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A Major Qualifying Project

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Authorship

All team members contributed to all aspects of the project, as well as to each section of the paper and therefore, the group wishes to decline the option of individual authorship.

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Abstract

Blood coagulation is an important aspect of hemostasis in the human body. Under normal circumstances, the blood coagulates using two different pathways, the intrinsic and the extrinsic. The extrinsic pathway works to counteract trauma but may lead to stroke forming clots. Components for an assay were created so that an assay could be designed to test the functionality of the proteins involved in the clotting cascade: Tissue Factor, factor VII, and factor X when used in conjunction with Template Directed Assembly (TDA) on a nickel-nitriloacetic acid (Ni-NTA) derived liposome.

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Chapter 1: Introduction

Blood clotting is a complex biological process where Tissue Factor (TF), factor VIIa (fVIIa), and factor X (fX) all play a role due to the initiation of the plasma coagulation cascade upon exposure of blood to something foreign. TF, fVIIa, and fX are integral membrane proteins, making them more difficult to express at high yields. Since the proteins are integral membrane proteins, project group used liposomes to mimic the membrane structure to make the assay more accurate. Without the liposome, cleavage and processing do not happen, making the assay less exact. Although procedures have been effective for reconstituting purified integral membrane proteins into phospholipid vesicles, these procedures are painstaking, time-consuming, and expensive (Waters and Morrissey, 2006). The design team developed an enhanced assay for blood clotting, and TF/fVIIa/fXa activity specifications, which requires development strategies for cloning, expression, purification, and assay which retain full biological activity.

Traditional expression of the integral membrane proteins has been high yield in bacteria. This technique is orders of magnitude less active than the intact, membrane-anchored protein that the design team used. A study done by Waters and Morrissey also showed that they were able to restore full biological activity to the isolated ectodomain of TF. The design team based the project off of this study. Waters and Morrissey engineered a hexahistidine tag onto the C-terminus of TF and used that in combination with membrane bilayers that contained nickel-chelating lipids. That allows for soluble TF to be attached to the membrane surface and for it to be able to bind fVIIa. The resulting fVIIa-TF complex supported fVII autoactivation and fX activation.

The project team used Blue Sky Biotech's exclusively licensed Template Directed Assembly (TDA) technology to anchor the protein onto a liposome. This method is useful in mimicking the membrane bound proteins found in the human body and has been shown to approach the full biological activity of the proteins (Waters, 2006). The TDA technology is able to simulate the adhesion of proteins to a bilayer and reproduce the clotting process. The incorporation of membranes and phospholipids is necessary for studying clotting *in vitro* because thrombin produced by the clotting cascade adheres to phospholipids that make up the bilayer or cell membrane within the body.

The unmet need of the project included the cost benefit. Others have tried this before and it did not work, so if a different approach is applied, there is a large demand for the product. The product also needs to be applicable to diagnostics and drug development. These are the two largest markets for the assay and the characteristics needed include that the assay is high-throughput and that it reproduces

full biological activity. The team defines high throughput as an individual being able to analyze more than 48 samples a day. Full biological activity is defined as the activity of the proteins in the human body.

The design team followed the book *Engineering Design: A Project Based Introduction* by Clive I. Dym and Patrick Little, which outlined the five-stage prescriptive model for design. The first stage was to meet with the client to identify objectives and revise the client statement. That allowed for clarification of the problem and construction of conceptual and preliminary designs. Engineering design tools were utilized, such as the work breakdown structure, linear responsibility chart, and objectives tree to organize and track the project.

The client statement indicated the need for an enhanced assay for blood clotting that retained full biological activity. To get a better idea of the problem and client statement, the design team researched topics that were related to the client statement. Based on the research and meetings with the client, the design team established objectives that were desired in the final design. The design team wanted the final assay to be accurate, cost effective, high-throughput, user friendly, and safe. The cost of the final diagnostic and drug discovery kit should cost no more than $9000 including disposables, the project must be completed by April 2012, and the design team must express TF, fVII, and fX in a cell line. The team then used the objectives and constraints to revise the client statement. A more detailed discussion of the objectives, constraints, client statement, and project approach can be found in Chapter 3.

After defining the design limitations in Chapter 3, the design team created several conceptual and alternative designs, which can be found in Chapter 4. Chapter 5 through chapter 8 reveals how the project was conducted, how the data was interpreted, and what the team recommended including alternative solutions and a deeper research analysis. The recommendations allow for future groups to make improvements on the design. The design team met regularly with the client to both receive feedback and update the client on the status of the project, and all documentation was submitted to the client at the completion of the project to complete the final design stage.

Chapter 2: Literature Review

Tissue factor (TF), factor VIIa (fVIIa) and factor X (fX) all play a role in blood coagulation due to the initiation of the plasma coagulation cascade upon exposure of blood, making it an essential defense mechanism in conveying hemostasis (Morrissey, Fakhrai, Thomas, 1987). Because of this, these proteins often represent those that could provide valuable insight into disease processes and treatment. A major difficulty is restoring recombinant proteins to their full biological activity, while retaining desirable expression, solubility, and handling characteristics. While progress has been made, these proteins have only been expressed in cells or in test tubes in the past. With new technology, our group will incorporate the proteins into liposomes using template directed assembly (TDA). TDA allows for a better assay because it does not ignore the protein structure's polarity and allows its relationship to subunits to be seen. The project group will develop an assay that can be utilized by companies with clinical clotting assays like hemostasis diagnostics, general screening, and anti-coagulant monitoring. In addition, this type of assay can also be utilized for drug discovery to make less toxic drugs for coagulation disorders or test the effects of drugs on the clotting process without using live test subjects. Some of the hurdles that our group will overcome will be to obtain full expression of the proteins and to develop a high-throughput system. Designing a high-throughput assay to measure activity and developing a purification scheme for the cloning and recovery of tissue factor proteins is promising and possible.

2.1 Applications of the Assay

Laboratories and medical companies often utilize clinical clotting assays. An assay is a test or analysis to measure the presence and activity of a drug, chemical, or other substance. Some common applications for assays are general screening, hemostasis diagnosis and anti-coagulant monitoring.

Assays are often used for general screening. They can be used in many applications because they are multifunctional and help to determine the most efficient way to solve a problem. They are useful for testing activity, measuring trends, testing problems, quantifying amounts and many other applications in organisms or organic samples. The IFN-γ Elispot assay is an example of an assay that is used in HIV vaccine research. While it does predict and map T-cell responses, it is not ideal because it is inefficient (Hendrik, 2009). There are many assays available and while they perform a basic function, they could be improved in many ways by improving efficiency and making them high-throughput.

Hemostasis occurs after an injury in order to prevent bleeding and maintain vascular system integrity while at the same time permitting blood flow through blood vessels. Hemostasis is a combination of interactions between the blood vessels, platelets and coagulation proteins (University of

Washington, 2005). Hemostatic response produces a platelet plug, a clot that is fibrin based, many white cells deposited at the site of the injury, repair and inflammatory responses, blood flow control and maintains the integrity of the vascular system (Pasi, 1999). There are specific tests and assays currently available to understand platelet function, vessel wall function, fibrinolysis, coagulation proteins and natural occurring inhibitors (University of Washington, 2005).

Coagulation in the arteries and veins is brought on by tissue factor after an injury. There are several assays for monitoring coagulation including clot-based assays and chromogenic assays. Clot-based assays are generally used on patients that may have bleeding abnormalities. Chromogenic assays are anti-factor Xa assays that are useful in measuring levels of heparin concentrations in the plasma. Anti-coagulant assays are used to monitor therapy. Common anticoagulant drugs are warfin, heparins, and thrombin inhibitors. Warfin is used commonly for the prevention of stroke, recurrent infarction and cardiovascular issues. Heparins promote the inactivation of thrombin and factor Xa which leads to clot breakdown, but the anticoagulant response is unpredictable. Direct thrombin inhibitors work by binding directly to thrombin to block its interaction with other substrates. Each of these anticoagulant drugs are commonly used in assays (Bates, 2005).

2.1.1 Clinical Applications for Stroke Patients

A stroke is the loss of blood flow to the brain due to either blockage or a clot in the walls of a major artery or blood vessel within the brain. Blockages and clots form due to arthrosclerosis, or the buildup of fat, cholesterol, plaque or other substances inside an artery or blood vessel. There are two types of strokes: ischemic and hemorrhagic. Ischemic strokes can be either embolic or thrombotic. Embolic strokes are when the clot or plaque buildup travels through the bloodstream from its origin to a blood vessel in the brain and creates a blockage or clot. Thrombotic strokes are caused by a blockage or clot in a major artery or in the heart that directly affects blood flow to the brain. Hemorrhagic strokes are caused by the bursting of blood vessels within the brain when the blood floods the brain causing the brain cells to die (National Stroke Association 2011).

Within the United States strokes are the third leading cause of death. There are on average 795,000 strokes a year in the United States and approximately 137,000 of those people die each year. Between the years 1996 and 2006 the overall stroke rate has decreased 33.5 percent and the overall stroke deaths have decreased 18 percent. When a stroke occurs approximately two million brain cells die per minute due to the loss of blood flow (National Stroke Association 2011).

There are two major approaches to pharmaceutically treating strokes. The first style is

thrombolytic drug therapy, which prevents the clot or plaque buildup. The major drug on the market is Activase® and it is used to treat ischemic strokes. To be effective the patient must be treated with this therapy immediately. The second style is the use of tissue plasminogen activator (tPA) which is a natural enzyme that is produced within the body that activates plasminogen into an enzyme that dissolves clots. This can be delivered intravenously by a doctor and, similar to thrombolytic drug therapy, treatment must be immediate to be of the greatest effect (National Stroke Association 2011).

Based on the statistics provided by the National Stroke Association, there is a clinical need to develop a high throughput tissue factor assay to test anti-clotting drugs similar to thrombolytic drug therapy. The development of this high throughput screening system could potentially increase the efficiency of research and development of anti-clotting drugs and testing the effectiveness of the drugs. Time is a major factor in the treatment of strokes and there is a need for faster acting anti-clotting drugs to help decrease the number of stroke deaths per year in this country.

2.2 Clotting Cascade

The clotting cascade begins when TF an integral membrane protein, is exposed to a serine protease, fVIIa. These two proteins form a complex called the Extrinsic Factor Xase, which activates two zymogens, factor IX (fIX) and fX. The resulted activated proteins, factor IXa and factor Xa, cleave and activate several more factor proteins in the blood coagulation process. FXa cleaves factor V (fV) and factor VIII (fVIII) forming the activated versions of each, factor Va and factor VIIIa. Factor IXa forms the Intrinsic factor Xase with factor VIIIa to produce more fXa. FXa forms the prothrombinase complex with factor Va, which activates prothrombin responses within the platelets (Butenas and Mann, 2001). Although several other factors are normally used within the clotting cascade, TF, fVII, and fX form what is known as the Tissue Factor Pathway (TFP) and are responsible for triggering the blood clotting cascade in both normal hemostasis and many thrombotic diseases (Waters and Morrissey, 2006). Since fIX, fVIII, fV, and other clotting factors act as promoters to the clotting procedure, they are not necessary for testing the core functionality of a blood clotting assay (Ruf et al., 1990). This process is illustrated in Figure 1.

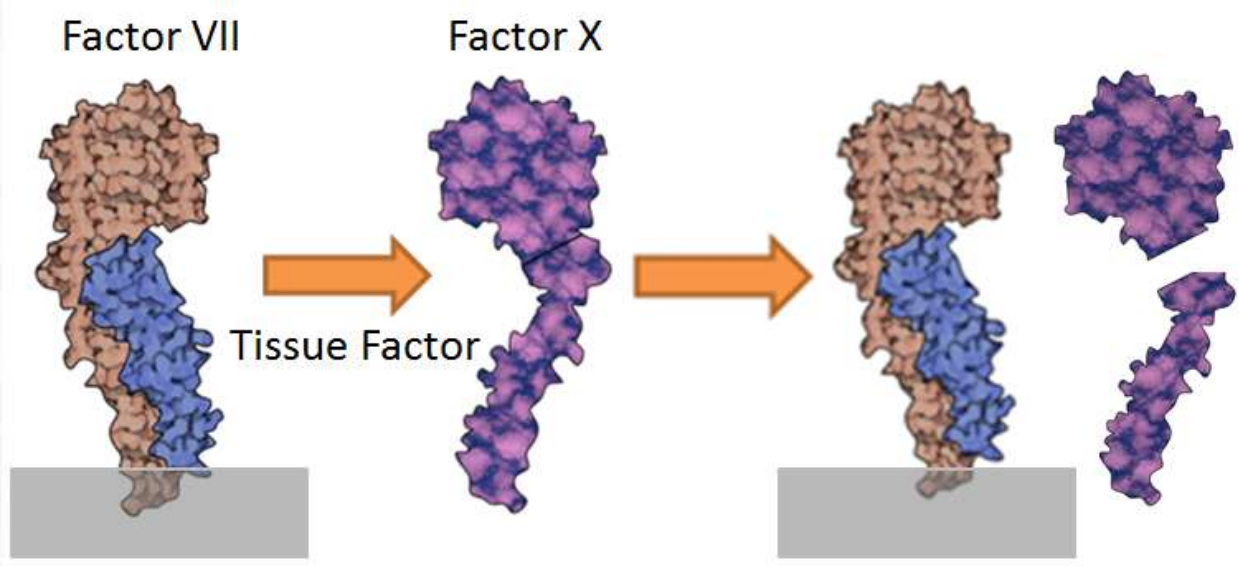


Figure 1: Clotting Cascade

2.2.1 Abnormalities within the Clotting Cascade

Although the coagulation of blood is a necessity for life, there are several deficiencies either through genetic or contracted means that cause a decrease in coagulation ability. A major reason that these diseases affect the clotting ability is by having a deficiency of one factor in the TFP. FVII and fX are the most affected factors in the TFP, but diseases affect fV and fVIII as well. When there is a lack of one specific factor in the extrinsic pathway the blood takes longer to clot than normal, which can cause severe problems in surgical or trauma cases. There are several conditions that result in mutations of the protein structure. Vitamin K deficiency affects the GLA binding sites of fVII and fX ultimately inactivating them. This effect can either be genetic or caused through the use of warfarin.

2.2.2 Tissue Factor

Tissue factor (TF) is a high-affinity cell-surface receptor and essential cofactor for the serine protease factor VIIa. Coagulation is initiated by the induction of TF expression. This is involved with the molecular pathogenesis of thrombogenesis, or the formation of blood clots, and is an effector of inflammation in cellular immune responses and cell-mediated inflammation. TF contains an uncommon sequence that is repeated three times, a tryptophan-lysine-serine sequence, that may represent a functional motif. TF has been proven to be a small cell-surface receptor and novel in its sequence, which is not like the known receptor-like protein cofactors for other vitamin K-dependent serine proteases of the coagulation cascade (Morrissey et al. 1987). TF has a single membrane-spanning domain (Rezaie, Fiore, Neuenschwander, Esmon, Morrissey, 1992) where, an mRNA sequence transcribed from a 6-exon gene encodes the amino acid sequence (Ruf et al. 1990). The phospholipid anchoring region of TF is an

essential component for the autoactivation of fVII since the isolated extracellular domain of TF does not support autoactivation of fVII. Soluble TF (sTF) is more often used *in vitro* because the native TF has prolonged clotting times in a standard clotting assay (Neuenschwander, Morrissey, 1992). sTF is highly water soluble and can be produced recombinantly in high yields, also making it more suitable for the standard clotting assay. The drawbacks of using sTF instead of native TF are that it cannot promote the conversion of fVII to fVIIa and also has reduced procoagulant activity (Waters et al. 2006).

2.2.3 Factor VIIa

Factor VIIa (fVIIa) is a vitamin-K dependent serine protease that is converted from fVII, an inactive zymogen, by a variety of proteases (Neuenschwander et al., 1995). TF promotes the activation of fVII, which is called TF-mediated fVII autoactivation, when in the presence of positively charged surfaces. This is one of the steps involved in triggering the clotting cascade (Fiore, Neuenschwander, Morrissey, 1992). The hydrolysis of a peptide bond between Arg and Ile in fVII results in a two-chain fVIIa molecule that is held together by a disulfide bond (Neuenschwander et al. 1992). Although the exact mechanism of how fVII and fVIIa bind to membranes is not known, the vitamin K-dependent carboxylation (GLA), epidermal growth factors 1 and 2 (EGF1; EGF2), and serine protease (SP) domains have been shown to be the primary binding sites for TF on the ectodomain of membranes (Morrissey et al., 2011 and Venkateswarlu et al., 2003).

2.2.4 Factor X

Factor X is activated by the TF-fVIIa cofactor (Fiore et al. 1992) and cleaved as a substrate. Ruf et al. found that fX is a more effective substrate on a membrane than free in solution (Ruf et al. 1990). FX has been shown to have a similar membrane binding mechanism as fVIIa (Venkateswarlu et al., 2003).

2.3 Production of Recombinant Proteins

Transcription is the production of a complementary mRNA chain from a chain of DNA. Translation is the use of the mRNA chain to produce proteins at a ribosomal site. The transcription and translation processes are different in prokaryotes, the bacteria cell-line, and eukaryotes, the mammalian and insect cell-lines.

In order to create the clone DNA template for transcription, an online program called DNAWorks was used. Then the DNA template was built by oligonucleotide synthesis. This method does not require a host cell or existing DNA templates to build the final template. De novo synthesis of the DNA template allows for a more flexible design process if different cell-lines are used for expression. Once the

template DNA is made the DNA was transfected into E. coli cells through a pFastBac, which produces large amount of the DNA for use in transfecting or transforming future cell cultures.

2.3.1 Prokaryotes

For prokaryotes, the transcription process starts with mRNA being directly transcribed from the DNA chain through the use of RNA polymerase. RNA polymerase binds to the DNA at different promoter sites. In prokaryotes, the strong promoter sites are the -10 sequence and the -35 sequence. The 10 and the 35 indicate how far upstream the promoter is from the start of the transcription site. Once the RNA polymerase binds to the promoter site, it starts to unwind the DNA double helix at the -10 sequence. The unwinding process allows for the addition of ribonucleotides to the DNA chain. At this point, the polymerase, DNA, and mRNA strand form the transcription bubble. The addition of ribonucleotides proceeds in the 5' to 3' direction of the DNA and ends at a termination sequence like the GC hairpin. RNA polymerase is only capable of unwinding the DNA about 17 base pairs, but the polymerase moves through the DNA chain unwinding incoming DNA and winding outgoing DNA. This elongation process allows for the production of mRNA longer than 17 base pairs in length (Raven, Johnson, Losos, & Singer, 2005).

2.3.2 Eukaryotes

The production of mRNA in eukaryotes is similar to prokaryotes, but has some distinct differences. Unlike prokaryotes, three different kinds of RNA polymerase exist. Each of the RNA polymerases has their own promoter sites and is used to produce different kinds of RNA. RNA polymerase II is used to produce mRNA. Its specific promoter site is called a TATA box, which consists of a TATAAA chain in the DNA sequence. A key difference between transcription in eukaryotes from prokaryotes is the presence of transcription factors and regulatory elements. The regulatory elements control the level of expression of different cellular and tissue developments in the cell's life (Asturias, 2003). Regulatory elements are not present in all mRNA constructs, but when they are, they bind to secondary promoter sites like the CAAT box. Transcription factors bind to the TATA promoter box and are necessary for the RNA polymerase II to start the unwinding process. In prokaryotes, the site is called the transcription bubble, but in eukaryotes, it is called the initiation complex. The initiation complex is more complex than the transcription bubble. In the transcription bubble, transcription factors are not necessary, but in the initiation complex a series of transcription factors are necessary. The main purpose for transcription factors is to control the rate of production for specific mRNA and protein strands (Locker, 2001). In bacteria, the mRNA and protein are produced indiscriminately. Before translation in

eukaryotes, posttranscriptional modifications are made to the mRNA strand produced from RNA polymerase II. There are two additions made to the ends of the mRNA strand before it heads to the translation phase of protein production. On the 5' end of the mRNA, a GTP or methyl-G cap is added, and on the 3' end of the mRNA, a series of adenines, a poly-A tail, is added. Through this process, the caps are added to prevent the breakdown of the mRNA by enzymes before translation (Raven, Johnson, Losos, & Singer, 2005).

2.4 Protein Expression Systems

Every method of producing recombinant proteins uses some form of transcription and translation, but the mechanistic differences between eukaryotic and prokaryotic cell lines leads to differing levels of expression, stability, and functionality. Commonly used protein expression systems include cell-free tubes, bacterial expression systems, mammalian transformation, and baculovirus expression systems. Commercially, a bacterial expression system is the most used, but the other methods have their own specific traits that make them more desirable for various functions.

2.4.1 Cell-free System

Cell-free systems are a relatively new process developed for producing recombinant proteins; however, the process uses some very similar principles such as transcription and translation. Several components of cells are used for cell-free tubes. The cell extracts necessary for cell-free tubes can be harvested from *E. coli*, wheat germ or rabbit reticulocytes by centrifugation at 30,000g (Shimizu, 2005). The supernatant collected is called the S30 fraction. Transcription and translation are still possible in this cell extract system. Centrifugation removes the DNA components of the cell, which allows for the introduction of another source of DNA. This source of DNA produces proteins through standard transcription and translation. The substrate particles used in the production of the protein must be replaced to allow for continuous production. The addition of a Histidine tag (HIS-tag) to the template DNA of the protein allows for nickel chelating resin to purify the desired proteins (Kim, et al., 2006). Cell-free tubes are useful for several reasons, especially in laboratory situations. Since there are no cells used in the process, cells do not need to be constantly cultured. This leads to more space available for other lab experiments and can be a large savings on incubators and other equipment. The lack of cellular organs in the tube leads to a higher concentration of ribosomes and other needed organelles for protein production. Cell-free tubes have had production rates as high as 650mg/L (Shimizu, 2005).

When using cell-free tubes, cell viability is no longer a large constraint of operation. This leads to a level of control over the operating temperatures of the cell-free tube. Also, since nutrients are

continuously added into the system, the amount of translation factors and amino acids can be kept at a high concentration to promote protein production. In cellular based expression systems, the concentration within the cell of amino acids and translation factors is monitored by the cell, but in cell-free tubes the concentrations are monitored by the user. The lack of cells in the process means that cell-free systems are capable of producing proteins that would otherwise kill cells.

The higher level of control also introduces some downsides of the cell-free tube. The number of materials added into the system can be well above 30. This high number leads to a high operating cost over time, but it also necessitates a more controlled environment so some nutrients are not added in excess (Kim, et al., 2006). Cell-free systems also have a very limited production capability and are limited to microgram levels of protein production.

2.4.2 Bacterial Expression Systems

Bacterial expression systems are capable of many feats considering their simplicity of use. Bacteria, largely *E. coli*, are easy to manipulate throughout the entire cloning process. The insertion of a plasmid, a circular ring of DNA, into *E. coli* is simple for several reasons. The primary reason is that the entire genome of *E. coli* is known and the promoter sites necessary to incite transcription are easily programmable into the inserted DNA (Verma, Boleti, & George, 1998). Certain plasmids are also responsible for creating antibiotic resistant strains of *E. coli*, which increase production efficiency and resilience to outside infection. Also by using plasmids with antibiotic resistance genes, the colonies that accepted the plasmid are able to grow in media that contain an antibiotic, thus removing the step of manually selecting specific strains of cells through plaque assays (Braoudaki & Hilton, 2004). Another beneficial trait for *E.coli* is the production rate. *E.coli* has the capability to grow at extremely high rates compared to other cell lines. This fast growth rate means that more of the protein can be produced in a shorter period of time. Bacteria also require very few nutrients or special techniques, relative to mammalian or insect cells, to grow to a substantial cell density. This allows for the very inexpensive manufacturing of large amounts of proteins (Verma, Boleti, & George, 1998).

Despite the apparent benefits of using *E.coli* or other bacterial expression systems, there are a large number of drawbacks. Different factors like protein complexity, protein specificity, and protein size can make the production in bacteria unfeasible or impossible. Protein complexity is a large factor in determining the use of bacteria or other methods for protein production. Bacteria are not capable of many types of folding, which can cause the proteins to be inactive. Other factors that play a part in protein complexity are also limited in bacteria. The ribosomes of E. coli and other prokaryotic cells are simpler and smaller than eukaryotic ribosomes and may not be able to correctly process the protein.

Even if the bacterial cell has the correct ribosomal subunit, it may not have a sufficient number to produce the protein efficiently compared to other cell types (Pharmingen, 1999).

In cases where protein folding and amino acid binding sites do not matter, there are limitations on the bacterial cell to make those proteins. In many cases, the protein can degrade quickly due to the presence of certain enzymes in the bacterial cell. Protein degradation is also affected by the larger levels of mutations and the decreased levels of folding. A mutation in the chemical structure of the protein can cause the protein to break down within the cell. Foreign protein production in bacterial cells can be hampered throughout the lifetime of the cell due to the presence of insoluble inclusion bodies. These inclusion bodies act as clogs and prevent ribosomes from being effectively used. Over time this causes the protein production to diminish (Verma, Boleti, & George, 1998).

Although bacteria are used more often for commercial scale production of proteins, there are several alternatives. Yeast expression systems are capable of overcoming certain problems that other bacteria have, while maintaining most of the benefits. The growth, lifetime of production, and complexity are the best features of yeast expression systems. Yeast is capable of growing at fast rates using simple media. Yeast cells can be grown in large numbers very quickly because the reproduction of the cells can be hastened using different signal sequences. The different signal sequences can shorten the resting period between reproductions, and once the desired level of growth is obtained, reproduction can be inhibited and the protein production can be promoted. The use of simple media is another benefit of using yeast cells. In some cell-lines, a complex series of nutrients must be added at the appropriate time or the cells could die. In yeast cells, the nutrients added are very simple and since the reproduction and production life cycle can be monitored, the kinds of nutrients necessary for some protein production can be saved for only periods of production (Verma, Boleti, & George, 1998). The possible complexity of proteins produced in yeast expression systems is better than that of bacterial expression systems but not as good as some other cell-lines. Yeast cells have the benefit of being able to fold more complex proteins while limiting the level of insoluble inclusion bodies and DNA mutation; however, yeast cells cannot perform some of the more complex folds that mammalian and insect cell expression systems can do. Other limitations associated with yeast cells are related to the promoter sites. The promoter sites for yeast cells are heterologous, meaning they need to be tailored specifically for yeast. This does not pose a large problem because the genome of most yeast cell-lines are known, but it does make the vector more difficult to design.

2.4.3 Mammalian Expression Systems

Mammalian expression systems are another expression system used to produce large amounts of protein. A major benefit of using mammalian expression systems is the capability of producing any naturally occurring protein. Mammalian cell types are able to translate and fold very complex proteins with very few drawbacks. Very specific peptide sequences are used which ensures that the proteins are created, folded and stabilized (Verma, Boleti, & George, 1998). Although mammalian cells can produce very complex proteins, they also have some limitations. Mammalian cell-lines have slow growth rates and take longer to produce proteins in large quantities. Another drawback is the amount of work necessary to prepare a cell-line for protein production. The different cell-lines require some very specific promoter sites and expression vectors. This requires a genetic engineer to undergo a series of trial-and-error tests before a final product can be used.

Insect expression systems can be broken down into two groups: transformation based and baculovirus-mediated. Both expression systems use the same transcription and translation process described previously, but the baculovirus-mediated expression system has additional components. Baculovirus expression systems use a double stranded DNA virus that carries the DNA of the protein into an insect cell. The baculovirus acts like a normal virus and splices its DNA into the cell's DNA. The virus is replicated and the protein is translated using the cell's own transcription and translation machinery. The produced viruses then lyse the cell and infect the surrounding cells. The produced protein remains in the cell until the cell is lysed and released into the medium (Raven, Johnson, Losos, & Singer, 2005).

2.4.4 Baculovirus Expression Vector Systems

The baculovirus expression vector system (BEVS) is the second most widely used method for producing large amounts of a protein. Proteins are produced by inserting a transfer vector of the protein into the genome of a virus. The virus then invades a cell and splices its DNA into the cell's DNA. In the splicing process, the virus prevents the expression of the host genes promoting the production of more viruses and, in BEVS, target proteins. The virus propagates using the cell's natural protein processes detailed above (Pharmingen, 1999).

BEVS are very common for several reasons. The infected cells can produce upwards of 500 mg/L of infection and several proteins can be produced at once in the same cell. *Baculoviridae* are also capable of carrying very large strands of DNA, well into the 100 kilobase range. Since the infection takes place in an insect cell, a large level of complexity can be achieved for protein folding and structure (van Oers, 2011). Another benefit of using the baculovirus infection to produce proteins is its price. Baculovirus expression systems are less expensive and more effective than most transformation based

expression systems. This is largely due to the ultimate death of the insect cells, the high level of protein production, and the ease of manipulating the *Baculoviridae* virus. The promoter site used is the polyhedrin promoter and it is inserted directly before the protein DNA (Verma, Boleti, & George, 1998).

There are a few limitations to BEVS. The death of the infected cells means that a whole new culture of insect cells has to be used for the next batch of protein expression. Another limitation is the specificity of the protein, meaning that the protein may be less effective that proteins produced in other expression systems. This is due to slight differences in the posttranslational processes of insect cells from mammalian or bacterial cells (Pharmingen, 1999).

2.5 Quantification Methods

In order to determine if an assay is positive or negative, quantitative and qualitative tests are performed. A quantitative test has a reproducible set of data that gives a magnitude to the level of expression in an assay. A qualitative test has a subjective or numberless association between positive and negative. Both methods have various benefits and drawbacks in determining clotting.

Blood is composed of three major components: plasma, red and white blood cells, and platelets. Plasma comprises about 55%, red blood cells are about 45% and the other 10% are made of platelets, white blood cells and other macromolecules by volume. Plasma is largely composed of water and nutrients (91%), and proteins (8%). The density of plasma is about 1025 kg/m^3 and the viscosity is about 1.6 centipoise (Wells, 1961). Plasma is a light yellow color that is darkened when clotting. For spectrophotometry, plasma has a discernible peak at 280 nm (Motrescu, 2006).

2.5.1 Quantitative Means

The methods of quantification deal with the absorption of the sample and measuring the change in the concentration of the proteins over time. The viscosity of the reaction is also quantifiable. If the viscosity was very similar to 1.6 centipoise, it did not clot. Means of qualifying include looking for color changes to determine if the sample could be clotted or not and differences in density also to indicate clotting. As the sample clots, the protein separates more from the water and becomes denser creating a discernible solid shape. Spectrophotometry measures absorption by directing a beam of light at a set wavelength through a sample and comparing the intensity of light received on the other side of the sample to the original intensity. Spectrophotometry is widely used and compatible with 96 and 384 well plates, but the machines are expensive, often costing over $15,000.

Another option for quantifying the clotting is using the flow rate of a liquid. If the after-assay sample passed through a tube, the flow rate could be measured. From that the viscosity and density can

be calculated due to the relationship between the Reynolds number of a liquid and the velocity. This alternative is less expensive than a spectrophotometer; however, it is less accurate and may not be compatible with use in automatable machines or 96 and 384 well plates.

2.5.2 Qualitative Means

Aside from the quantifiable means of measuring the level of clotting, there are also the qualitative means. Qualitative means are important in order to obtain a direct measure of the clotting reaction and see each enzymes activity. After a period of time a color change should be noticeable when observing the well plate. The assay should change from clear to white, based on the known color change of the clotted plasma. This difference in color could be more noticeable if a color-coded sheet was created to show a scale detailing color differences. This design would be easy to use on larger samples; however, for a 96 or 384 well plate mistakes would occur more often.

Another qualitative design is the use of a digital picture system. By uploading photos of the assay to a computer, a computer program could determine changes in the color of the samples. This process builds on the last design by removing the technician and decreasing the possible sources of error. Although this would be a more accurate system than personally observing every well of the plate, a camera would be expensive to integrate into an automation machine.

The density is another good property to build off of for qualitatively determining the clotting of the assay. When blood and plasma clot, they become denser due to higher protein interactions. The change in density could be utilized by introducing a buffer in between the density of the clotted proteins and the density of the non-clotted proteins. By separating the clotted and non-clotted proteins, a technician or alternative device could count the clotted wells more easily. The drawback of this method is that it could create problems with low levels of clotting and cause there to still be a need for a person present to determine if the proteins clotted. This process may also require special well plates due to the small volume of 96 and 384 well plates.

Chapter 3: Project Strategy

The team was initially given a timeline for deliverables, a general client statement and a rough project outline. Using the prescriptive design process (Dym and Little 2009), the team refined the client's problem statement and suggested several objectives, constraints and functions. The preliminary client statement was:

> *To design a high-throughput assay to measure activity of Tissue Factor, Factor VII, and Factor X and purification design strategies for cloning, expression, and recovery of tissue factor proteins. Develop operating parameters appropriate for the type of assay.*

After meeting with the client, a list of objectives, constraints, specifications and a final client statement were approved or revised. A Gantt chart (Appendix A) was generated highlighting the deliverables and the timelines given by the client and setting specific milestones for research and assay development. After some testing the client and team will choose the assay method that fulfills the design requirements best.

3.1 Objectives and Constraints

To keep the project focused, the design team created a list of general objectives to conceptualize the design. These objectives were gathered and developed through conversations with the client and background research.

In the design of the tissue factory assay, safety is important. The user should be protected and there should be no threat of danger. The safety of the environment should also be taken into consideration. The device should also be user friendly, which means it should be easy to use, as well as require minimal interaction. Another important objective is that the device is cost effective, operating costs and unit costs are an area of consideration. The tissue factor assay will be high-throughput and will run more tests in less time. Another important objective is that the device be accurate. The tissue factor assay should produce the desired volume per well. It should also produce consistent activity readings. The project team created an objectives tree, as seen in Figure 2 below. The design team also created a pairwise comparison chart with the client, as seen in Table 1 below.

The constraints of the project include a budget of $156 per team member. This budget is set in place by WPI and is a standard for every Major Qualifying Project. The cost of the diagnostic and drug discovery kit should cost no more than $9000 including disposables. Time is also a constraint for the design team since the project must be complete by April 2012. Lastly, the design team must express

Tissue Factor, factor VII, and factor X in a cell line. That is a constraint since the assay will not be able to be completed without the expression of all three coagulation proteins.

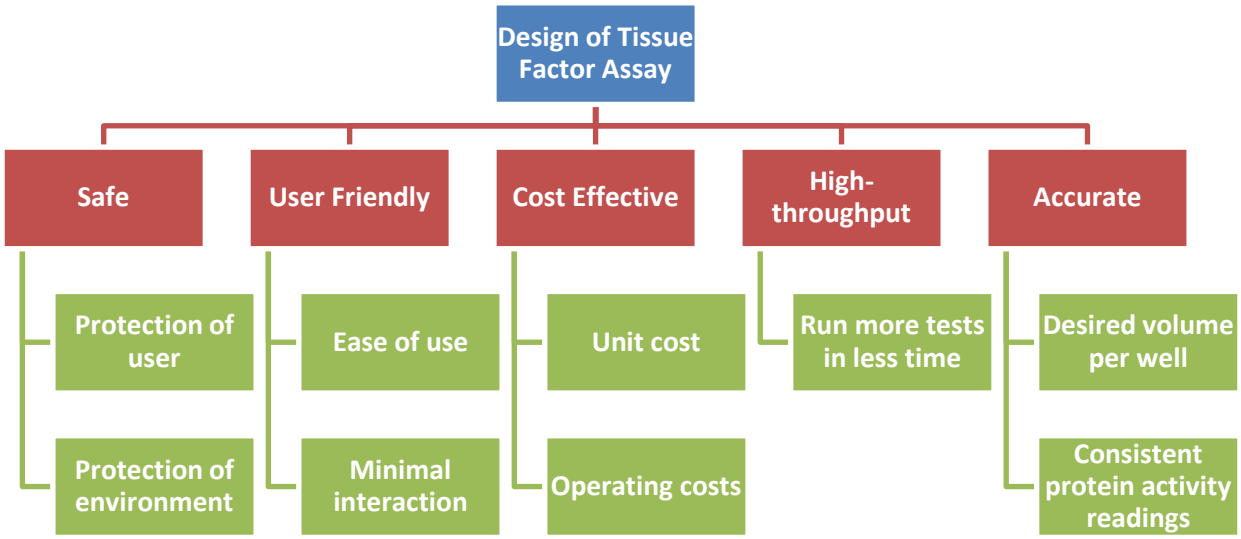


Figure 2: Objectives Tree

Table 1: Pairwise Comparison Chart

	Safe	User Friendly	Cost Effective	High-throughput	Accurate	Total
Safe	-	0	0	0	0	**0**
User Friendly	1	-	0	0	0	**1**
Cost Effective	1	1	-	1	0	**3**
High-throughput	1	1	0	-	0	**2**
Accurate	1	1	1	1	-	**4**

3.2 Revised Client Statement

Based on the design teams meeting with the client and research, the group revised the initial client statement.

> *The goal of this project is to develop an enhanced assay for blood clotting, and TF/fVIIa/fXa activity specifications. This requires development of strategies for cloning, expression, and purification of these proteins, and incorporation into an assay with full biological activity.*

3.3 Project Approach

The project team used the client statement to brainstorm design alternatives and draft a plan of methods to be used. The team then used the planned experiments along with deadlines provided by the advisor and client to develop a detailed Gantt chart (Appendix A), linear responsibility chart (Appendix B) and a work breakdown structure (Appendix C). The B term experiments include the design of the proteins Tissue Factor, Factor VII and Factor X, and culturing of the SF9 baculovirus constructs. This encompasses reverse translation, ordering of the proteins, gene synthesis and cloning into an expression vector, fluorescent labeling, and transfection of cells to get an intact P1 virus for each of the three proteins. The team will then move to the assay development portion of the project, where an assay will be designed and tested to measure clotting times. The project team's design steps fit into the objectives of the project because the protein design will be user friendly with the sequences being completed as well as cost effective and high-throughput since they will be able to be produced rapidly once the sequence is optimized. The assay will be developed to be high-throughput as well as accurate since the client will be able to run more tests in less time as well as have consistent protein activity readings. From there, the project team made a process flow diagram (Appendix D) that represents the progression of the project.

For B term, the project team started by ordering the proteins from Blue Sky Biotech. A word document was assembled that comprised of the amino acid sequence, the accession numbers, and HIS-tag location (Appendix F). The project team also used reverse translation to read protein sequences and turn them back to RNA to design the oligonucleotides. The reverse translation allowed for the project team to control the production of the protein including the location of transcription factors. After that, the team began the gene synthesis and assembled the oligonucleotides using modular progression that consisted of constructing the recombinant proteinsusing the technique of splicing by overlap extension by the polymerase chain reaction (SOE-PCR). SOE-PCR is a cost-effective tool for recombinant gene

construction. The proteins were then tagged by 5-(pentafluorobenzoylamino)fluorescein di-β-D-galactopyranoside (PFB-FDG), which is a green-fluorescent PFB-F dye (Invitrogen by Life Technologies, 2011). Subsequently, the SF9 baculovirus cell line was transfected to introduce the nucleic acids into the cells using a virus as the carrier or by using liposomes that transfect by fusing to the cell membrane and releasing the DNA into the cell. Homologous recombination then occurred, where nucleotide sequences were exchanged between two similar or identical molecules of DNA. After that happened, the project group had an intact P1 virus that can be cultured and used to extract proteins from, which will lead into the assay development portion of the project that will take place in C term.

Chapter 4: Alternative Designs

This chapter describes the alternative designs of the project. It also discusses the need of the project as well as if the project is feasible, how the project is modeled, and preliminary data.

4.1 Alternative Designs

There are two elements that defined the alternative designs for the expression of the TF, fVII, and fX proteins: host cell line and location of His-tag attachment onto the protein chain. The choices for the host cell line were *E.coli*, insect cells, and mammalian cells. Each cell line has advantages and disadvantages that have been discussed in the background research and will be reiterated in later sections, as appropriate. The team will use the host cell line to culture the proteins to build up the TDA liposome that will be used in the expression system. There are two ideal locations that are possible alternatives for attaching the His-tags: the N-terminus of the protein sequence or the C-terminus of the protein sequence.

4.2 Needs Analysis

A list of needs was required in order to properly create an assay to measure the clotting ability of blood. This list encompassed the needs of the required product and the wanted product of the assay. Since the assay was designed for two purposes, the required and wanted features of the end product were be split into three different categories: drug discovery items, diagnostic items, and collective items. Drug discovery and diagnostics were the two largest markets for a clotting assay and were the focus of improvement. The characteristics needed for both the drug discovery and diagnostic applications of the assay included the ability to be high throughput and the ability to have full biological activity. High throughput for this project was defined as being automated and producing more than the current speed of analyzing samples. Due to time restraints, the automation of the assay was not included in the design objectives, but the capability of automation was included. Current methods are able to analyze less than 48 samples per day so the analysis of more than 48 samples a day was required, but the analysis of hundreds of samples a day was desired.

Another requirement included in both drug discovery and diagnostics was the reproduction of full biological activity. Full biological activity was defined as the activity of the proteins in the human body. It was a requirement of the project sponsor, Dr. Scott Gridley of Blue Sky Biotech, that the Template Directed Assembly (TDA) method be used. TDA is a method of attaching a protein to a liposome that is useful in mimicking the membrane bound proteins found in the human body and has been shown to approach the full biological activity of the proteins (Waters, 2006). The incorporation of

membranes and phospholipids was necessary for studying clotting *in vitro* because thrombin produced by the clotting cascade adheres to the phospholipids that make up the bilayer or cell membrane within the body. TDA technology has the ability to mimic the adhesion of proteins to a bilayer and simulate the clotting process. To the design team's knowledge, there is no other assay on the market at this time that can replicate the results that a TDA based assay can potentially produce.

Measuring the activity of the proteins is especially useful for drug development. In drug development, it is important to have specific numbers to show the effect that a drug has on the clotting ability of blood, since a small alteration in the clotting ability of a drug could pose a dangerous risk to the patients. Comparing the effect that drugs have on the clotting assay to a control sample is also important. In the case of this project, the control sample for the assay will be an assay test without the addition of drugs or chemicals. Testing drugs is a long and laborious process using current methods, due to the time necessary to test a sample and the small testing size of current automated assays, so a decrease in the testing time and an increase in the number of samples was desired.

A diagnostic kit can be used to give a quantifiable piece of data to assist in the diagnosis and treatment of diseases or conditions. For this assay to be successful in a diagnostic kit, it needs to provide a precise number for the clotting ability of patient blood. Without the specific level of activity of the clotting proteins, doctors and technicians could not use the assay for diagnostic purposes. If possible, the diagnostic kit should be faster and less expensive than the available kits.

4.3 Functions and Specifications

The final design of the clotting assay must meet several different criteria, including being high throughput, accurate, and efficient. In order to achieve these objectives, the protein host, screening platform, quantification of clotting, and marker must be chosen. Before the assay can be developed the proteins need to be created. The proteins are created using a baculovirus that carries the protein DNA and replicates the proteins when the virus is replicating. The DNA for the proteins is engineered to control codon bias, optimize 2° structures, and include or exclude restriction sites to facilitate cloning. DNAworks, a website from the National Institute for Health, was used to design each gene (output in Appendix F and Appendix G).

For this assay, high throughput is considered to be hundreds of samples in a day with the potential to be automated. The current standard is 48 samples a day for existing clotting assays, so any amount higher than 48 is an improvement over existing means. 96 well plates and 384 well plates will be tested to run more assays at a time. Automating the assay would lead to a large increase in number of samples that can be run in the same period of time because several automated assays could be run at

the same time with the same number of technicians present. Automation would be particularly useful for drug discovery where thousands of samples would run in order to determine the best treatment methods for clotting disorders.

The accuracy of the test is another area of improvement. Using the TDA, the clotting cascade and the proteins may be measured at nearly full biological activity, improving the reliability that the assay is reflective of the clotting process occurring in the body. The addition of the membrane to the assay is responsible for this increase in activity. The membrane is simulated Template Directed Assembly with TDA liposomes. Nickel nitrilotriacetic acid (Ni-NTA) is covalently attached to the lipids. The liposomes are created from the lipids, which act as the membrane. The His-tag has a negative charge that binds to the positively charged Ni-NTA thus binding the proteins to the membrane. Since the assay can mimic the clotting process in the human body, it is better for diagnostic tests and drug development, because even small changes in clotting ability could cause irreparable damage or serious health complications.

Improving efficiency is a beneficial exploration because it reduces cost and testing errors. In order to reduce the cost, the assay will be conducted in a 96 well plate or a 384 well plate instead of test tubes. The larger sampling platform and fewer materials needed would reduce costs and allow for a variety of samples to be run simultaneously. Reducing the cost of the assay is important because current assays produced by Blue Sky Biotech cost $5000 to $9000. Providing inexpensive assays would lead to a lower cost for the patient and insurance companies for diagnostic testing and a lower development cost for pharmaceutical applications.

The most vital part of the assay is the quantification of the protein activity, specifically, the correlation of the activity of fVII and fX to the activity of the entire clotting cascade. In order to measure the activity of the two proteins, a specific kind of spectrometer called a coagulometer is used to to measure the absorbance of the sample at a wavelength of 405nm. The clotting assay should take between 20 and 150 seconds depending on the concentration of the protein in the solution. For drug discovery, the samples could take shorter or longer to clot than the control samples due to the addition of chemicals or drugs to alter the coagulation time.

4.4 Conceptual design

The project team brainstormed several alternative designs for generating a working high throughput tissue factor protein assay. The process for designing this assay was based off of a previous study conducted by Waters and Morrissey (Waters and Morrissey, 2006); however, several variables were taken into account for the design process for our specific assay. Factors that must be taken into

account when determining the final design were: the host cell type to produce the proteins, the ideal harvest time of the transfected cells, and a micro plate size that working assays will use.

The project team researched available host cell lines in order to determine which to use to produce the TF, fVII, and fX. *E.coli* was used in the Waters and Morrissey study and is an ideal host for fast and efficient production of proteins with minimal care; however, the lack of protein stability due to errors in folding and possible degradation within the culture due to overproduction made *E.coli* an unfeasible host. Mammalian and insect cell lines were considered as possible hosts for producing the proteins because both cell lines have the capability to produce any naturally occurring protein without folding or structural errors which is beneficial since our project was designed around growing up TF, fVII, and fX into liposomes. Another benefit of these cell lines is that they are both capable of folding complex proteins and maintaining the stability of the proteins. The main drawbacks of using mammalian cells as hosts is that the growth rate is too slow for the demands of this project and more extensive care and preparation is needed when using this cell line. Insect cells have a slightly higher growth rate which can be increased substantially with the addition of heavy metals to the nutrients (Verma, Boleti, & George, 1998). Dr. Gridley confirmed the use of insect cell lines, specifically the baculovirus expression system, as the host cell line to use for producting our proteins.

In order to make this system high throughput, the project team determined that the system needed to be able to operate within a micro plate. There were two possible options for plate designs to house the system: 96 well plates and 384 well plates. The 384 well plates were the ideal choice for developing a high throughput system, which our project defines as being able to test hundreds of samples for per day. Dr. Gridley stated that 96 well plates would be satisfactory for the project; however, it would be simple to scale the assay to be used in 384 plates, which would provide higher cell concentrations.

4.5 Feasibility Study

The team determined that the project was feasible to complete and manufacture based on the paper published by Waters and Morrissey because they were able to restore full biological activity to the isolated ectodomain of tissue factor (Waters et. al, 2006). The project team designed an assay that is similar to the process outlined in the literature. The project team decided that the clotting cascade proteins, Tissue Factor, factor VII, and factor X, were necessary to design and test the assay. The cost of doing that is expected to exceed $21,000. That number was determined by looking at the Blue Sky Biotech website to see how expensive it is to create each of the proteins. For one protein it generally

costs between $7000 and $8000 and since the project team needs Tissue factor, factor VII, and factor X, the total cost is between $21,000 and $24,000. This exceeds the budget that the project team was allotted by WPI, but with the help of Blue Sky Biotech, the necessary experiments will be able to be performed. It is also feasible for the project to have results by the deadline of April 2012, as shown by the timeline of experiments and due dates.

The first design met all of the feasibility requirements. The proteins were feasible because the truncated sequences were proven to be sufficient in previous studies. The project group also verified that the proteins were stable and had a long half-life by using the web programs ProtParam and Protein Calculator. The web programs allow for the protein sequence to be computed for various physical and chemical parameters such as molecular weight, theoretical pI, amino acid composition, extinction coefficient, estimated half-life, and instability index. From the web programs, the group found that TF has a theoretical isoelectric point (pI), of 7.11. FVII was found to have a theoretical pI of 7.06 and fX was found to have a pI of 6.05. All of the proteins have an estimated half-life of over 30 hours in mammalian reticulocytes, which was considered to be adequate for the current purpose. Also, all of the proteins contain cysteine, lysine, and tryptophan, so the group can use them for fluorescent dye experiments. TF's instability index is 32.14, which classifies the protein as stable. The only major concern with the feasibility study was that the instability index of 47.33 for fVII and the instability index of 40.46 for fX, which classifies the proteins as unstable. The cell line that Blue Sky Biotech proposed for us to use, SF9 insect cells, also proved to be the most feasible. Concerns the project team has for the proteins include getting an acceptable expression and ensuring that they are the correct protein. Through collaboration with Blue Sky and extensive research, we found this cell line to be the easiest and most viable with which to express our protein. The team was most concerned with getting accurate expression of the proteins and obtaining sufficient protein to be able to run the assay to get acceptable data. A 96 well plate was used. The team also determined a protocol that includes taking the compounds that are known to work, creating an intact reaction, adding other compounds, looking at how effective the clotting is and creating a standard curve. Concerns included using an appropriate control for the experiments, getting consistent data, and making the assay high throughput.

4.6 Modeling

(See Appendix H for model of TDA Liposome structure)

4.7 Preliminary Testing

In order to begin successful testing, the project group ran preliminary tests on the proteins used and on the assay.

4.7.1 Preliminary Tests on Proteins

The proteins being used, TF, fVII, and fX were tested in order to verify that they were the most effective proteins for our assays. The project group tested the proteins using western blots. Western blots determine the weight of the proteins and also measure how much of each protein is present within the samples. Western blots separate proteins by gel electrophoresis, transfer the proteins onto blotting paper while retaining their separation pattern, incubate the blot, add an antibody to bind to the proteins, and then locate the antibody (Overview of Western Blotting, 2011). Once westerns blots had been conducted for TF, fVII, and fX, the project group determined how successful they were by seeing how much of each protein was present in our samples. If each protein is adequate, the team will use them for further testing.

4.8 Description of Experimental Methods Used to Test Design Alternatives

The team identified which host cell line was best to use between the *E. coli,* insect cells, and mammalian cells by doing research on which would best mimic the actual clotting cascade and which would be the most feasible for our allotted time and budget. It was decided that mammalian cells would take too long to express the desired amount of protein and that *E. coli* did not mimic the clotting cascade as well as the baculovirus constructs could.

It was also important to identify the location of the His-tag attachment onto the protein chain. The two possible alternatives for attaching them to the sequence are at the N-terminus or C-terminus. Through research and discussions with Dr. Gridley, the group decided that the ideal location of the His-tag would be the C-terminus.

The design team was not able to experimentally test either of the two design alternatives listed above because of the constraints of time and cost. It would be too expensive to transfect mammalian, insect, and *E. coli* with the proteins. It also would be too expensive to attach the His-tag to either end of the protein to test which is more appropriate for the project.

The team will test the design alternatives of plates used and quantification methods for the assay. A 96 well plate will be tested in order to see if it can output accurate and acceptable data. In addition, tests will be run to determine which design alternative for quantification method is easiest and

most accurate. This will be tested by performing the assay and creating a standard curve using the alternatives of plate reader, density measurement, and viscosity measurement.

4.9 Conclusions from tests

Using the ProtParam and Protein Calculator web programs, the project group was able to analyze the sequences used for Tissue Factor, Factor VII and Factor X. Based on the program, the team determined if each of the proteins were stable or not. Table 2 shows the data from the ProtParam and Protein Calculator programs.

Table 2: Table with Data from ProtParam and Protein Calculator

	Tissue Factor	Factor VII	Factor X	Based on:
Theoretical pI	7.11	7.06	6.05	ProtParam
Estimated pI	7.52	7.34	6.48	Protein Calculator
Est. half-life	30 hours	30 hours	30 hours	ProtParam
Instability Index	32.14	47.33	40.46	ProtParam
Number of cysteines	5	26	24	Protein Calculator
Extinction co w/out disulfides	1.5875	1.2514	0.9235	Protein Calculator
Extinction co with disulfides	1.5944	1.2808	0.949	Protein Calculator
Stability	stable	unstable	unstable	ProtParam

The estimated half-life is 30 hours for each Tissue Factor, Factor VII and Factor X in mammalian reticulocytes. The extinction coefficient with and without disulfides is at a wavelength of 280. In addition, each of the proteins contains cysteine, lysine and tryptophan, meaning they could all be used for fluorescent dye experiments. The sequences used for Tissue Factor, Factor VII and Factor X can be found in Appendix F.

4.9.1 Tissue Factor

For Tissue Factor, it was determined by ProtParam that the theoretical pl (isoelectric points) was 7.11. The Protein Calculator estimated the pl to be 7.52. The estimated half-life is 30 hours in mammalian reticulocytes. Tissue Factor contains cysteine, lysine and tryptophan, which suggests that it could be used for fluorescent dye experiments. Tissue Factor contains five cysteines. For Tissue Factor, the instability index is 32.14, which means that it can be classified as a stable protein. The extinction coefficient at a wavelength of 280 is 1.5875 without disulfides and 1.5944 with disulfides. The sequences used for Tissue Factor can be found in Appendix F.

4.9.2 Factor VII

In terms of Factor VII, the ProtParam determined the theoretical pl to be 7.06. The Protein Calculator estimated the pl to be 7.34. The estimated half-life was also found to be 30 hours in mammalian reticulocytes. As with Tissue Factor, Factor VII contains cysteine, lysine and tryptophan. This means that Factor VII could also be used for fluorescent dye experiments. There are 26 types of cysteines in Factor VII. The instability index is 47.33, which means that the protein is classified as an unstable protein since the value is over 40. The extinction coefficient at a wavelength of 280 is 1.2514 without disulfides and 1.2808 with disulfides. The sequences used for Factor VII can be found in Appendix F.

4.9.3 Factor X

For Factor X, the theoretical pl was 6.05 based on the ProtParam program. The Protein Calculator estimated the pl to be 6.48. The estimated half-life is also 30 hours in mammalian reticulocytes. Factor X also contains cysteine, lysine and tryophan, like Tissue Factor and Factor VII. Factor X was found to have 24 cysteines. The instability index rated Factor X at 40.46, meaning it is classified as an unstable protein since the value is over 40. The extinction coefficient at a wavelength of 280 is 0.9235 without disulfides and 0.9490 with disulfides. The sequences used for Factor X can be found in Appendix F.

4.9.4 Restriction Sites

Based on DNAworks, the team found the restriction sites to be:

- BamHI
- EcoRI
- HindIII
- KpnI

- NcoI
- NotI
- XbaII
- XhoI

4.10 Choice of Final Design

The project team's final design choice was to purify the proteins from a *baculovirus* expression system. Each protein had an oligohistidine tag (His-tag), SGGGAAGHHHHHHHHSG, attached to the N-terminus. Using predictive models (ProtParam and Protein Calculator), half-lives for these proteins in an expression system and theoretical pI (isoelectric points) were generated. The half-life for each of the proteins within a mammalian system is 30 hours. The pI ranges from these programs are as follows. TF has a pI range of 7.11 to 7.52. fVII has a pI range of 7.06 to 7.34. fX has a pI range of 6.05 to 6.48. In addition, using these predictive models the overall stability of the proteins was predicted and only TF produced an instability index that indicated acceptable stability. fVII and fX had slightly elevated instability indexes; however, these were still believed to be acceptable values. TF was used to build up the TDA liposomes using a procedure defined by Smith and Morrisey (Smith and Morrisey, 2004). The TDA liposomes were generated to have a diameter of approximately 100nm. A transmembrane containing nickel chelating heads will enable direct interaction with His-tags. His-tags were attached to RTK structures that will attach to the transmembrane and allow expression of whole model in various systems.

Diagnostic kits and drug discovery kits will be created using the liposomes and purified proteins along with other components. Standard elements of the kits include: printed protocol for either diagnostic application or drug discovery application, a supply of 96-well plates, HBSA, 1M calcium chloride, purified proteins (stored at -80° C), and TDA liposomes (stored at approximately 4° C). In general, kits will be customized to suit client needs in terms of quantity and application.

Chapter 5: Design Verification

This chapter describes the raw results of the project as well as how the experiments were conducted.

5.1 Gene Assembly

Three genes were synthesized *in vitro* without the need of initial template DNA samples by using oligonucleotide synthesis from digital genetic sequences. First, oligonucleotides were designed for the respective coagulation proteins using the online programs ProtParam and Protein Calculator. The results of the programs displayed that the proteins would likely be stable. Next, oligonucleotides were ordered on a 96 well plate with 10nm of oligonucleotides in each well. They were then resuspended in 100µL of water. After resuspension, 10µL of each were mixed together and added to PCR to be spliced together using overlap extension. The first PCR became a template for the second PCR to ensure that all residual PCR primers and unincorporated nucleotides were removed. From there, the sequence was amplified. An agarose gel electrophoresis was used to check whether the PCR generated the anticipated DNA fragment. The size of the PCR products was determined by comparing where it was on the gel with a molecular weight marker that was run alongside the PCR products. The DNA was viewed using UV light. The band was then purified and cloned into the pFastBac1 vector, where it was transformed in *E. coli* to get a number of colonies. The bacmid complemented a lacZ deletion that was present on the chromosome to form blue colonies. Transposition of the gene of interest from the pFastBac1 donor plasmid into the bacmid disrupted the lacZ sequence, which caused the recombinant bacmids to appear as white colonies. The plasmid was then prepped and the bacmid DNA was formed.

5.2 Cell Maintenance

Maintenance of the Sf9 cell lines was conducted over a two week period. During that time the cell stocks were split three times at 72 hour intervals to ensure the cells were maintaining proper densities, viabilities, and diameters. All cell counts and measurements of stocks were taken using a Cedex Cell Analyzer. The media used for this step was Gibco Sf900 III. Gibco Sf900 III was chosen because it promotes a high yield for Sf9 and Sf21 cell lines and has no mammalian origins. These are good qualities for a media to have when being used in conjunction with insect cell lines. The density or Viable Cell Concentration (VCC) determined the amount of media used per split. This was calculated using Equation 1:

Equation 1: Calculating Media quantity

$$\frac{Total\ Volume\ of\ solution\ (mL)}{Viable\ Cell\ Concentration\ (\frac{cell}{mL})}\ X\ Target\ Cell\ Concentration\ (cell/mL)$$

The total volume of the solution for the new flask was 60mL and the target cell concentration was $5x10^5$ cell/mL. The aim was the cell viability of the stocks greater than 97% to ensure that the cells were healthy and competent. The cell diameter needed to be maintained above 16µm. If the cell diameter was less than 16µm, it would indicate possible contamination of stock. After the third split the stocks were transfected. Table 3 shows the mean VCC, viability, and average diameters of the four flasks of stock that were maintained by the team.

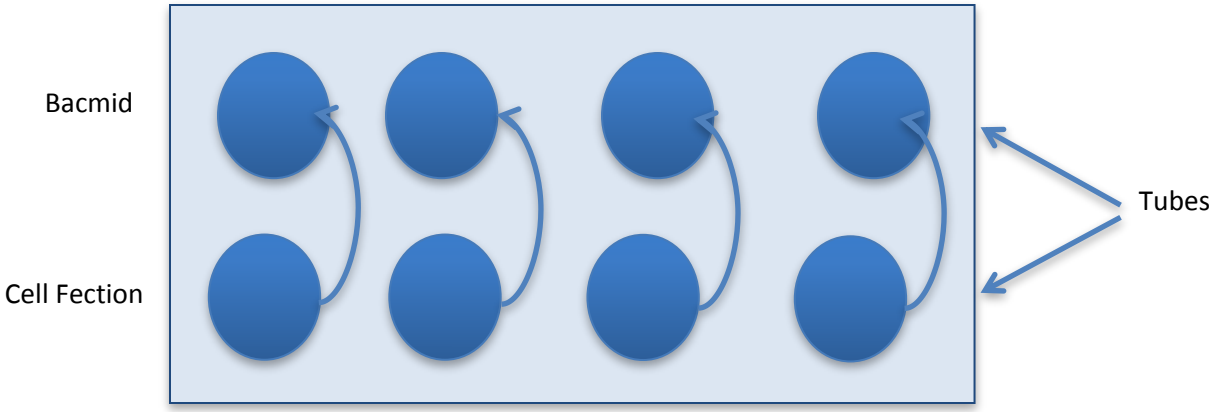
Table 3: Cedex Readings of Stock Splits

Sf9 CEDEX readings			
	Viable Cell Concentration ($1x10^5$ cell/mL)	Viability (%)	Average Diameter (µm)
Split 1	21.89	97.4	16.3
Split 2	56.47	99.2	16.1
Split 3	38.24	99.6	16.1

5.3 Transfection

In order to transfect the cells, the team obtained a Transfer Sheet, which provided the plasmid information, protein size, protein tag, etc. The bacmids were stored at 20°C and the Opti-Mem1 was kept at 4°C. The transfection reagent used was Cellfectin II. Materials needed were two sterile 2mL tubes, 750mL media (in each tube), 45mL of Cellfectin II and 10µL of bacmid. Figure 2 below shows a schematic of how the tubes should be arranged inside the hood.

Figure 3: Set-up of hood during transfection



When performing the transfection, the Cellfectin II was inserted into the tubes that were closest to the clinician and the bacmid was inserted into the tubes that were farthest from the clinician. The clinician worked left to right in order to avoid contamination.

Next, the tubes were incubated for five minutes. After, the Cedex counted the cell stock. P1 was considered expression. At this point, 36mL was added to the flasks. All material were transferred from the Cellfectin II tube to the bacmid tube (front tube to back tube). The procedure was carried out in this order to insure that no bacmid was lost in the process.

Incubation occurred for 30 minutes in order to permealize the DNA and allow liposome/DNA complexes to form. After a half hour, all the material was taken from the tubes to 36mL of Sf9 at $1X10^{6}$. At that point, the full volume had been added to the stock that had been knocked back. After 72 hours, the allotted time for reactions to occur, the viruses could be made. Another Cedex count was taken at the 72 hour time point.

The viable cell concentration, total cell concentration, viability, and average diameter for the team's P1 transfections can be seen in tables 4, 5 and 6.

Table 4: Cedex readings 96 hr. post-transfection of pFastbac1-TF

P1 Transfection				
	Viable Cell Concentration ($1x10^5$ cell/mL)	Total Cell Concentration ($1x10^5$ cell/mL)	Viability (%)	Average Diameter (µm)
Flask 1	17.34	28.65	60.5	19.6
Flask 2	11.31	20.70	54.7	19.02

Table 5:Cedex readings 96 hr. post-transfection of pFastbac1-fvII

P1 Transfection				
	Viable Cell Concentration ($1x10^5$ cell/mL)	Total Cell Concentration ($1x10^5$ cell/mL)	Viability (%)	Average Diameter (µm)
Flask 1	19.77	28.27	70	19.6
Flask 2	15.89	22.89	69.1	19.66

Table 6:Cedex readings 96 hr. post-transfection of pFastbac1-fx

P1 Transfection				
	Viable Cell Concentration ($1x10^5$ cell/mL)	Total Cell Concentration ($1x10^5$ cell/mL)	Viability (%)	Average Diameter (µm)
Flask 1	19.09	27.26	70	19.62
Flask 2	19.03	24.14	78.9	19.71

5.4 Virus Stock Production

Once the sf9 cells were transfected with the bacmid, the cells were grown until a viability of 60%-70% was reached. This viability has been shown to be the optimum period to harvest transfected cells because the cells were lysed by the baculovirus. It is necessary to harvest the cells at viabilities above or about 50% because the baculovirus begins to die when there are not enough cells to be infected. In order to harvest the sf9 cells, the flasks were spun in a centrifuge at 1500g at 4°C to separate the media from the cells. Western blots and SDS-PAGE gels were run on the cell pellet and the media from the centrifugation to ensure that the target proteins had been produced. TF and fX were expressed in the western blots, but fVII was not expressed in either the media or the soluble fraction runs of the western blot. The separated media from the centrifugation was filtered through a 0.45 micron filter tubes further to ensure that no cells or macromolecules were present. The media was then diluted to form the final virus stock.

5.5 Infection

Sf9 cells were used as hosts to create the proteins. The cells were grown to a density of about 14.5×10^5. A dilution of 1:1000 of the virus stock was used to infect each flask. This resulted in about 500µL of virus stock per flask. Three flasks were prepared for each baculovirus infection at 500mL in order to determine the best time to harvest the proteins. The flasks were harvested at 24, 48, and 72 hours. Centrifugation was used to harvest each flask. The media and collected cells were tested using western blot and SDS-PAGE to ensure protein production prior to purification. The western blots showed that there was significant expression of TF and fX in the soluble fractions of the cells, but there was little or no production of fVII. The optimal time to harvest was determined to be 48 hours for fX and 72 hours for TF. There was some concern with fX and whether or not it is the inactive or active version of the protein. The His-tag and the molecular weights were checked to ensure that it was fX and not fXa. TF was not released into the media so a more complex purification process would be used. It was decided that FVII would be grown in a mammalian expression system. With failure in the mammalian system, fVII could be purchased.

5.6 Expression of Proteins

The level of expression of each protein was determined using western blotting. The western blots showed that TF and FX were successfully expressed using BEVS; however, FVII did not significantly express in BEVS and a mammalian expression system was used instead. The western blots for TF and FX are seen in Figure 3 below. TF and FX both had a good expression in the BEVS. FX was shown to degrade

significantly within the cells. The western blots for FVII are seen in Figure 4. FVII also degraded within the mammalian expression system.

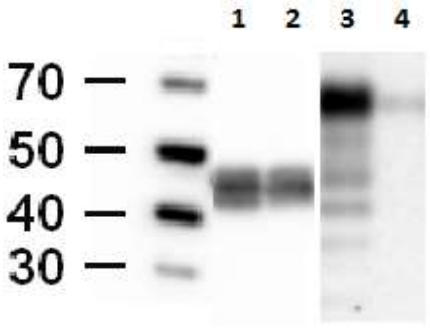


Figure 3: Column 1 and 2 show the expression for TF in sf9 cells. Column 1 is the whole cell lysate and column 2 is the soluble fraction of the cells. TF is seen at about 40 kDa. Column 3 and 4 show the expression of FX in sf9 cells. Column 3 is the whole cell lysate and column 4 is the soluble fraction. FX is seen at about 70 kDa. Column 3 indicates that FX degrades in the cell.

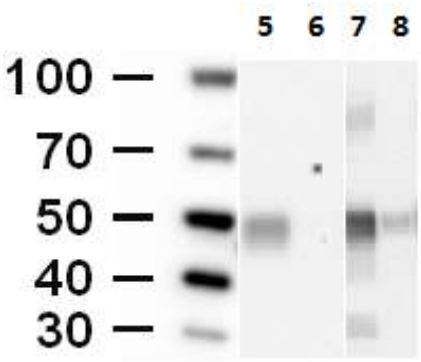


Figure 4: Column 5 and 6 show the expression of FVII in sf9 cells. Column 5 is the whole cell lysate and column 6 is the soluble fraction. Column 5 and 6 are the expression of FVII in 293F mammalian cells. Column 5 is the whole cell lysate and column 6 is the soluble fraction. FVII is seen at about 50 kDa. Column 3 shows that FVII degrades in the cell.

5.7 Enrichment of Proteins

A standard Immobilized Metal Affinity Chromatography (IMAC) was used to enrich the proteins from the cell media. The procedure for an IMAC is outlined in Appendix I. 75mM, 150mM, and 300mM washes of imidazole were used to elute TF and fX from the column. A 1M concentration of imidazole was used to wash the column and remove all proteins. Figure 5 shows the peak elution of TF and fX. TF was shown to have a high level of enrichment at the 150mM imidazole solution. FX had a mixed level of purity meaning that some of the elutions had fX and another protein.

FVII was enriched with Benzamidine added to the cell media. The benzamidine was used to prevent proteases from degrading fVII. Figure 6 shows that fVII did not enrich using 100mM, 200mM, 300mM, and 500mM imidazole elutions with a final wash of 1M. The final wash of the column at 1M imidazole shows a significant amount of fVII, indicating that the optimal concentration of imidazole for enrichment is in between 1M and 500mM.

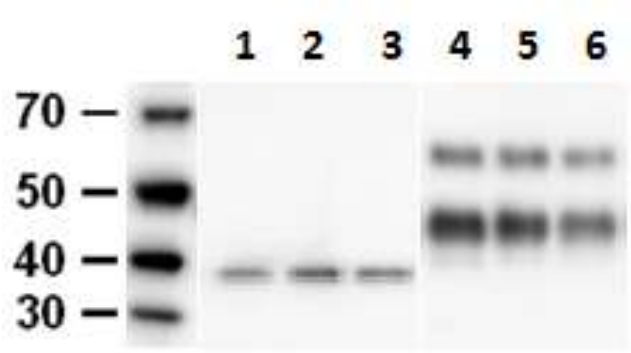


Figure 5: The best elutions of TF and fX. TF is seen in Lanes 1, 2, and 3. The protein is the expected size for TF. FX is seen in Lanes 4, 5, and 6. The protein shows a high level of impurities. The top line matches the expected size for fX.

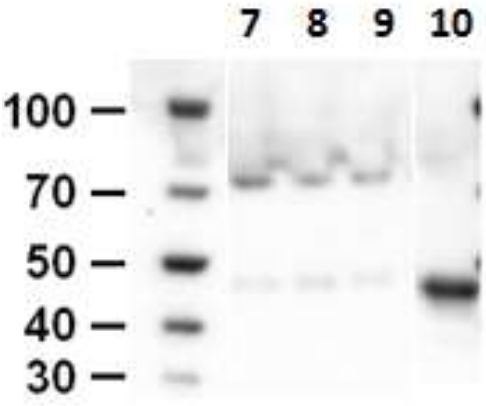


Figure 6: The enrichment of fVII. FVII did not enrich at 500mM solutions of imidazole but did wash out with the 1M wash. Lanes 7, 8, and9 show the best elutions at 200mM, for 7 and 8, and 300mM, for 9.

Chapter 6: Discussion

The following chapter will discuss and evaluate the overall application and influence of this project. It is important to understand the advantages and benefits of the development of the proteins and the design of the coagulation assay.

6.1 Host Cell Line Choice

The expression of the proteins within insect cells as opposed to the more common bacteria cells was because the insect cells were more capable of handling the complex structure of the proteins. Due to complications and challenges associated with the Factor VII protein, mammalian cells were used instead of insect cells. The reason insect cell lines were the first choice over mammalian cells was because of the cost of the cells and the time required to complete the transfection and harvest of proteins expressed in the mammalian cells.

6.2 Template Directed Assembly Liposome

The decision to use template directed assembly (TDA) liposome as the membrane for the proteins is what makes this project novel. TDA liposomes allow for the coagulation assay to accurately mimic the biological function and environment of proteins in the human body better than any other *in vitro* coagulation assay currently on the market. This is achieved by both the make-up of the proteins and the liposomes. The proteins were assembled with His-tags attached to the sequence prior to cloning. These His-tags are negatively charged and the surface of the liposomes is coated with DOGS-Ni-NTA lipids which are positively charged. Due to the opposing polarities of the components, the protein fragments are allowed to align correctly on the surface of the liposome and concentrate into protein clusters that will enhance the high-throughput possibilities of the assay.

6.3 Coagulation Assay Selection

The creation of a coagulation assay was not achieved due to time restrictions and the complications of the factor VII expression and enrichment.

6.4 Economic Impact

If the coagulation assay is successful then it has the potential to become one of the most accurate assays on the market that could be used for high-throughput screening whether it be for diagnostic or drug related purposes.

6.5 Societal Influence

The capability to test for diagnostic and drug delivery would allow for better treatments and faster diagnoses of patients and that it would be a huge help with embolisms as well as other diseases related to clotting.

6.6 Political Ramifications

There were no evident political ramifications associated with this project and no controversial products or materials were used during testing or for the final deliverable.

6.7 Ethical Concerns

The project team used recombinant proteins instead of proteins harvested from human plasma. This alleviates any concerns that there may be for the origin of the cells and proteins used. All products and testing for this project were conducted by the project team and staff of Blue Sky Biotech.

6.8 Health and Safety Issue

For this project there were no considerable safety and/or health issues. During each protocol proper laboratory etiquette and safety was observed. This consisted of using protective gloves, laboratory coats and the fume hoods.

6.9 Sustainability

The development of the proteins and assay was based on commonly used lab equipment and that the usage of these materials would not require any special development, just gathering from outside venders.

6.10 Manufacturability

The overall manufacturability and reproducibility of these proteins and the assay are very high. The protocol for expressing and enriching the proteins was optimized by the project team through experimentation and researching similar protocols, specifically from Waters and Morrissey. The use of His-tags on the protein enabled the enrichment of them using an IMAC system to be extremely efficient. In addition, the His-tags allowed the use of TDA liposomes to act as the membrane the proteins would anchor to.

Chapter 7: Final Design and Validation

The final design was chosen through various test trials to determine the best conditions to express the proteins at as well as the ideal enrichment level. The entirety of the protocol used to achieve the final design can be viewed in Appendix I. All three proteins were harvested at several time points: 24 hours, 48 hours, and 72 hours following Blue Sky Biotech's protocol. Western blot analysis showed that the proteins TF and fX were found to have the best solubility factor at 72 hours and 48 hours respectively within the baculovirus expression vector system. Western blot analysis of fVIIa showed poor solubility in the baculovirus expression system and therefore a mammalian expression system was substituted to express fVIIa. Within the mammalian expression system, samples were harvested at 24 hour intervals for up to 168 hours. Data revealed that samples harvested at 120 hours showed the least amount of degradation with protein expressed at the appropriate size and activity. Once all of the proteins were harvested the chosen samples were all purified. The protocols used for protein purification are presented in in Appendix I.

Chapter 8: Conclusions and Recommendations

TF and fX were able to be expressed in the BEVS and purified. fVII was able to be expressed in a mammalian construct. Due to time constraints and the difficulties with fVII expression and purification, the project group was not able to test the assay protocol.

The project group recommends that expression should be optimized for Factor X and Factor VII. Although expression was detected for these proteins, the process needs to be adapted since not having ideal expression could be problematic for the future commercialization of the assay. Adapting the expression of Factor VII in mammalian cells could lead to a higher fraction of the protein in the soluble fraction of the cell, which could the purification of the protein more efficient. Slight modifications to the host cell line, media, or protein sequence could enhance the protein solubility or prevent proteases from denaturing the protein. A similar problem existed for the production of Factor X. Factor X was present in the soluble fraction of the cell, but the expression levels within the insect cells were not ideal. Expression was sufficient for the development of the assay, but boosting the amount of protein per culture would be desired for commercial production of the assay. Factor Xa was also expressed in the cell, which could cause problems when trying to recreate the coagulation assay *in vitro*. A significant portion of Factor Xa could skew the results to show activation when none is observed.

For the assay portion of the project, the team recommends that to test the effectiveness of the clotting cascade, an assay should be done to test combinations of varying concentrations of each protein. In addition, different doses of anti-clotting and clotting drugs should be tested versus the proteins to quantify and qualify the how effective the assay is at simulating biological responses *in vitro*. To quantify the activity of the proteins the team recommends the use of a microplate reader to measure the optical density (OD) versus different concentrations of one of the proteins when added to control concentrations of the other two.

Currently the proposed assay design is not designed for multiple purposes. In order to maximize the use of the assay for the diagnostic or drug development field, it the team recommends that testing should be taken further. For diagnostics, the assay must be evaluated at a clinical level in order to verify that doctors can diagnose clotting disorders effectively. For drug development, the assay needs to be tested using many different drugs that are known to have an effect on the clotting ability of blood.

The tissue factor pathway studied in this report is not the only clotting pathway for blood. The intrinsic pathway uses a few different proteins, Factor V, Factor VIII and Factor IX, to control the clotting

of blood without the rupturing of the endothelial membrane. Although several different diseases affect the tissue factor pathway, the intrinsic pathway is affected by several more. Hemophilia, van Willebrand's disease, and amyloidosis are three such diseases. By expanding the assay to include the intrinsic pathway, the assay could be used for more versatile diagnostic testing. Overall, the coagulation assay should be optimized for diagnostic and drug development uses.

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Appendix

Appendix A

Table 7: Gantt Chart

Task Name	Duration	Start	Finish
A Term Deliverables	**22 days**	**Sat 9/10/11**	**Fri 10/7/11**
B Term Deliverables	**34 days**	**Tue 10/25/11**	**Fri 12/9/11**
C Term Deliverables	**32 days**	**Thu 1/12/12**	**Fri 2/24/12**
Introduction	**22 days**	**Sat 9/10/11**	**Fri 10/7/11**
Literature Review	**38 days**	**Sat 9/10/11**	**Mon 10/31/11**
Design Section	**22 days**	**Sat 9/10/11**	**Fri 10/7/11**
Methodology	**126 days**	**Fri 10/7/11**	**Fri 3/30/12**
B Term	**70 days**	**Fri 10/7/11**	**Thu 1/12/12**
Order protein	1 day	Mon 12/5/11	Mon 12/5/11
Reverse Translation	11 days	Tue 12/6/11	Tue 12/20/11
Gene Assembly	14 days	Tue 12/20/11	Fri 1/6/12
C Term	**37 days**	**Thu 1/12/12**	**Fri 3/2/12**
Cell Maintenance	8 days	Fri 1/20/12	Tue 1/31/12
Transfection of Cells	5 days	Tue 1/31/12	Mon 2/6/12
Virus Stock Production	2 days	Mon 2/6/12	Tue 2/7/12
Infection	4 days	Fri 2/10/12	Wed 2/15/12
Western Blots and SDS PAGE	4 days	Thu 2/16/12	Tue 2/21/12
Redo and Harvest fX at 48 hrs	9 days	Tue 2/21/12	Fri 3/2/12
Harvest TF at 72 hrs	9 days	Tue 2/21/12	Fri 3/2/12
Produce fVII in Mammalian	9 days	Tue 2/21/12	Fri 3/2/12
Research	**111 days**	**Fri 10/28/11**	**Fri 3/30/12**
Grow Cell Lines with Baculovirus	23 days	Fri 1/20/12	Tue 2/21/12
Test Assay According to Methodology	22 days	Thu 3/1/12	Fri 3/30/12
Adjust for Compatibility with Automation Machines	6 days	Fri 3/23/12	Fri 3/30/12
Results	**112 days**	**Sat 10/29/11**	**Mon 4/2/12**
Discussion	**23 days**	**Thu 3/1/12**	**Mon 4/2/12**
Final Paper			Mon 4/2/12
Final Presentation			Thu 4/19/12
Final Deliverables			Mon 4/23/12

Appendix B

Table 8: Linear Responsibility Chart

Column1	Chris	Jetta	Alex	Brie	Client	Advisor
1.0 Introduction	1	2	2	2	4	5
2.0 Literature review	2	1	2	2	4	5
3.0 Design section	2	2	1	2	4	5
4.0 Understand client statement	1	1	1	1	4	5
5.0 Develop objectives tree	2	2	2	1	5	5
6.0 Analyze function requirements					3	5
7.0 Generate alternative designs	2	2	1	2	5	5
8.0 Evaluate alternative	1	1	1	1	4	5
8.1 Select preferred design	1	1	1	1	3	5
8.2 Weigh objectives	1	1	1	1	4	5
9.0 Project management	2	1	2	2	2	4
Key:						
1= Primary responsibility						
2= Support/work						
3= Must be consulted						
4= May be consulted						
5= Review						
6= Final approval						

Appendix C

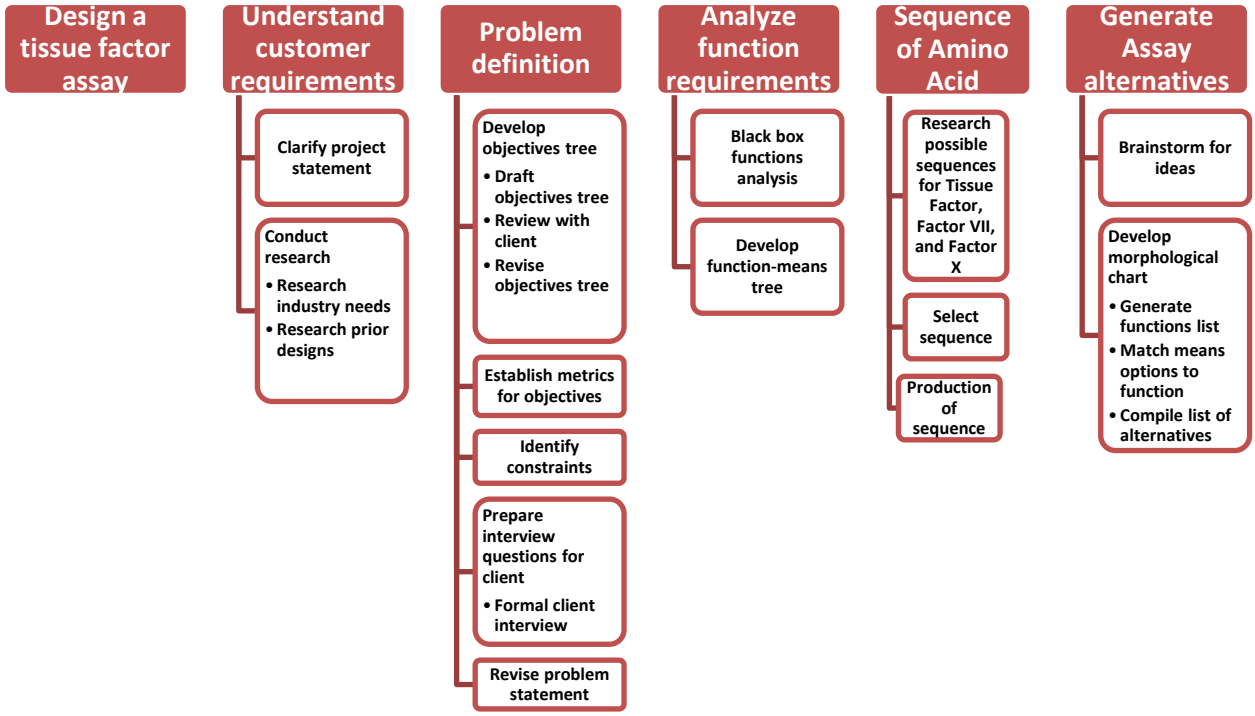

Figure 4: Work Breakdown Structure

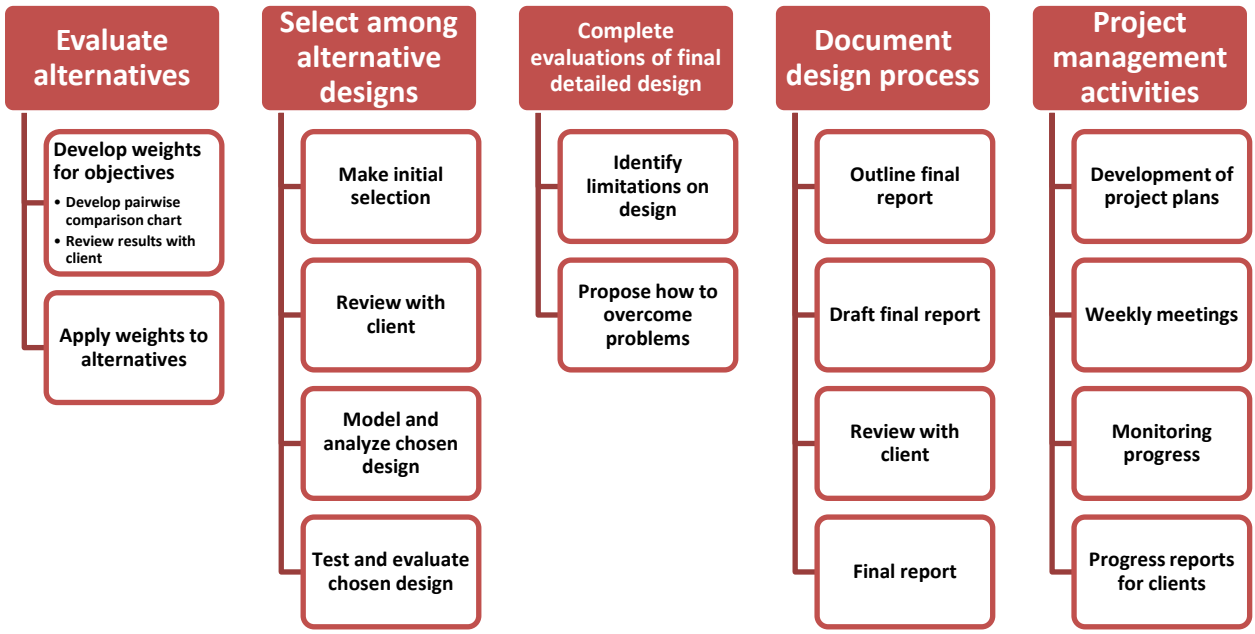

Figure 5: Work Breakdown Structure

Appendix D

Cloning steps for each protein:

Chromogenic Assay Steps:

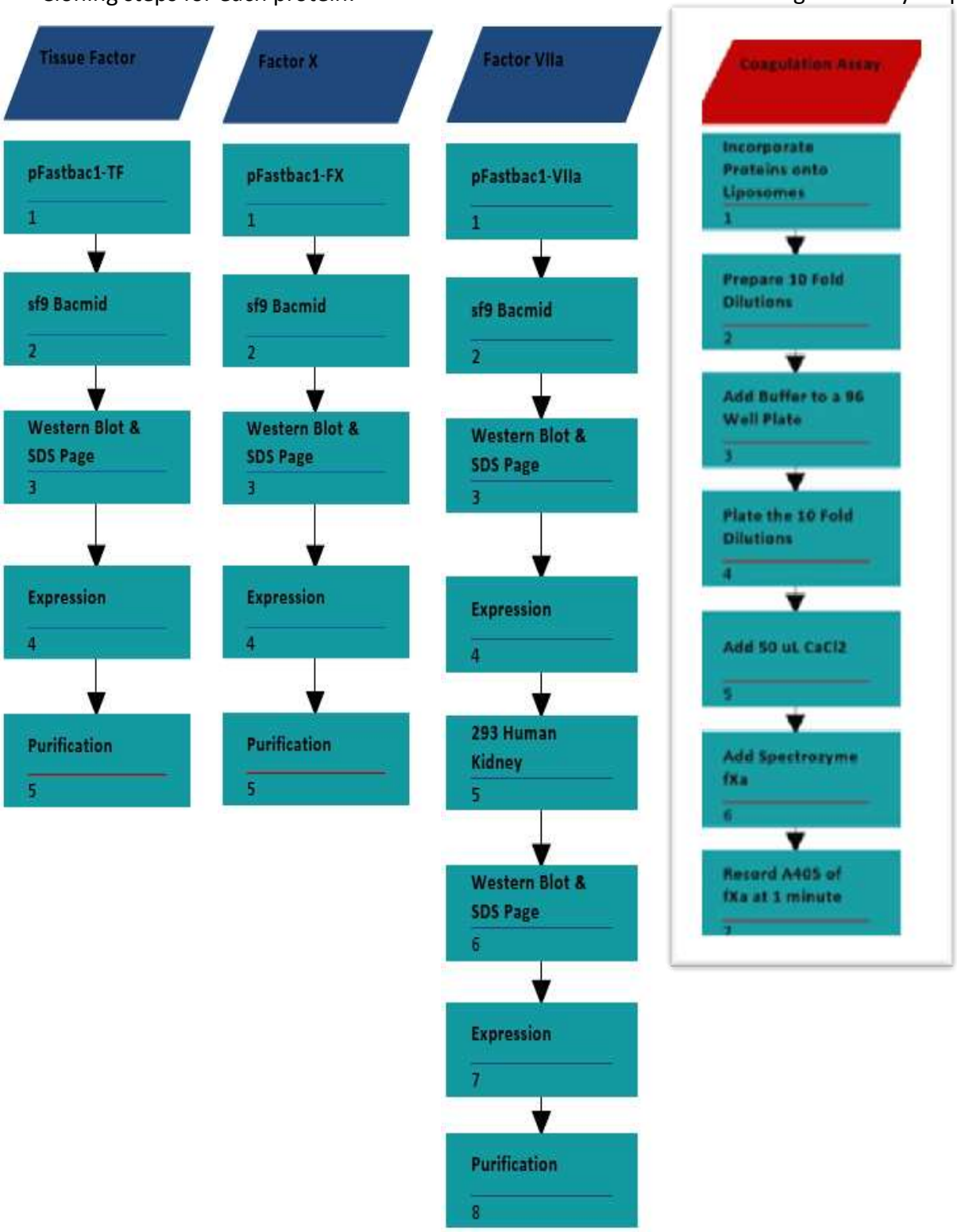

Figure 6: Process Flow Diagram

Appendix E

Table 9: Design Budget Table

Budget Table		
Item	Reason for buying	Amount Spent
***Nothing bought in A term**		
***Nothing bought in B term**		
Chromogenic Substrate Xa	Component for assay	$389.28

Appendix F

Sequences Used

Tissue Factor

METPAWPRVP RPETAVARTL LLGWVFAQVA GASGTTNTVA AYNLTWKSTN FKTILEWEPK PVNQVYTVQI STKSGDWKSK CFYTTDTECD LTDEIVKDVK QTYLARVFSY PAGNVESTGS AGEPLYENSP EFTPYLETNL GQPTIQSFEQ VGTKVNVTVE DERTLVRRNN TFLSLRDVFG KDLIYTLYYW KSSSSGKKTA KTNTNEFLID VDKGENYCFS VQAVIPSRTV NRKSTDSPVE CMGQEKGEFR EIFYIIGAVV FVVIILVIIL AISLHKCRKA GVGQSWKENS PLNVS SGGGAAGHHHHHHHHSG

Factor VII

MVSQALRLLC LLLGLQGCLA AGGVAKASGG ETRDMPWKPG PHRVFVTQEE AHGVLHRRRR ANAFLEELRP GSLERECKEE QCSFEEAREI FKDAERTKLF WISYSDGDQC ASSPCQNGGS CKDQLQSYIC FCLPAFEGRN CETHKDDQLI CVNENGGCEQ YCSDHTGTKR SCRCHEGYSL LADGVSCTPT VEYPCGKIPI LEKRNASKPQ GRIVGGKVCP KGECPWQVLL LVNGAQLCGG TLINTIWVVS AAHCFDKIKN WRNLIAVLGE HDLSEHDGDE QSRRVAQVII PSTYVPGTTN HDIALLRLHQ PVVLTDHVVP LCLPERTFSE RTLAFVRFSL VSGWGQLLDR GATALELMVL NVPRLMTQDC LQQSRKVGDS PNITEYMFCA GYSDGSKDSC KGDSGGPHAT HYRGTWYLTG IVSWGQGCAT VGHFGVYTRV SQYIEWLQKL MRSEPRPGVL LRAPF SGGGAAGHHHHHHHHSG

Factor X

MGRPLHLVLL SASLAGLLLL GESLFIRREQ ANNILARVTR ANSFLEEMKK HLERECMEE TCSYEEAREV FEDSDKTNEF WNKYKDGDQC ETSPCQNQGK CKDGLGEYTC TCLEGFEGKN CELFTRKLCS LDNGDCDQFC HEEQNSVVCS CARGYTLADN GKACIPTGPY PCGKQTLERR KRSVAQATSS SGEAPDSITW KPYDAADLDP TENPFDLLDF NQTQPERGDN NLTRIVGGQE CKDGECPWQA LLINEENEGF CGGTILSEFY ILTAAHCLYQ AKRFKVRVGD RNTEQEEGGE AVHEVEVVIK HNRFTKETYD FDIAVLRLKT PITFRMNVAP ACLPERDWAE STLMTQKTGI VSGFGRTHEK GRQSTRLKML EVPYVDRNSC KLSSSFIITQ NMFCAGYDTK QEDACQGDSG GPHVTRFKDT YFVTGIVSWG EGCARKGKYG IYTKVTAFLK WIDRSMKTRG LPKAKSHAPE VITSSPLK SGGGAAGHHHHHHHHSG

Appendix G

Oligonucleotides

Tissue Factor

1 ATGGAAACCCCAGCGTGGCCCCGTGTCCCTAG
2 GTTCGAGCAACGGCGGTTTCGGGCCTAGGGACACGGGGCC
3 CCGCCGTTGCTCGAACCTTGCTGCTGGGCTGGGTGTTTGC
4 TGCCGCTGGCACCGGCCACCTGAGCAAACACCCAGCCCAG
5 CGGTGCCAGCGGCACGACGAACACCGTGGCTGCCTACAAC
6 AAGTTGGTCGACTTCCAGGTCAGGTTGTAGGCAGCCACGG
7 ACCTGGAAGTCGACCAACTTCAAGACCATTCTGGAGTGGG
8 CTGGTTCACGGGCTTGGGCTCCCACTCCAGAATGGTCTTG
9 CCAAGCCCGTGAACCAGGTGTATACGGTGCAGATCAGCAC
10 TTTTCCAATCGCCGCTCTTCGTGCTGATCTGCACCGTATA
11 AAGAGCGGCGATTGGAAAAGCAAGTGCTTCTACACCACTG
12 GGTCAGATCGCACTCCGTATCAGTGGTGTAGAAGCACTTG
13 ACGGAGTGCGATCTGACCGATGAGATCGTGAAGGATGTGA
14 CGTGCGAGGTAGGTCTGTTTCACATCCTTCACGATCTCAT
15 ACAGACCTACCTCGCACGCGTATTCAGCTACCCTGCTGGC
16 GCGCTACCGGTGCTCTCCACGTTGCCAGCAGGGTAGCTGA
17 AGAGCACCGGTAGCGCAGGCGAGCCCTTGTATGAGAACTC
18 GTAGGGGGTGAACTCGGGGGAGTTCTCATACAAGGGCTCG
19 CCCGAGTTCACCCCCTACCTGGAGACCAACCTCGGCCAAC
20 CTGTTCGAAGCTCTGAATGGTGGGTTGGCCGAGGTTGGTC
21 ACCATTCAGAGCTTCGAACAGGTGGGCACCAAGGTGAACG
22 CGTCCGCTCATCTTCCACGGTCACGTTCACCTTGGTGCCC
23 GTGGAAGATGAGCGGACGCTGGTCCGCCGGAACAACACTT
24 GAACACGTCGCGGAGCGACAAAAAAGTGTTGTTCCGGCGG
25 GCTCCGCGACGTGTTCGGCAAGGACCTGATCTACACGCTG
26 CGAGCTGCTCTTCCAGTAGTACAGCGTGTAGATCAGGTCC
27 CTACTGGAAGAGCAGCTCGTCCGGAAAGAAGACCGCCAAA
28 CAGGAACTCGTTTGTGTTGGTTTTGGCGGTCTTCTTTCCG
29 CCAACACAAACGAGTTCCTGATCGATGTGGATAAGGGCGA
30 TGCACGCTGAAGCAGTAGTTCTCGCCCTTATCCACATCGA
31 CTACTGCTTCAGCGTGCAGGCAGTCATCCCCTCGCGCACC
32 GGGAATCAGTGCTCTTGCGGTTAACGGTGCGCGAGGGGAT
33 CGCAAGAGCACTGATTCCCCCGTGGAGTGCATGGGCCAAG
34 GATCTCGCGGAACTCGCCTTTCTCTTGGCCCATGCACTCC
35 GCGAGTTCCGCGAGATCTTTTACATTATCGGCGCGGTCGT
36 TCACGAGGATGATCACTACGAAAACGACCGCGCCGATAAT
37 TCGTAGTGATCATCCTCGTGATAATCCTGGCCATCTCGCT
38 CGCCAGCCTTGCGGCATTTGTGCAGCGAGATGGCCAGGAT
39 CCGCAAGGCTGGCGTGGGCCAGTCGTGGAAAGAGAATAGT
40 GCTGCTCACGTTGAGGGGACTATTCTCTTTCCACGACTGG
41 CCCTCAACGTGAGCAGCGGCGGTGGCGCTGCGGGTCACCA
42 GCCGCTATGATGATGGTGGTGATGATGGTGACCCGCAGCG

Factor VII

1 ATGGTAAGCCAGGCCCTGCGACTGCTGTGCCTCCTGTTG
2 CGGCGAGGCATCCCTGCAAGCCCAACAGGAGGCACAGCAG
3 GGGATGCCTCGCCGCTGGTGGCGTGGCGAAAGCCAGTGGC
4 CCACGGCATATCGCGGGTCTCTCCGCCACTGGCTTTCGCC
5 CCGCGATATGCCGTGGAAACCCGGTCCCCACCGAGTGTTC
6 CGTGTGCTTCTTCCTGGGTTACGAACACTCGGTGGGGACC
7 CCAGGAAGAAGCACACGGTGTATTGCACCGAAGGCGTCGC
8 GCAATTCCTCCAGAAAGGCATTTGCGCGACGCCTTCGGTG
9 GCCTTTCTGGAGGAATTGCGTCCTGGCTCGTTGGAACGCG
10 AGCTGCACTGCTCCTCTTTACATTCGCGTTCCAACGAGCC
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21 GCGTCAATGAGAATGGAGGTTGTGAGCAATACTGCAGCGA
22 GGAGCGCTTCGTGCCGGTGTGATCGCTGCAGTATTGCTCA
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24 CAGCTGACTCCATCCGCCAGCAGGGAATAGCCCTCGTGGC
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55 CACCTGGTACTTGACCGGCATCGTATCGTGGGGCCAGGGT
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57 GTTGGCCACTTCGGCGTATACACACGGGTCAGCCAATACA
58 CATCAGTTTCTGCAGCCACTCAATGTATTGGCTGACCCGT
59 AGTGGCTGCAGAAACTGATGCGCAGCGAACCGCGTCCCGG
60 GCTGAAGGGAGCGCGCAGGAGAACGCCGGGACGCGGTTCG
61 CGCGCTCCCTTCAGCGGTGGAGGTGCGGCAGGCCACCACC
62 GCCGCTGTGGTGATGATGATGATGGTGGTGGCCTGCCG

Factor X

1 ATGGGTAGGCCCTTGCACTTGGTACTCCTCAGCGCC
2 AGCAGCAGGAGTCCGGCCAAGCTGGCGCTGAGGAGTACCA
3 CCGGACTCCTGCTGCTGGGTGAGTCCCTGTTCATCCGCCG
4 GCGAGTATGTTGTTCGCTTGTTCTCGGCGGATGAACAGGG
5 AAGCGAACAACATACTCGCCCGTGTAACGCGGGCCAACTC
6 GCTTCTTCATCTCCTCCAGGAACGAGTTGGCCCGCGTTAC
7 CCTGGAGGAGATGAAGAAGCATCTCGAACGGGAGTGCATG
8 TCCTCATAGCTGCAGGTTTCTTCCATGCACTCCCGTTCGA
9 GAAACCTGCAGCTATGAGGAGGCACGCGAAGTGTTTGAGG
10 AAAACTCATTGGTCTTATCGCTGTCCTCAAACACTTCGCG
11 CGATAAGACCAATGAGTTTTGGAACAAGTACAAGGATGGC
12 GGGCTGGTCTCGCACTGATCGCCATCCTTGTACTTGTTCC
13 AGTGCGAGACCAGCCCCTGTCAGAACCAGGGCAAATGTAA
14 TGTATTCGCCGAGGCCGTCTTTACATTTGCCCTGGTTCTG
15 CGGCCTCGGCGAATACACCTGCACCTGCCTCGAAGGCTTT
16 ACAGCTCGCAGTTCTTGCCTTCAAAGCCTTCGAGGCAGGT
17 GCAAGAACTGCGAGCTGTTCACTCGCAAGTTGTGCAGCCT
18 ACTGGTCGCAGTCGCCGTTATCGAGGCTGCACAACTTGCG
19 GGCGACTGCGACCAGTTTTGTCACGAGGAGCAGAACTCCG
20 CCGCGCGCGCACGAGCACACAACGGAGTTCTGCTCCTCGT
21 CGTGCGCGCGCGGTTATACGCTGGCTGACAACGGCAAGGC
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45 GTTCCGCATGAATGTGGCACCAGCATGTCTGCCAGAGCGC
46 TCATCAGGGTACTCTCAGCCCAATCGCGCTCTGGCAGACA
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63 CGGGGTCTCCCGAAAGCAAAAAGTCACGCTCCCGAGGTCA
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Appendix H

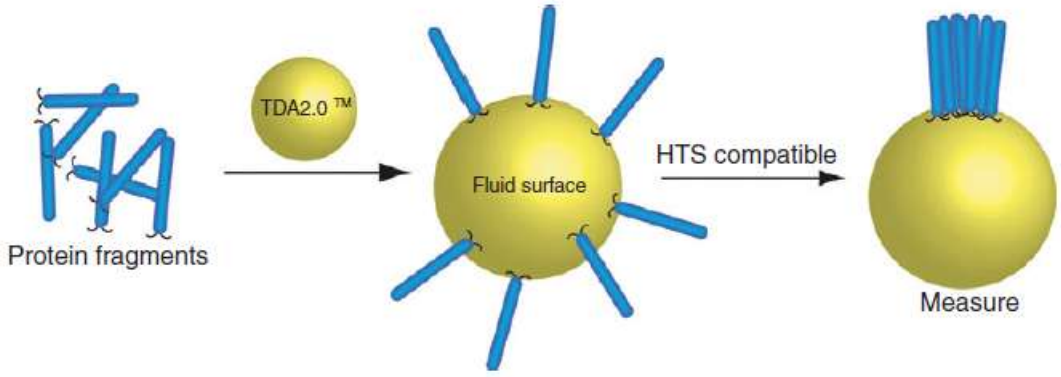


Figure 7: TDA process

Protein fragments bind to the template surface and are able to move in the same manner as they would in a cell due to the fluid surface of the TDA (Gridley, 2010).

Appendix I

1 Baculovirus Expression Vector Systems Procedure

The baculovirus expression vector system (BEVS) consists of three major steps: growing the insect cells, introducing the baculovirus, and purifying the proteins. There are several different methods to accomplish each of these steps; however, due to the cost of these methods, there is only one that will be utilized for this project. For growing the insect cells a suspension culture will be used; for the baculovirus preparation, the Concert high purity plasmid purification system will be used; and for the protein purification, the purification of intracellular proteins will be used. The following procedures were taken from 'Invitrogen Life Technologies' "Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques."

1.1 Insect Cell Growth

The following protocol will be used for the growth of insect cells in a suspension medium.

Table 10: Materials Needed for Growth of Insect Cell in Suspension

Materials and Equipment	Amount
Disposable Erlenmeyer Flasks	125-, 250-, or 500-ml
Glass Spinner Flasks	125- or 250-ml
Orbital Shaker for Erlenmeyer Flasks	Shaking speed of 150 rpm
Stirring Platform	90-100 rpm
Pluronic F-68, 10%	10ml/L

Either the orbital shaker or the stirring platform may be used to grow the cells. The glass spinner flasks are used with the stirring platform and the Erlenmeyer flasks are used with the orbital shaker.

1.2 Procedure

1. The following procedure is taken from Protocol 3: Maintaining Suspension Cultures from "Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques."Maintain the orbital shaker or stirring platform in a 27°C ± 0.5°C, nonhumidified, non-CO2 equilibrated, ambient-air regulated incubator or warm room. For cultures already adapted to and maintained in suspension culture, set orbital shaker at 135 to 150 rpm and spinner platforms at 90 to 100 rpm.
2. Dilute a 1- to 2- ml sample of sf9 cells from a 3- to 4-day old suspension culture to 3 X 10^5.

3. Add 10 ml/L of Pluronic F-68 if the cells are in serum-supplemented cultures. This is done to prevent cellular damage to shear forces.
4. Incubate cultures until they reach 2×10^6 to 3×10^6 viable cells/ml. To maintain consistent and optimal cell growth, subculture suspension cultures twice weekly.
5. Once every 3 weeks, gently centrifuge the cell suspension at 100 × g for 5 min. Resuspend the cell pellet in fresh medium to reduce the accumulation of cell debris and metabolic byproducts.

2 Baculovirus Purification and Transfection

Before the transfection of the baculovirus may take place, a series of steps must be taken to ensure that the baculovirus is purified and carrying the DNA for Factor III, Factor VII, and Factor X. Protocols 6, 7, and 8 from "Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques" will be used to purify the baculovirus and prepare them for transfection.

3 Bacmid DNA Isolation

A Concert high purity plasmid purification system will be used to purify the baculovirus DNA for a Bac-to-Bac baculovirus expression system. Several materials are needed for this purification process.

Table 11: Materials Necessary for Bacmid DNA isolation

Materials and Equipment	Amount
LB kan, gent, tet broth	2 ml
Shaking water bath	250 rpm
Cell Suspension Buffer (E1) with RNase A	0.4 ml
Cell Lysis Solution (E2)	0.4 ml
Equilibration Buffer (E4)	2 ml
Wash Buffer (E5)	2 x 2.5 ml
Elution Buffer (E6)	0.9 ml
Column	
Microcentrifuge	
Isopropanol	0.63 ml
Ethanol 70%	1 ml

TE Buffer (TE)	40 µl
Luria Agar Plates (Miller's Formulation)	

3.1 Procedure

1. Before beginning: Verify that no precipitate has formed in Cell Lysis Solution (E2.) If the solution E2 is too cold, the SDS will precipitate out of solution. Note: Make sure you have added RNase A to Cell Suspension Buffer (E1.)
2. Column Equilibration: Apply 2 ml of Equilibration Buffer (E4) [600 mM NaCl, 100 mM sodium acetate (pH 5.0), 0.15% Triton X-100] to the column. Allow the solution in the column to drain by gravity flow.
3. Cell Harvesting: Pellet 1.5 ml of an overnight culture. Thoroughly remove all medium.
4. Cell Suspension: Add 0.4 ml of Cell Suspension Buffer (E1) [50mM Tris-HCl (pH 8.0), 10 mM EDTA, containingRNase A at 0.2 mg/ml] to the pellet and suspend cells until homogeneous.
5. Cell Lysis: Add 0.4 ml of Cell Lysis Solution (E2) [200 mM NaOH, 1% SDS]. Mix gently by inverting the capped tube five times. Do not vortex. Incubate at room temperature for 5 min.
6. Neutralization: Add 0.4 ml of Neutralization Buffer (E3) [3.1 M potassium acetate (pH 5.5)] and mix immediately by inverting the tube five times. Do not vortex. Centrifuge the mixture at top speed in a microcentrifuge at room temperature for 10 min. Do not centrifuge at 4°C.
7. Column Loading: Pipet the supernatant from step 12 onto the equilibrated column. Allow the solution in the column to drain by gravity flow. Discard flow-through.
8. Column Wash: Wash the column two times with 2.5 ml of Wash Buffer (E5) [800 mM NaCl, 100 mM Sodium acetate (pH 5.0)]. Allow the solution in the column to drain by gravity flow after each wash. Discard flow-through.
9. Plasmid DNA Elution: Elute the DNA by adding 0.9 ml of Elution Buffer (E6) [1.25 M NaCl, 100 mM Tris-HCl (pH 8.5)]. Allow the solution in the column to drain by gravity flow. Do not force out remaining solution.
10. Plasmid DNA Precipitation: Add 0.63 ml ofisopropanol to the eluate. Mix and place on ice for 10 min. Centrifuge the mixture at top speed in a microcentrifuge at room temperature for 20 min. Carefully discard supernatant. Wash the plasmid DNA pellet with 1 ml of ice cold 70% ethanol and centrifuge for 5 min. Carefully and fully pipet off the ethanol wash. Air dry the pellet for 10 min.

11. Purified DNA: Dissolve the pelleted DNA in 40 µl of TE Buffer (TE) [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]. Allow DNA to dissolve for at least 10 min on ice. To avoid DNA shearing, pipet DNA only 1-2 times during resuspension. Bacmid DNA can be stored at -20°C, but avoid repeated freeze/thawing. Use 5 µl of this bacmid preparation for transfection of insect cells.

4 Cationic Liposome-Mediated Transfection

This process is taken from Protocol 7 and requires the following materials:

Table 12: Materials Necessary for Cationic Liposome-Mediated Transfectio

Materials and Equipment	Amount
Sterile Tubes 12 x 75 mm	2 per well
Tissue Culture Plates 6 wells	
Cell Fectin Reagent	1.5 to 9 µL
Penicillin/Streptomycin/Neomycin 0.5X	
Sf9 cells at 5 x 10^5 viable cells/ml	
Sf-900 II SFM or Express-Five SFM	6 ml per well

4.1 Procedure

1. In a 6-well tissue culture plate, seed 9 × 105 Sf9 cells per well in 2 ml of Sf-900 II SFM or 9 × 105 BTI-TN-5B1-4 cells per well in 2 ml of EXPRESS-FIVE SFM (with antibiotics).
2. Incubate the plate at 28°C for at least 1 h to allow cells to attach.
3. In two 12 × 75-mm sterile tubes, prepare the following solutions.
    a. Solution A: For each transfection, dilute 1 to 2 µg baculovirus DNA and 5 µg transfer vector of choice into 100 µl Sf-900 II SFM or EXPRESS-FIVE SFM without antibiotics.
    b. Solution B: For each transfection, dilute 1.5 to 9 µl CELLFECTIN Reagent into 100 µl Sf-900 II SFM or EXPRESS-FIVE SFM without antibiotics.
4. Add Solution B to the tube containing Solution A, mix gently, and incubate at room temperature for 15 min.
5. While lipid/DNA complexes are forming, wash the Sf9 cells from step 2 once with 2 ml per well of Sf-900 II SFM without antibiotics.
6. Add 0.8 ml Sf-900 II SFM to each tube containing lipid/DNA complexes. Mix gently. Aspirate the wash medium, and overlay the diluted lipid/DNA complexes onto the washed cells.

7. Incubate for 5 h in a 27°C incubator.
8. Remove the transfection mixture. Add 2 ml Sf-900 II SFM or EXPRESS-FIVE SFM (containing antibiotics) per well or dish and incubate at 27°C for 72 h.
9. Harvest the virus from the cell culture medium at 72 h post-transfection.

5 Creation of Virus Stock

Before the proteins are purified from the transfected insect cells, a stock of the baculovirus will be created and stored for future use. Protocol 9 will be used to create the stock.

Table 13: Materials to Create a Virus Stock

Materials and Equipment	Amount
Sealable Plastic Container (4x8x8 in.)	1
Tissue Culture Plates, 6-well	
Sf9 cells at 5 x 10^5 viable cells/ml	
Serum-free or Serum Supplemented insect medium	
Centrifuge	

5.1 Procedure

1. Seed each well with 2 ml Sf9 or BTI-5B1-4 cell suspension, at 5 × 105 viable cells/ml in fresh medium.
2. Mark the plates containing plaques below putative recombinants. For assistance in identifying recombinants, see Identifying the Plaques.
3. Under sterile conditions, remove plugs of the overlay from the selected plaques. Transfer one plug to each well of a multi-well plate.
4. Incubate the plate in a humidified chamber at 27°C.
5. Examine the wells daily for signs of infection and absence of polyhedra.
6. At day 4 or 5, harvest the supernatant. At this point, you may screen and confirm that the recombinant viruses are producing the gene of interest.
7. Following Protocol 8, replaque 10-1 to 10-3 dilutions of these supernatants. Note: It is not necessary to prepare the full range (10-1 to 10-8) of serial dilutions. Repeat the plaque purification of the recombinant virus twice and determine virus titers.

8. Amplify confirmed purified producers in either monolayer or shaker infections at a multiplicity of infection (MOI) of 0.1 to 0.01 as described in Protocol 10. Store stocks at 4°C for up to 1 year, protected from light.

6 Purifying Recombinant Protein

Factors III, VII, and X are all intracellular proteins so a process that removes the cells from the desired proteins is used. Since the proteins have HIS-tags, a special form of chromatography called Immobilized Metal Affinity Chromatography (IMAC) can be used. Before IMAC is used the proteins need to be separated from cells. This is typically done by lysing the cells and separating the dead cells from lighter components through centrifugation. The following procedure is adapted from "Immobilized metal affinity chromatography of DNA," an article about the combined use of PCR and IMAC for the production of oligonucleotides.

Table 14: Materials Necessary to Purify the Recombinant Proteins

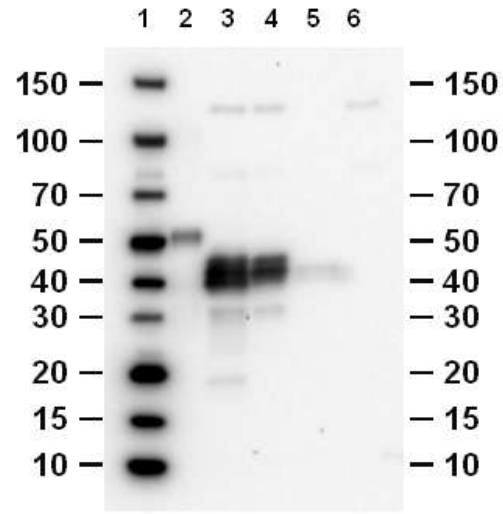
Materials and Equipment	Amount
Chromatography Column	1
1.5 ml Eppendorf Tubes	
Ni^{2+} -NTA-Agarose Resin	Bed Volume of Chromatography Column
Binding Buffer (6 M guanidine HCL, 10 mM Tris-HCL)	1.150 ml
Washing Buffer (10 mM Tris-HCl, 5 mM imidazole)	1 ml
200 mM Imidazole	1.2 ml
100 mM Ethanolic 1,10-phenanthroline	30 µl
3 M aqueous NaOAc	30 µl
Ethanol Absolute	900 µl
Ethanol 80%	200 µl
TE Buffer	50 µl
Centrifuge	

6.1 Procedure

1. In a 1.5 ml Eppendorf tube, suspend the protein recovered from cell lyses in 150 µl of binding buffer. Heat the mixture at 90°C for 5 minutes.
2. In a separate 1.5 ml Eppendorf tube, add 250 µl (or the bed volume of the chromatography column) of Ni^{2+} -NTA-Agarose Resin and 1 ml of binding buffer.
3. Add the protein supernatant to the suspended resin and mix for 1 to 1.5 minutes by pipetting or vigorous shaking.
4. Transfer the mixture to the chromatography column. Collect the flow-through of the column in an Eppendorf tube.
5. Mix the flow-through by pipetting and split it into several Eppendorf tubes at 300 µl a piece. Set these tubes aside for later. These are the unbound fractions.
6. Wash the resin with 1 ml of washing buffer. The wash is discarded.
7. 1.2 ml of the 200 mM imidazole solution is passed through the column to collect the tagged proteins. Collect the flow-through and split it into Eppendorf tubes at 300 µl a piece. These are the bound fractions.
8. Add 30 µl of 100 mM ethanolic 1,10-phenanthroline and 30 ml 3 M aqueous NaOAc to the bound fractions.
9. Add 900 µl of absolute ethanol to the unbound and bound fractions. Vortex the mixtures briefly and chill them for 30 minutes on dry powdered CO_2.
10. Centrifuge the tubes for 30 minutes at 16,000 g.
11. Remove the supernatant and wash the remaining pellet with 200 µl 80% ethanol.
12. Add 50 µl of TE buffer to each tube. Keep in storage at 4°C. The concentration of the proteins can be determined by UV spectrometry.

Appendix J

Western Blot Data for TF:



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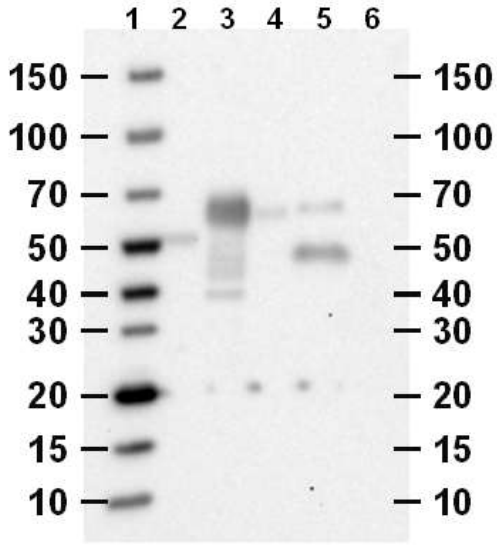

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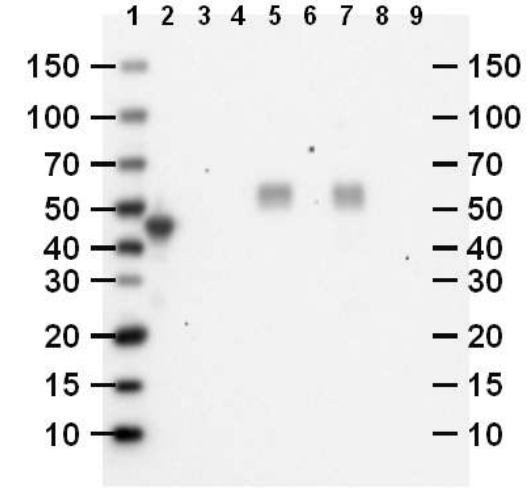
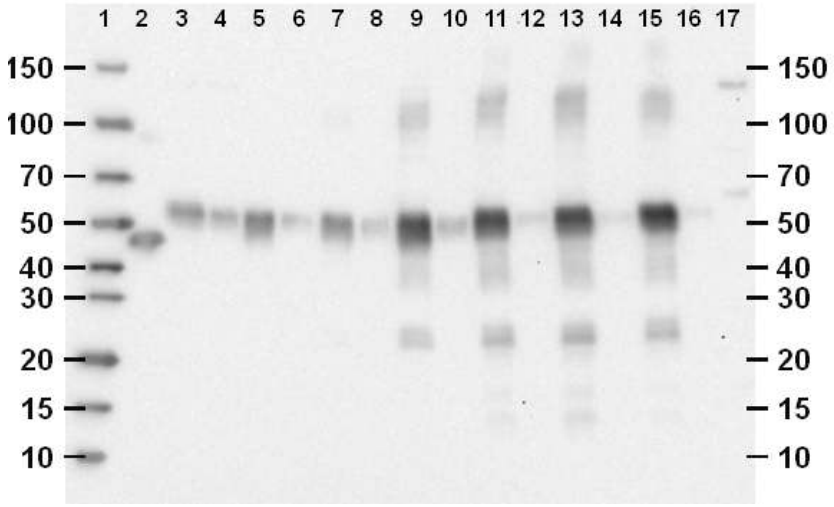
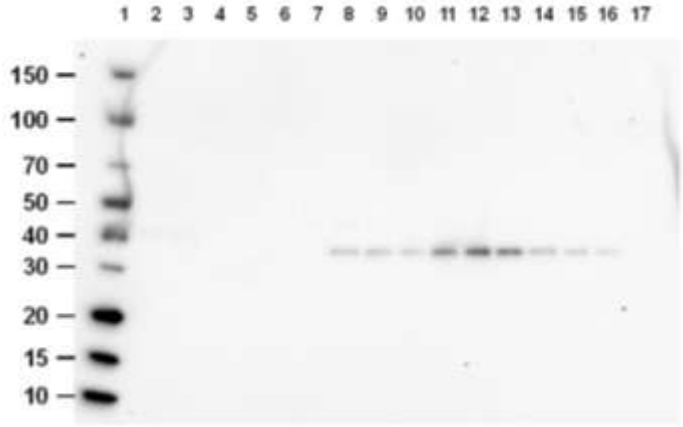


Western Blot Data for FVII in 293 Mammalian Cells:



Appendix K

Western Blot of TF Enrichment:



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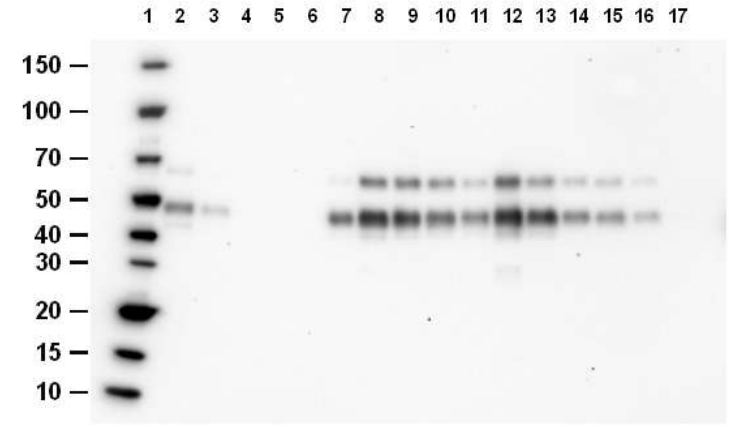

Western Blot of FVII Enrichment: