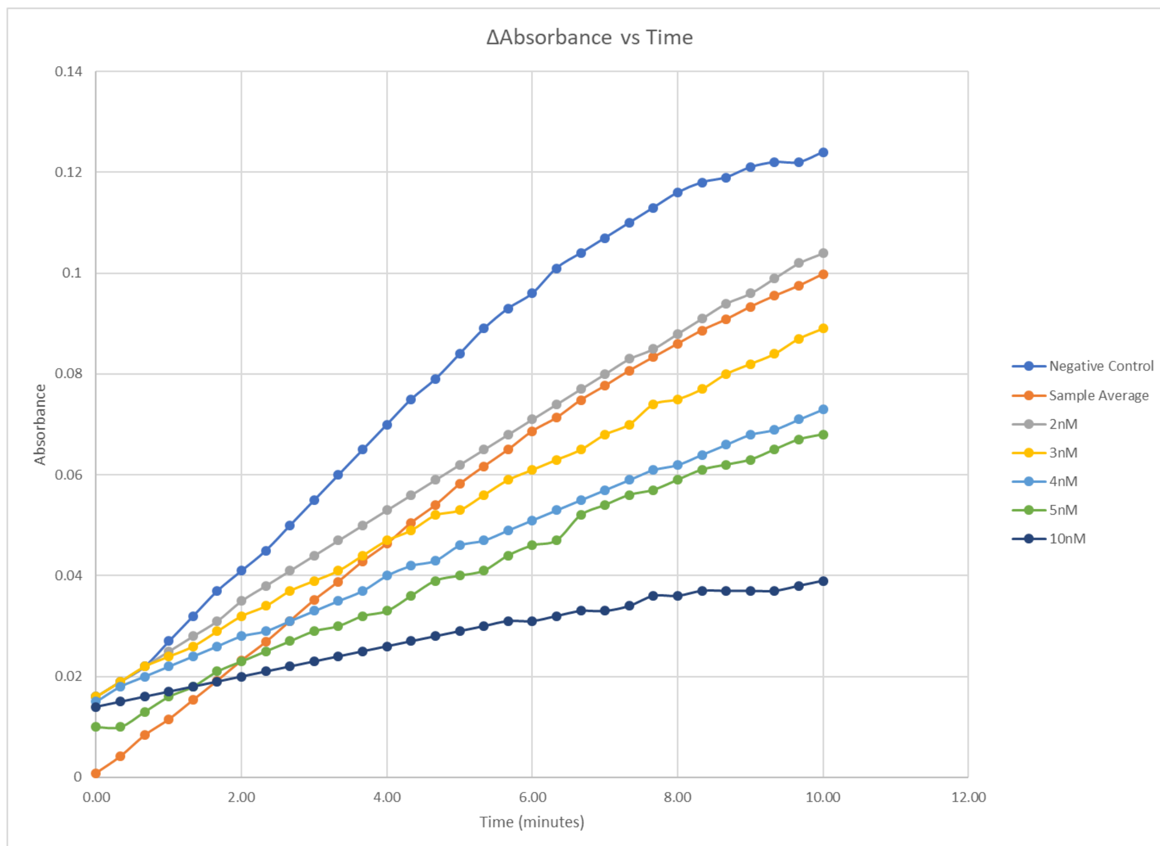

Figure 5. Purified AT activity as measured by ability to inhibit thrombin cleavage of a chromogenic substrate. Elution fractions were pooled, and salt and urea were removed by dialysis. Initial reaction velocity was higher than controls. This may be due to non-glycosylated ATs higher affinity for heparin which increase's ATs rate of reaction.

Discussion

The goal of this research was to develop and assess the viability of a prokaryotic production system for human antithrombin III with the intention of utilizing purified antithrombin in the capture of heparin, specifically non-anticoagulant heparin, for use as a possible treatment in sepsis. The recombinant AT was desired to act as a component piece in the further purification of non-anticoagulant heparin. By preferentially binding high anticoagulant heparin fractions AT would be used to isolate the clinically relevant heparin for use in the treatment of sepsis. The scope of this project was to refine and optimize the production method in the defined time frame and, if possible, assess the benefits and drawbacks of both the "tried and true" prokaryotic expression host (if it proved functional) versus a pioneering cell-free method. Additional secondary project goals were established to attempt a second novel production method utilizing a cell-free methodology and undertake various structural analysis of the purified samples to begin a rigorous definition of the product so that it may be acceptable to regulatory bodies in the use of biologics development. This project was successful in accomplishing the primary defined goal:

assessment of the viability of a prokaryotic production method for human antithrombin III was completed. However, the projects secondary success criteria were not met, for several reasons. Primarily the project team decided to prioritize further achievement on primary goal. This led to an iterative development lifecycle with no clear ending, and therefore secondary goals of the project were deferred in favour of pursuing more rigorous refinement of the primary production method in the prokaryotic vector. Eventually, the decision to remain focused on the primary project goals resulted in the highest yield batch of the project, but the results cannot be compared to alternative methodologies nor can the identity of the product be confirmed or analysed by such rigorous means as mass spectroscopy.

In making a final assessment of the projects primary goal – the viability of a prokaryotic production system – it is useful to consider the framework in which the product will be used. The AT itself is desired to be a component piece of a system intended to produce medical grade unfractionated heparin for use in treatment of sepsis. Therefore, it is necessary that the AT preferentially bind unfractionated heparin as efficiently as possible to maximize yields. Also, due to ATs short half-life (approx. 70 hours) the viability of the production method should be assessed from a timeliness perspective. Finally, for use in medical grade biologics production the purified AT must meet certain regulatory controls for purity and identity. The protocol to produce AT as developed here created a quantity of final product, whether this quantity is of commercial value when compared to its cost basis is difficult to determine in simple "milligrams produced vs time/money spent" terms and is better left to the pharmaceutical company which seeks to employ the method. The efficacy of the recombinant AT in selectively binding unfractionated heparin has been attested to in literature, and the scientific basis is sound. As the recombinant product here is produced in prokaryotes, and therefore not subjected to post-translational modification, it is produced in an entirely un-glycosylated form. This form aids in the selection of the beta-heparin binding efficiency as there is no charge interactions from the more glycosylated beta-heparin. If it was determined that this production method was viable for commercial use another project would need to be undertaken to determine the reliability and efficiency with which the recombinant AT binds the fraction of heparin desired. At the benchtop scale the timeliness of the procedure can be deceptive when considering a more functional pharmaceutical production. One 1L production batch with this protocol takes approximately 48hours (20 man-hours) from culture seed to purification collection (not including purity assays). Increasing volumes of production would not significantly increase production cycle lengths, and additionally considerations of batch-feed bioreactor cycles as opposed to batch-batch cycles could create a sustainable production method for generating fresh AT within the 70hour viability window. However, secondary steps for utilizing

the produced AT, such as binding it to a stationary phase column gel matrix, were not in the primary scope of this project and therefore the time needed to complete those steps is unknown. It would be an oversimplification to simply state, at present, that the production protocol developed from this project is either “good/viable” or “bad/not viable” as the variable relative to those determinations are not known and not part of the scope of the project to reveal. However, it is possible to reflect on whether the developed protocol is as efficient as it could be on its own. To that end, the lessons learned in the 7 months spent developing it have been very revealing as to the nature of recombinant protein production process optimization. It is hard to determine whether something is close to the goal without crossing the goal line. Therefore, I believe the process is close to being as complete as possible but (a few) successive development cycles would still be beneficial to concluding definitively.

This data should be held in comparison to alternative approaches to the production of the desired protein. In this project a secondary goal was established to compare the prokaryotic production method with a novel cell-free expression system. While the prokaryotic production can be considered novel on its own regarding this protein of interest it is a well-established production method in the industry and is supported by years of research. Less well established, and less well defined, is cell-free expression systems which utilize the harvesting of protein producing molecules from simple *E. coli* cultures and processing of those materials to obtain only the necessary components of protein production. Essentially “skipping the middleman” of protein production in *E. coli* by harvesting the necessary cellular machinery and utilizing it in solution. This method is preferable as it circumvents the prokaryotic expression systems need to create life-maintaining products which in this context are considered as contaminants or unwanted by-products of our production cycle. Cell-free expression consists of two phases - production of the cellular components for use in POI production and the production of POI in a subsequent phase. To generate the necessary cellular components an *E. coli* expression vector culture is grown to confluence and then harvested to obtain ribosomes, polymerases, tRNA, and subcellular components while removing cellular debris such as the bacterial genome, cell walls, and other cellular organelles not involved in protein production. The relevant components are then transferred to a solution containing the necessary molecular cofactors and a plasmid containing the gene for the POI is added to the solution. The harvested cellular machinery goes to work creating the POI from the provided genetic material until the available substrates are depleted. The solution can then be easily purified with available established methods such as Heparin-sepharose column purification, which would theoretically result in significantly increased purity as the ratio of product to by-product is increased by magnitudes since unwanted cellular

components are no longer transcribed. Unfortunately, this method was not assessed in this project because the criteria for moving from the initial method, prokaryotic system, to the novel method, cell-free, was not defined prior to project beginning.

The major outcome of this research was the development of a repeatable production method for recombinant antithrombin in a prokaryotic expression vector. While this may sound facile at first the necessary refinement of the process is time consuming and laborious. As previously displayed in a process flow diagram a specific procedure was used to develop the protocol over time. This process enabled the evaluation of the protocol at each step so that each hurdle was overcome as it was identified through practice. However, when a particular process in the total protocol failed it was not always immediately clear what corrective or preventative actions were required to overcome the hurdle. For example, early in the project soluble AT in crude cell extract was not properly binding the Ni-NTA column. At first it was believed that possibly the 8M urea content in the binding buffer was impacting the 6xHis tag binding capability to the substrate. A batch cycle was run with 2M urea, but identical results were obtained as the previous 8M. So, another option was explored, returning the protocol to the 8M urea state but with lower imidazole content in the binding buffer to reduce binding inhibition of the target protein. Eventually, the Ni-NTA chromatography proved unreliable and was removed from the protocol all together. But for a significant amount of time it had remained as the leading purification method for obtaining the POI and significant time was spent in optimizing the procedure until it was decided that overall, it was not a viable solution. Since the key stakeholders of the project intend to use the methodology in a larger scheme to produce medical grade heparin to treat sepsis it is an important finding that Ni-NTA is *not* an ideal part of the purification protocol. While time spent discovering how best not to accomplish something may appear, at first glance, unproductive, it is an essential part of developing a rigorous biotechnological production method. After exhaustive testing it can be reasonably determined that Ni-NTA does not represent an ideal solution to the problem of purifying recombinant AT from a complex mixture and a new approach can utilized. In this case, heparin-sepharose chromatography proved at once more suitable to the task. Overall the primary goal of this project, the development and assessment of a production method for recombinant antithrombin, was thoroughly explored. More than 10 production cycles with numerous intermediate assessments contributed to a deep understanding of the technical constraints in producing a viable human protein from *E. coli* cultures which could be isolated in very mild solutions and used for other applications. The natural progression of this project would be to evaluate the cell-free

expression system outlined herein and asses the hypothesis that a cell-free expression system would eliminate the final challenge of the prokaryotic method explored here.

APPENDIX

PROTOCOLS

E. COLI TRANSFORMATION PROTOCOL

1. For DH5α host strain, prepare vector by adding 20 mL miliQ water and heating for 40 min at 42°C.
2. Add 0.5 mL miliQ (control), 0.5 mL undiluted plasmid stock (25 mM) and 0.5 mL of 100x diluted plasmid to 100 mL DH5α and put on ice for 35 min.
3. Incubate at 42°C for 1min and put directly on ice for 5 min to shock.
4. Shake strain at 37°C, 275 RPM for one hour in a volume of 1 mL LB with 37ug/mL kanamycin.
5. Centrifuge for 3 min in 4°C at 3478 rcf and carefully decant supernatant.
6. Redissolve pellet and plate bacteria on LB plates with appropriate antibiotic

HISTRAP NI-NTA CHROMATOGRAPHY PROTOCOL

1. Regenerate pre-pack GE HiFlow column with 10mg of nickel sulfate dissolved in 10mL miliQ water.
2. Equilibrate column with 5 column volumes (CVs) of Binding Buffer.
3. Run crude cell harvest over column.
    a. Collect flow through.
4. Wash column with Binding Buffer to remove unbound proteins until A280 reading nears zero. Continue for 5 more CVs.
    a. Collect a sample.
5. Elute column with Elution Buffer until A280 measurement reaches zero.
    a. Collect elution fractions in 1.5mL LoBind protein tubes. Keep cold!
6. Wash column with 10CVs dH20.
7. Store column in 20% ethanol.

HEPARIN-SEPHAROSE CHROMATOGRAPHY PROTOCOL

1. Wash pre-packed HiTrap HP heparin-sepharose column with 5CVs dH20.
2. Regenerate with 10 CVs of Regeneration Buffer.
3. Wash with 5 CVs dH20.
4. Wash with 5 CVs of Binding Buffer.
5. Blank NanoDrop with Binding Buffer.
6. Load Sample onto column.
    a. Collect flow through.
7. Wash with Binding Buffer until A280 reading = 0 to remove unbound contaminates.
    a. Collect a sample.
8. Wash with 10 more CVs of Binding Buffer.
9. Elute with 60/40 Binding Buffer to Elution Buffer solution until A280 reading = 0
    a. Collect a sample.
10. Wash with 5 CV's of 60/40 Solution
11. Elute with Elution Buffer until A280 = 0
12. Collect fractions
13. Rinse with 10 CVs dH20
14. Store in 20% Ethanol

Purification Protocol

1. From transformed BL21(DE3) stock frozen in 50% glycerol begin selective agar growth plate with 50 µg/ml chloramphenicol and 30 µg/ml kanamycin.
    a. Incubate overnight at 37°C.
2. Select one positive colony and inoculate 5mL LB starter culture with 50 µg/ml chloramphenicol and 30 µg/ml kanamycin, incubate in 37°C with shaking until $OD_{600} > 0.6$
    a. Transfer 5mL culture to 150mL culture, repeat incubation.
    b. Transfer 150mL to 1L culture.
3. When 1L culture reaches $OD_{600} = 0.6$ induce with 1mM IPTG (100 mM IPTG stock)
    a. Induce at 28°C with shaking overnight.
4. Centrifuge at 3428rcf for 30 minutes, 4°C, in pre-massed centrifuge tube.
5. Decant supernatant and mass pellet.
    a. Collect sample of Supernatant and Pellet
6. Resuspend in 10mL Binding Buffer per 1g pellet weight.
    a. Save sample of resuspension
7. Freeze resuspension completely.
8. Thaw and sonicate resuspension, 5 x 12amp for 30 second followed by 30 second rest in ice bath.
9. Centrifuge again 3428 rcf for 30 minutes at 4°C to remove remaining cellular debris.
10. Decant supernatant.
11. Load sample over chromatography column according to protocol.
12. Perform dialysis to remove urea content.
13. Run SDS-PAGE followed by Coomassie Brilliant Blue staining and Western Blot analysis.
14. Evaluate activity with anti-IIa test.

Anti IIa activity

1. Preheat 96 well plate to 37°C
2. Per Sample Well
    a. Add 40uL of 20IU Heparin
    b. Add 40uL of Sample (AT)
    c. Incubate for 1 minute
    d. Add 40uL of 1nM Thrombin
3. Place plate on preheated plate warmer
4. Add S-2238 substrate to all sample wells simultaneously with multichannel pipette
5. Begin plate read protocol
    a. Every 30 seconds for 15 minutes with shaking
6. Repeat procedure with known AT concentrations for reference curve

Reagents

Heparin-sepharose Buffers

Binding Buffer	Elution Buffer
100mM Tris	100mM Tris
10mM Citric Acid	10mM Citric Acid
50mM NaCl	2M NaCl
6M Urea	6M Urea

Ni-NTA Buffers

Binding Buffer	Elution Buffer
20mM NaHPO4	20mM NaHPO4
500mM NaCl	500mM NaCl
2mM imidazole	200mM imidazole
6M Urea	6M Urea

Anti IIa Buffer

HNBSA

HEPES	20mM
NaCl	100mM
BSA	5mg/mL

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