7-2014

Characterization of Lipid-Anchored Inhibitor Rulers as a Measure of Enzyme Topography in Coagulation Enzyme Factor Xa

Robin C. Conley

Philadelphia College of Osteopathic Medicine, conleyrc@acomedu.org

Follow this and additional works at: http://digitalcommons.pcom.edu/biomed

Part of the Biochemistry, Biophysics, and Structural Biology Commons, and the Medicine and Health Sciences Commons

Recommended Citation


This Thesis is brought to you for free and open access by the Student Dissertations, Theses and Papers at DigitalCommons@PCOM. It has been accepted for inclusion in PCOM Biomedical Studies Student Scholarship by an authorized administrator of DigitalCommons@PCOM. For more information, please contact library@pcom.edu.
Characterization of Lipid-Anchored Inhibitor Rulers as a Measure of Enzyme Topography in Coagulation Enzyme Factor Xα.

By:

Robin C. Conley

Department of Basic Sciences

Philadelphia College of Osteopathic Medicine, Georgia Campus

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Biomedical Science

May 2013
PCOM Biomedical Sciences Degree Programs

Signatory Page for Master's Thesis

We approve the thesis of Robin Christina Conley.

Kimberly J. Baker, PhD
Assistant Professor of Biochemistry
Thesis Advisor

Francis E. Jenney, Jr., PhD
Associate Professor of Biochemistry

Harold Komiskey, PhD
Professor of Neuroscience, Physiology, and Pharmacology

Brian M. Matayoshi, PhD
Graduate Program Director and Professor of Physiology
Table of Contents

I. List of Figures
II. List of Tables
III. Abstract
IV. Introduction
   i. Hemostasis
   ii. Platelets
   iii. Vasculature Walls
   iv. Coagulation Factors
   v. The Blood Coagulation Cascade
   vi. Extrinsic Pathway
   vii. Intrinsic Pathway
   viii. Cell-Based Model of Coagulation
   ix. Factor Structure
   x. Cascade Regulation
   xi. Traditional Therapies and Treatments
   xii. The Role of Kunitz Inhibitors
   xiii. The Importance of Factor X
   xiv. fXa Topography

V. Research Design
   i. Current Information and Fluorescence Studies
   ii. Preliminary Studies and Results
      a) Proof-of-concept using lipid-tethered inhibitors
      b) Construction of a base vector
      c) Expression and purification of an active inhibitor
      d) Relipidation of recombinant proteins

VI. Specific Aims
   i. Specific Aim 1
   ii. Specific Aim 2

VII. Materials, Methods, and Results
VIII. Discussion
IX. Acknowledgements
X. Appendices
   i. Appendix 1: Truncated DNA of pET11d vector system with HindIII, Ncol, and BamHI sites
   ii. Appendix 2: DNA and protein sequence of BPTI
   iii. Appendix 3: Primers
   iv. Appendix 4: Plasmids
   v. Appendix 5: Sequencing Data

XI. References
List of Figures

I. GPI Complex
II. TF Interaction on the Cell Surface during Coagulation
III. Factor Complexes
IV. General Overview of Hemostasis
V. TF/fVIIa Complex on Membrane Surface
VI. Overview of Cell-Based Coagulation—Initiation, Propagation, and Amplification
VII. FVIIa Ribbon Diagram
VIII. General fXa structure
IX. FXa Catalytic Head Ribbon Diagram
X. BPTI Protein Structure
XI. N-acyl, s-diacylglycerol moiety group
XII. EA3K Linker Structure
XIII. Projected Graphic Map of p-LAGC-EAK10-BPTI
XIV. Graphic DNA Map of pET11d Vector System
XV. Verification of pET11d DNA Purification
XVI. Restriction Digest with Ncol
XVII. A and B. Alkaline Phosphatase-treated DNA
XVIII. Oligonucleotide Synthesis
XIX. Annealed LAGC and LINKER Oligonucleotides
XX. Observation of Transformation Plates
XXI. Ligation 1 Plate 1
XXII. Vector Map of Predicted Recombinant DNA including BamHI site
XXIII. Pre-screening of Ligation Colonies
XXIV. Data Analysis of Recombinant DNA
XXV. Graphic Map of p-LAGC-EAK10
XXVI. Annealed BPTI Product
XXVII. 9:1 Insert-Vector Ratio Plate
XXVIII. Ligation Colonies digested with XhoI
XXIX. Ligation Plates G, H, and p-LAGC-EAK10 without insert
XXX. Restriction Digest with XhoI
List of Tables

I. Serine Proteases and their Function

II. Non-enzyme Coagulation Factors and their Function

III. Cascade Regulation

IV. Restriction Digestion with Ncol

V. Dephosphorylation of pET11d + Ncol + HindIII

VI. Annealing Components of Oligonucleotides

VII. Ligation of pET11d Backbone with LAGC and EA3K Linker

VIII. Transformation Plates

IX. Ligation Colony Count

X. Restriction Digest with BamHI

XI. Pre-screening Colonies with BamHI

XII. p-LAGC-EAK10 Restriction Digest with XbaI

XIII. p-LAGC-EAK10/XbaI Restriction Digest with Ncol

XIV. p-LAGC-EAK10/XbaI/Ncol Restriction Digest with AP-CIP

XV. Ligation of BPTI into p-LAGC-EAK10/XbaI/Ncol

XVI. Ligation Attempt 1

XVII. Ligation Attempt 2

XVIII. Ligation Attempt 3

XIX. Ligation Attempt 4

XX. Ligation Attempt 5

XXI. Ligation Attempt 6
Abstract:

The blood coagulation cascade is activated under various circumstances such as an injury. This system involves a tightly regulated series of events. The enzymes involved assemble with their respective cofactors on lipid membranes to reach their full pro-coagulant complex potential. The coagulation cascade is divided into intrinsic and extrinsic portions, both of which converge into a common pathway with the activation of factor X ($\text{FX}_a$). $\text{FX}_a$ is a very important part of the blood coagulation cascade because its activation is primarily responsible for thrombin generation. Activation of $\text{FX}_a$ alone is, however, insufficient to produce a fully active thrombin. For this to occur, $\text{FX}_a$ must form a complex with its protein cofactor Factor $V_a$ and its protein substrate prothrombin on a phospholipid surface. This suggests that the topography of the enzyme on the lipid surface changes when in a fully pro-coagulant state. However, the topography of $\text{FX}_a$ alone and in complex with these cofactors is poorly understood. Kunitz-type protein inhibitors, such as basic pancreatic trypsin inhibitor (BPTI), are globular proteins, which inhibit serine proteases such as $\text{FX}_a$. These small protein inhibitors fit just inside the active site of the enzyme, making them ideal candidates with which to study the active site of $\text{FX}_a$. The goal of this project is to gain understanding of the topography of fully active $\text{FX}_a$ by using lipid-anchored BPTI with linkers that act as molecular rulers to measure the range of reactive heights of the active site of $\text{FX}_a$ in its pro-coagulant complex on a phospholipid surface.
Introduction

Hemostasis

Hemostasis is the process responsible for initiating and terminating the blood clotting mechanism upon injury (1). It is a tightly regulated and dynamic process by which bleeding as a result of vessel damage is stopped and, ultimately, returned to normal (1-3). The hemostasis process works to maintain blood in a fluid, clot-free state under normal conditions, form a solid clot to block bleeding upon injury, and then return blood to a fluid state upon repair of that injury. This process involves a subset of processes including blood clotting, platelet activation, and vascular damage repair (3). Coagulation is the first integral part of this process as it is the formation of blood clots which assist with sealing the injury and returning blood flow to normal (2-4). The coagulation cascade includes three major components some of which circulate in the blood in a deactivated state and become activated upon vascular injury. These components are platelets, vessel endothelium, and coagulation factors. Platelet activation is important in hemostasis for sealing vessel damage and accelerating the coagulation system overall. Vessel repair is a result of the hemostatic plug composed of fibrin and platelets that seals the damage as well as endothelial components which repair the tissue (3). To understand more about the blood coagulation cascade, it is important to investigate the blood components involved more closely.

Platelets

Platelets are specialized blood cells that play an important role in hemostasis (2, 5, 6). The structure of both the un-stimulated and stimulated platelet is important in its
functioning. In a deactivated state, they are small, disk-like, non-nucleated cell fragments of megakaryocyte cytoplasm in the blood (6). When activated, platelets structure morphs from smooth and discoid to rough cells with pseudopodia. As the term suggests, these pseudopodia are feet-like projections which serve as puzzle-like sites of attachment for other platelets and fibrin. The cytoplasm is surrounded by a plasma membrane constructed in an open canalicular system (6). This system transports nutrients in and out of the cell. The plasma membrane is composed of over 30% phospholipids and nearly 60% proteins. The phospholipids are organized as a bilayer with polar head groups on both the external and internal layers in addition to a hydrophobic center. Neutral phospholipids like phosphatidylcholine are oriented on the external layer while anionic phospholipids are oriented internally. These phospholipids include phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine (7). Tasks assigned to the phospholipids include solubilizing cholesterol such that cholesterol can maintain the fluidity of the phospholipid bilayer. A small percentage of carbohydrates are present in the membrane in the form of glycoproteins and glycolipids.

The outer layer of the bilayer contains a glycoprotein complex known as the GPI complex. There are subgroups of glycoproteins involved in this complex: GPI\textsubscript{a}, GPI\textsubscript{b}, and GPII\textsubscript{b}/GPIII\textsubscript{a}. These proteins work together to attach platelets to collagen and bind von Willebrand factor. This step-wise process involves the platelet exposure to coagulation-inducing proteins, adhesion, and cellular reorganization. A well-characterized coagulation factor named von Willebrand factor (vWF) attaches to a receptor on the GPI
complex during platelet adhesion. vWF is a multimer produced by endothelial cells and megakaryocytes which is composed of over 200,000 subunits connected by disulfide bonds which functions with coagulation factor VIII to assist in platelet adhesion (5, 8, 9). This complex plays a necessary role in the conformational changes and overall activation (i.e. pseudopodia, increases in surface area) platelets undergo after stimulation (10).

GPIα is a receptor protein. It attaches to extracellular collagen exposed at the site of injury. Next, GPIβ then becomes exposed to vWF. Another structural shift in platelets occurs which allows for GPIIb and GPIIIa to bind with vWF and collagen. This process is depicted in Figure I.
Figure I. GPI Complex. Depicted above is the phospholipid bilayer of a platelet cell containing GPIa, GPIb, GPIIb, and GPIIIa subunits and the step-wise platelet activation process. Adapted from (20)
The internal membrane of platelets has been likened to the sarcoplasmic reticulum of muscle cells because they contain dense tubing, mitochondrial membranes, and storage granules (11). These storage granules serve to release necessary components to platelet adhesion when needed and are divided into different categories including α, δ, and λ types. Enzymes including phospholipase A and cyclooxygenase assist in the transfer of \( \text{Ca}^{2+} \) from membrane stores within the platelet to the cytosol encouraging secretion from the storage granules (5). The \( \alpha \)-granules contain fibrinogen, which upon vessel injury is later cleaved to become fibrin necessary for the hemostatic plug. Alpha granules also contain proteins like β-thromboglobulin. One of its functions is to assist in megakaryocyte maturation and therefore in platelet production. The \( \delta \) granules also known as electron-dense storage granules contain metabolically-inactivated adenosine diphosphate or ADP (an agonist in platelet activation), serotonin (a common neurotransmitter), and calcium (metal ion involved in aggregation). Lambda granules contain lysosomal enzymes such as acid hydrolase important for the breakdown of waste materials (12).

Activated platelets undergo conformational changes as a result of contact with endothelial components. Under normal circumstances, the endothelial lining of the vessels is inert and platelets are not stimulated to adhere. When the blood vessel is damaged, platelets are exposed to subendothelial components including collagen, tissue factor, fibronectin, and glycosaminoglycans. Then, platelets undergo adhesion in which they attach to these macromolecules in subendothelial tissues at the point of injury. This collection of cells forms what is referred to as the ‘adherent layer’ (7). In response
to the initial platelets’ activation, other platelets aggregate to form a hemostatic plug (2, 6).

Aggregation is subcategorized into primary and secondary stages. Primary (1°) aggregation is a reversible process that arises from the stimulation (i.e. signaling) other platelets receive from the adherent layer. While platelets are aggregating with one another, they are also undergoing a process called secretion which stimulates the irreversible secondary (2°) aggregation. Like many biochemical processes, aggregation requires divalent cations such as Mg$^{2+}$ or Ca$^{2+}$ to function properly (6). Platelets release several vasoactive amines and other important substances, including but not limited to ADP, β-thromboglobulin, epinephrine, and platelet-derived growth factor (6). Epinephrine, a hormone and neurotransmitter, binds to α-adrenergic receptors on the platelet surface thereby encouraging stimulation (13). The process is broken down into two major steps. First, storage granules cluster at the center of each platelet surrounded by microtubules. Then, the granules travel along the microtubules to the surface of the cell and fuse to the surface membrane and release their contents. The amount of substances released is dependent on the agonist involved as established by Kinlough et al. (14). Secretions such as these trigger other platelets in the area to adhere and continue forming the hemostatic plug.

Plug formation and pro-coagulant activity on platelet surfaces assists in the generation of the thrombin burst which leads to fibrin formation. Fibrin complexes with the platelets to form the fibrin clot in the final stages of coagulation. As wound healing takes place, both the platelet aggregate and fibrin clot are broken down over time in a
process called fibrin clot retraction (2, 6, 13, 15, 16). This process is also mediated by platelets. All of these components are part of an intricate stimulatory signaling pathway, which communicate messages to the platelets upon injury. There are also inhibitory components as well. One such pathway would be the adenylate cyclase enzyme complex. Adenylate cyclase and cyclic adenosine monophosphate (cAMP)-dependent phosphodiesterases work closely with one another to inhibit platelet function. These enzymes recruit the second messengers to attach to receptors on platelet surfaces sending signals to essentially de-activate each platelet cell (17).

**Vasculature Walls**

In addition to platelets, the vascular wall is an important component of blood coagulation. In general, blood vessels contain 3 layers: tunica intima, tunica media, and tunica adventitia (18). The tunica intima is the innermost layer composed of endothelial cells. The tunica media is the smooth muscle layer, which also contains elastic tissue as well. The tunica adventitia is composed of connective fibrous tissue. When the vessels are damaged, endothelial components such as tissue factor (TF) come in contact with platelets in the blood thereby activating them (2). Tissue factor, also known as factor III, is a protein that becomes exposed to intracellular blood components upon injury. It will be discussed further in subsequent sections. Coordinated interactions among these tissue components, plasma proteins, and platelet receptors lead to the primary hemostatic plug, which seals the damaged region of the vessel (2).

Ashford et al completed an electron microscopy study on particular endothelial components and the role they play in coagulation (19). The study was completed *in vivo*
in rats in which the vascular wall was manipulated and known endothelial components introduced, altered, or removed via purification.

Microscopic evidence indicates that platelets in proximity with fibrin were visibly altered and showed loss of granulation. Conversely, even with vascular injury, platelets that are not in close proximity to the fibrin remained intact. After a length of time neutrophils (known inflammatory responders) were recruited to the injured area. The study investigated activity around disrupted endothelium in the presence and absence of ADP, collagen, and thrombin. Data suggested that adding ADP induced platelet aggregation. Evidence indicated that collagen adheres rapidly to platelets after trauma. It also suggested that collagen encouraged the release of ADP. Another interesting finding was that cAMP exhibited inhibitory effects on platelets. Overall, the study surmised that the endothelium plays a passive barrier role separating circulating procoagulants in the blood from tissue-embedded activators necessary for the hemostatic plug.

Other endothelial components include vWF and fibrinogen. As mentioned, vWF is a large multimeric plasma protein, which mediates platelet adhesion by binding them to the collagen at injury sites on the damaged vessel (2, 6). Riddel et al (2) explored theories in blood coagulation by reviewing multiple studies that compared the accepted extrinsic/intrinsic model to the cell-based model theories proposed more recently. This publication describes vWF as an indispensable part of platelet-platelet interaction. Its primary role is to increase the efficiency of platelet attachment to one another. Its
secondary role is important for the survival of coagulation factor VIII described further in the [Non-Enzymatic Factors](#) section below.

*Coagulation Factors*

**Serine Proteases**

The coagulation factors are proteins developed by hepatocytes and are another portion of the cascade necessary to form a clot (20). Early work by Orwen, Biggs, and MacFarlane in the 1940s demonstrated that many of the coagulation factors are the zymogen form of serine proteases important in clot formation that required activation to function properly.

Coagulation proteins are proteases activated by proteolytic cleavage. Many of these proteins are vitamin-K dependent. Vitamin K is necessary for $\gamma$-carboxylation of glutamate residues on the protease necessary to allow attachment to the membrane. The process of $\gamma$-carboxylation involves the reduction of vitamin K to vitamin KH$_2$ by the enzyme, quinone reductase (15). Vitamin KH$_2$ serves as a co-factor for carboxylase enzymes within hepatocytes that add a carboxyl group to the glutamate (Gla) domain on these serine proteases (15, 20-22). Cleavage is a biochemical process by which one enzyme cleaves a portion of another enzyme adjusting its chemical composition converting it from an inactive state to an active one. Serine proteases are proteins which activate one another by this process. Because there are so many coagulation factors and more being discovered all the time, this publication will focus on discussing a select few in an effort to provide a general overview of their diverse characteristics.
Table I contains an abbreviated list of known serine proteases and their main functions (20).

<table>
<thead>
<tr>
<th>Coagulation Factors: Serine Proteases</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II Prothrombin</td>
<td>When cleaved becomes thrombin</td>
</tr>
<tr>
<td></td>
<td>Cleaves fibrinogen (\rightarrow) fibrin</td>
</tr>
<tr>
<td>Factor VII Proconvertin</td>
<td>Forms complex with Tissue Factor to partially activate fX and FIX</td>
</tr>
<tr>
<td>Factor IX Christmas Factor</td>
<td>Partially activates fX</td>
</tr>
<tr>
<td>Factor X Stuart-Prower Factor</td>
<td>Cleaves prothrombin (\rightarrow) thrombin</td>
</tr>
<tr>
<td>Factor XI Plasma Thromboplastin Antecedent</td>
<td>Partially activates fIX</td>
</tr>
</tbody>
</table>

Table I. Serine Proteases and their Functions

The activation of these factors leads to the initiation of thrombin cleavage.

Thrombin, a serine protease, is the activated form of coagulation factor II. Its main responsibility is to cleave soluble fibrinogen, also known as factor I, converting it to insoluble strands of fibrin. Fibrin sticks together with blood components including platelets to seal the injured site. Previous work has established that hemostatic constituents among vertebrates, including prothrombin, are conserved suggesting that any vertebrate with a circulatory system contains these factors within that system (3, 23).

Non-enzymatic Factors

Factor III, often called Tissue Factor, was mentioned earlier to complex with fVII in order to partially activate both fIX and fX. It is produced by endothelial cells and is, therefore, categorized as a member of the extrinsic pathway. Two distinct coagulation
pathways—extrinsic and intrinsic—work together to maintain hemostasis upon injury.

After each participant of pathway has been activated and performed its task, the cascade enters a third pathway called the Common Pathway in which the components necessary to generate thrombin are fully activated. Factor VII complexes with non-enzyme participant TF and performs its duties on the surface of TF-bearing cells (20).
Figure II. TF Interaction on the Cell Surface during Coagulation. Depicted above is the interaction of tissue factor, factors Va, VIIa, and Xa with the endothelial cell surface. Adapted from (20)
This complex serves as a partial activator of factors IX and X respectively.

Factor VIII is another factor necessary for the activation of fX. Factor VII complexes with fIX such that fX is cleaved to a fully active state. vWF is critical for fVIII survival. FVIII is a labile co-factor meaning that it is easily degraded. vWF complexes with FVIIIa prolonging its presence in the blood so that it may attach to fIXa for fX activation. All three factors work together to cleave fII for a burst of thrombin generation.

Pro-accelerin or factor V is a member of the prothrombinase complex necessary for fX to cleave fibrinogen. The interaction between fVa and fXa can be seen in Figure III. It is activated by trace levels of thrombin to catalyze its attachment to fX on cell membranes (20). Further information on the integral role of fV is discussed below.
Figure III. Prothrombinase Complex and fVIIIa/fIXa Complex. FVα. Adapted from (117).
Factor IX<sub>a</sub> activates FX partially by forming a complex with its co-factor fVIII then attaching to FX on the surface of platelets and endothelial cells. Its role is critical in encouraging the clotting cascade into the common pathway. In 1952, Rosemary Biggs and her colleagues published an article in the *British Medical Journal* regarding fIX (24). At that time, many of the common characteristics associated with coagulation factors were just being recognized. Biggs *et al* performed several case studies on patients exhibiting the blood disorder, hemophilia, but were unresponsive to traditional hemophilia treatments. These cases were all similar in that their bleeding issues could not be rectified with anti-hemophilic globulin (fVIII), Ca<sup>2+</sup>, or fVII—known to be critical to coagulation. The ultimate finding was that all cases were deficient in an unknown substance present in normal blood fractions. The unknown substance was identified as Christmas Factor named for case study 1 (24). Now, fIX and Christmas factor are synonymous with one another (20). Further studies by these researchers and others established that hemophilia can manifest in the deficiency of fVIII or fIX. In addition to cleaving FX, fIX<sub>a</sub> must diffuse away from cell surfaces before the coagulation process can reach the explosion of thrombin necessary for fibrin deposition. FX<sub>a</sub> marks the starting point for the common pathway and plays the largest role in thrombin generation. More detail on fX and its participation in the coagulation cascade are included in *The Common Pathway and the Cell-Based Model* section.

Fibrinogen, also known as factor I, is a soluble zymogen that is cleaved by fII<sub>a</sub> or thrombin. This cleavage catalyzes chemical changes within its composition such that it
becomes fibrin, an insoluble protein that cross-links with platelets to become the final clot in coagulation (20).

Ca\(^{2+}\) is a divalent cation necessary for many biochemical reactions within the body. It has also been given the title factor IV for its participation in the coagulation cascade. In particular, Ca\(^{2+}\) is a necessary co-factor for most, if not all, coagulation factors to perform their respective tasks. Table II is a compilation of non-enzyme factors and their main function.

<table>
<thead>
<tr>
<th>Coagulation Factors: Cofactors</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor I Fibrinogen</td>
<td>When cleaved becomes Fibrin and forms hemostatic plug</td>
</tr>
<tr>
<td>Factor III Tissue Factor</td>
<td>Receptor and co-factor that complexes with fVII(_a)</td>
</tr>
<tr>
<td>Factor IV Ca(^{2+})</td>
<td>Divalent cation/Cofactor important in catalysis of proteolytic cleavage</td>
</tr>
<tr>
<td>Factor V Pro-accelerin</td>
<td>Cofactor and member of Prothrombinase Complex</td>
</tr>
<tr>
<td>Factor VIII Anti-hemophilia A</td>
<td>Cofactor that complexes with fIX(_a) Deficiency leads to Hemophilia A</td>
</tr>
</tbody>
</table>

Table II. Non-enzymatic Factors and their Functions

**Factor Regulation**

Normal blood flow is maintained as long as factors in circulation are not exposed to endothelial components. The system is a tightly regulated culmination of positive and negative feedback loops. Each factor circulates in zymogen form which keeps them from activating each other unnecessarily, but there are other constituents in the blood as well as endothelial cells within vessel walls that regulate certain factors even when vessel injury has taken place. For instance, thrombomodulin (TMB) is an endothelial surface
receptor and its corresponding ligand is thrombin (fIIα). Binding of thrombin to TMB triggers the activation of protein C (APC) (20) APC then complexes with Protein S to remove fVα and fVIIIα respectively. The removal of each of these protein factors results in the shutdown of the clotting process by triggering the inactivation of the fVα and fVIIIα. Other factor regulators include tissue plasminogen activator (tPA) which cleaves plasminogen. Plasminogen is a zymogen involved in clot breakdown discussed in more detail in The Blood Coagulation Cascade section.

The Blood Coagulation Cascade

As discussed, the blood coagulation cascade (BCC) is activated by varying stimuli (2). An injury to the skin that pierces past the epidermal layer and reaches the vasculature sets the cascade in motion. Blood coagulation by definition is the cessation of blood loss from a damaged vessel (2). Blood coagulation is a tightly regulated series of events that involve the many enzymes and co-factors mentioned previously. These enzymes work in assembly with their respective cofactors on phospholipid membranes of platelets and endothelial cells to reach their full pro-coagulant complex potential (22, 25, 26). The early models of the BCC were established within blood vessels as a series of clotting factor activation events occurring in separate processes known as the extrinsic and intrinsic pathways. These models were established to explain the mechanisms of coagulation. Figure IV is a general overview of the coagulation cascade.
Blood Vessel Injury $\rightarrow$ Platelet Activation $\rightarrow$ Thrombin Generation

Fibrinolysis $\leftarrow$ Fibrin Clot Formed $\leftarrow$ Fibrinogen $\rightarrow$ Fibrin

Figure IV. General Overview of the Blood Coagulation Cascade.
**Extrinsic Pathway**

The extrinsic pathway is ignited when disruption occurs to the endothelium on the outside of the vessel wall. The endothelium contains TF, a glycosylated intrinsic membrane protein. TF, when bound to coagulation factor VIIa, is a powerful activator of the extrinsic pathway. It is found all over most tissues except the lining of the circulatory vessels. It is not released from the endothelium without stimuli from its constituents such as cytokines (27). Exposure to clotting factors in the plasma results only if there is damage to these vessels exposing endothelial components to the blood directly. Because this pathway will only be initiated when the plasma-derived fVII is exposed to the TF which exists outside the blood vessel, this cascade is known as the extrinsic pathway (27, 28).

A classic way to measure the total reaction time for the extrinsic pathway was by using prothrombin time (PT) experiments (20, 28, 29). Quick et al developed the prothrombin test in the 1930s as a means of measuring anti-coagulation (29). At that time, the historical 1905 Morawitz et al study had based the coagulation process to be centered around 4 plasma proteins (2, 30). Quick’s technique used patient plasma samples to measure the consumption of prothrombin when exposed to thromboplastin which is a lab reagent and protein that converts prothrombin to thrombin. Thromboplastin is a mixture of phospholipids and tissue factor (2). In the early 60s, Glover and Kuzell further developed the technique with the goal of improving anti-coagulant therapies. Experiments for measuring changing levels of prothrombin were done by the Quick one-stage plasma method using whole blood (29). Glover et al
employed whole blood from the finger, adjusting measurement temperatures to 37°C (body temperature), and altering the collection of blood with the administration of thromboplastin (29).

In addition to TF, the extrinsic pathway involves at least four other factors including prothrombin, Ca$^{2+}$, factor X, and thrombin (27, 28). However, this model only works if the other components located outside the vessel are involved. This is evident in patients with hemophilia, because their blood could have all four elements present and still be unable to successfully clot. Although the extrinsic pathway explained a great deal about the cascade, research has determined that it cannot work to clot the blood alone. Perhaps the best example of this research is the fact that TF works with FVIIa which circulates in the blood (31). It also does not explain why plasma has the capability to clot without extrinsic components.
Figure V. TF/FVIIa Complex on membrane surface. Adapted from (20).
**Intrinsic Pathway**

Classic participants in the intrinsic pathway include the following: fV, fVIII, fIX, Ca$^{2+}$, and fXI. The pathway begins when thrombin converts fXI to fXI$\alpha$. Then, fXI$\alpha$ cleaves fIX to make it available for attachment of fVIII. While it is now known that neither pathway is sufficient to seal the vessel disruption on its own, the distinction of these pathways remains the widely accepted model in the scientific community. As mentioned, fVIII$\alpha$, also known as anti-hemophilic factor, serves as the intrinsic co-factor for fIX$\alpha$ in the activation of fX$\alpha$. FVIII$\alpha$ catalyzes attachment of its associated components to the membrane surface. The phospholipid membrane of TF-bearing cells and platelets acts as the arena for these activities and Ca$^{2+}$ as a reaction catalyzer. This is further depicted in Figure V as the area where platelet activation is controlled. The intrinsic pathway is postulated to maintain its extrinsic counterpart (3, 20). Initial techniques used to study the intrinsic pathway and establish it as a viable part of coagulation is to measure the activated partial thromboplastin time (aPTT). This test was developed in a similar manner to the Quick prothrombin test. Partial thromboplastin is a reagent with similar components to thromboplastin with the exception of TF. This process indicates how fast the intrinsic pathway works with respect to exhausting the factors involved. In other words, aPTT measures the efficiency of the intrinsic pathway. Both the extrinsic and intrinsic pathways occur concurrently and once complete unite into a common pathway.
The Common Pathway & the Cell-Based Model

The common pathway is the process by which factor X catalyzes the activation of pro-thrombin (fII). These events that take place after pro-thrombin becomes thrombin. The common pathway is accepted as a true account of fibrin deposition. However, recent studies in blood coagulation suggest a cell-based model describes the process more accurately (2, 27, 28, 32). The new model focuses greatly on more complicated interactions among vessel endothelium, platelets, and coagulation factors.

Since information regarding coagulation has become more expansive, the cell-based model suggests that coagulation occurs in 3 phases (20, 27, 32). The first phase of the new cell-based model is Initiation. This step begins at the moment TF is exposed to the blood. As the platelets aggregate, TF forms a complex with fVII on endothelial cell surfaces. The TF/fVIIa autocalyzes itself to a fully activated state and then partially activates fIX and fX. As a form of regulation, this complex is diffused away by a special serine protease inhibitor called warfarin discussed in the Kunitz Inhibitors section (32). FXI also diffuses away to another anionic phospholipid membrane surface initiating the next phase of the cell-based model, Amplification. Amplification is earmarked by the use of thrombin (fII) as an activator for non-enzymatic factors V and XI. FXI is important for breaking up the complex between fVIIIa and vWF. This step is critical as fVIIIa is a necessary co-factor for fIX. This step is carried out on the surfaces of the platelets that were initially exposed to TF.

The initiation and amplification phases are the priming phases as every factor involved is not fully activated. During the final phase, every factor becomes activated.
The third phase is called **Propagation**. FVIIIa/fIXa forms a complex with fXa on platelet membranes (called the Tenase Complex) partially activating it and prompting it to then couple with fVa (20). The result is a burst of thrombin. Thrombin generation at high levels cleaves large amounts of fibrinogen into fibrin. Fibrin monomers (or subunits) clump together with platelets and form a “soft” clot. FXIIIa, also known as the enzyme transglutaminase, seals this soft clot by cross-linking the platelet/fibrin mix. Once this cross-linking is complete, the hard clot is formed.
Figure VI. Initiation, Propagation, & Amplification Phases of the Cell-Based Coagulation Model. Adapted from (20).
Breakdown of the clot is an important step in coagulation as well as it marks the stopping point of the clotting cascade. Fibrinolysis occurs when the clot is disintegrated after the vessel disruption is rectified. Plasminogen is a zymogen that is activated by tPA to become plasmin. Tissue plasminogen activator (tPA) and single-chain urokinase plasminogen activator (scuPA) are enzymes necessary to activate plasminogen (20). Plasmin will then cleave fibrin and trigger clot destruction. This step is another form of regulation mechanisms for the maintenance of hemostasis. Protein C and protein S are both participants in the proteolytic deactivation of factors Va and VIIIa. This process will be further explained in the cascade regulation section. Plasminogen activator inhibitor-1 (PAI-1) and a2-antiplasmin are proteins that serve as negative regulators of this process.
Figure VII. Factor VIIa—catalytic head, 2 EGF Domains, and Gla domain. Adapted from (116) and PDB ID: 1KLJ.
Factor Structure

As described, there are over a dozen clotting factors circulating through the blood. These factors each play a significant role in coagulation. Many of them share a structure that contains a catalytic head (similar to trypsin), two epidermal growth factor-like domains, and a Gla domain.

Although they are similar in structure, they are distinct in their degree of specificity for each of their respective co-factors and substrates. In order to understand our purpose in examining fXa, it is important to examine some of the other coagulation factors and their particular role in the BCC cascade. The 1940s marked an era in which many researchers began to focus more closely on the coagulation process (reviewed in (27)). Clotting factors were assigned numbers that correspond with their sequence of discovery rather than their point of interaction within the BCC (23, 33). Factor Vα is activated by proteolytic cleavage. As seen in Figure III, Factor Vα serves as co-factor for fX. It is very homologous with fVIIIα, which interacts with the anionic phospholipid surface and requires proteolytic cleavage as well (34). Upon complex formation with fVα and prothrombin (also known as the prothrombinase complex) on negatively charged phospholipid membranes, fXα converts prothrombin to thrombin in the final stages of the coagulation cascade (15, 22, 35-38). Although fX is capable of some activity prior to the assembly of the prothrombinase complex, that capability is minimal. This prothrombinase complex enhances the catalytic efficiency of fX greatly because it improves the $K_m$ (Michaelis’ constant) and $K_{cat}$ of the substrate activation reaction (38). $K_m$ improvement is credited to the binding of the Gla domain and prothrombin on the
membrane surface. Yang et al conducted a study regarding fVα and its contribution to
the improvement of $K_{\text{cat}}$. Their findings indicated that among its other tasks fVα causes
conformational changes of the Na$^+$ binding loop of fX. Researchers constructed fX
mutants with critical residues Glu-217, Lys-224, and Arg-221a replaced with an alanine
to study the catalytic function of fX as well as its interactions with the metal ion Na$^+$. After replacing these residues, Na$^+$ interaction was markedly impaired. However, in the
presence of fVα, the defect in fX catalytic efficiency appeared to be improved. By
binding both fX and prothrombin, fVα stabilizes the enzyme-substrate complex and
provides ideal docking capabilities for other necessary participants (38). Na$^+$ plays an
important modulatory role in the structural and chemical functioning of many of the
serine proteases involved in coagulation (39). Figure III is a depiction of how fVα
complexes with fX to activate it.

Cascade Regulation

Regulation of the BCC is of extreme importance that involves a delicate balance
of all the components described here. This process is that of a negative and positive
feedback loop which works to seal any disruption and maintain blood fluidity. Changes
are seen in blood fluidity as a result of such external or internal stimuli problems as
vessel injury, or increases in blood pressure, or fluctuations in hormones (40, 41). These
increases in blood pressure can occur as a result of cholesterol buildup or chemical
imbbalances like sodium or coagulation factors. Table IV is a compiled list of known
cascade regulators.
Coagulation Regulation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombomodulin (TMB)</td>
<td>Receptor, when thrombin is attached coagulation activity is suppressed</td>
</tr>
<tr>
<td>Protein C (APC)</td>
<td>Serine protease activated by TMB/thrombin complex; APC/APS complex dissolves fVα and fVIIIα</td>
</tr>
<tr>
<td>Protein S (APS)</td>
<td>Co-factor for Protein C; See above</td>
</tr>
<tr>
<td>Tissue Plasminogen Activator (tPA)</td>
<td>Activates plasminogen for hard clot breakdown</td>
</tr>
<tr>
<td>Single-chain Urokinase Plasminogen Activator (scuPA)</td>
<td></td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Cleaves cross-linking for hard clot breakdown</td>
</tr>
</tbody>
</table>

Table III. Cascade Regulation

Protein C and protein S play important roles in regulation when activated as well.

The APC/APS complex is responsible for the dissolution of fVα and fVIIIα respectively (40).

It is triggered by the attachment of thrombin to one of its receptors called thrombomodulin (TMB). FVα and fVIIIα function have been described and are critical to the continuation of the clotting cascade. Without their presence, blood clotting activity is suppressed.

Traditional Therapies & Treatments

When the regulation processes of blood described previously are not controlled properly, the result presents in a number of issues and diseases. In addition to physiological regulation, diet and genetics both play an integral role in blood regulation.

Although this paper will go into detail on a few of the diseases associated, the focus of
poor regulation of the process is narrowed to genetic de-regulation which can lead to issues including hemophilia or von Willebrand disease.

Hemophilia

Hemophilia is a rare, genetic bleeding disorder in which blood does not clot properly (42). It is an X-linked recessive disorder caused by a functional deficiency in fVIII or fIX. A deficiency in fVIII results in Hemophilia A while deficiency in factor IX leads to Hemophilia B (24, 43, 44). Each of these factors is a member of the intrinsic pathway necessary for the activation of fX. Deficiencies in either or both result in an abnormal or non-existent clot formation (24, 44). Patients suffering from this disease usually exhibit excessive bleeding post-injury no matter how minor in many places including the skin, brain, or joints (43, 44). One of the most popular courses of treatment is replacement therapy (42). The clotting factors for which the patient is deficient are replaced in the blood by infusion. Though these factors are harvested in donated blood and have a relatively low ability to incite an immune response in foreign blood, complications can occur including antibody formation against foreign clotting factors. Immune responses can destroy them before they have a chance to work (15, 45). Clotting factors circulate in the blood and are therefore susceptible to being exposed to other organisms circulating in the blood including viruses and consequently can be affected by them. While these complications are serious, they are rare as the screening process for donated blood is rigorous and thorough (4, 25). Other treatments are available for hemophiliacs including but are not limited to gene therapy as well as anti-fibrinolytic medicines.
**Factor V<sub>α</sub> Leiden Mutation**

The Factor V<sub>α</sub> Leiden mutation is another example of a coagulation disorder. Patients inherit the FVL allele which contains a missense mutation. A single G nucleotide is replaced with an A, which alters its functioning (46, 47). The mutation alters the arginine at the 506 position and replaces it with a glutamine. This single residue polymorphism causes fV<sub>α</sub> resistance to the degradation that takes place during the APC pathway mediated by thrombomodulin. The Arg-506 is one of the three cleavage sites protein C uses to degrade fV<sub>α</sub> during the cessation of the cascade. Ultimately, fV<sub>α</sub> becomes resistant to degradation and also gains the ability to perpetuate the cascade. FVL can cause a number of anomalies including venous thromboembolism, deep vein thrombosis, pre-eclampsia, pulmonary embolism, and miscarriage. Van Mens *et al* reported a high prevalence of FVL at 5% in Caucasians (46). The review study these researchers completed was based on understanding the evolution of FVL as well as its advantages and disadvantages. Disadvantages were already mentioned. However, some of the advantages reported by van Mens included decreases in intrapartum blood loss in affected females giving birth, decreased menstrual blood loss, patient survival of severe sepsis, and fecundity (46).

**Anti-coagulants**

This study is focused on the inhibition of fX<sub>a</sub>, which essentially inhibits the coagulation cascade. Therefore, it is important to note how anti-coagulants work. Ticks and leeches naturally produce these anti-coagulation agents, which have powerful
mechanisms to prevent blood from clotting. They do so as a method of feeding on the
blood of its host. Physicians often use anti-coagulants during surgical procedures to
prevent the blood from clotting as a result of an incision to the body or other induced
trauma to the circulatory vessels. Also, they are used as treatments for patients plagued
with thrombotic disorders such as atherosclerosis or prone to strokes (43).

Among some of the most commonly used anti-coagulants are coumarins (48).
These agents work by blocking the activity of vitamin K. Warfarin, a vitamin-K
antagonist, is the most used anti-coagulant in medicine to date (20). Warfarin is a
member of the coumarin family. It is such a great anti-coagulant that it is used as
pesticide, more specifically as a rat poison. As previously stated, serine proteases
including fI, and fX are vitamin-K dependent and susceptible to inhibition by warfarin.
Warfarin decreases γ-carboxylation of Gla residues in fX and other coagulation factors
by blocking the mechanism of the enzyme, vitamin K reductase, needed to replenish
active vitamin K (20). Vitamin K reductase regenerates the vitamin in hepatocytes.
Without a steady stream of active vitamin K, γ-carboxylation is blocked preventing
coagulation factors from docking on membrane surfaces. While it is the most frequently
used clot inhibitor, there are a few issues associated with its effectiveness. For example,
its effects take up to 3 days to act. Additionally, this substance has a lack of specificity in
regard to clotting factors. Its role as a vitamin-K antagonist makes warfarin difficult to
use because of its varying dose requirements and narrow therapeutic range. Its high risk
for serious bleeds especially is what makes proper dosing very difficult. It has been used
to treat deep vein thrombosis and atrial defibrillation (49). For this reason, it is often
distributed alongside a serine protease inhibitor known as heparin. Further discussion on the significance of heparin can be found in a sub-section below. Though using these anticoagulants is useful, one drawback is that both of them lack specificity—they decrease the activity of every component requiring vitamin K.

**The Importance of Factor X**

Factor X is a very important part of the blood coagulation cascade because its activation is primarily responsible for thrombin generation. For the purposes of this study, a particular focus is being placed on fX. As discussed, thrombin is necessary for the conversion of fibrinogen to fibrin. It is activated by the factor VIII and IX co-factor complex.

Proteolytic activation within the intrinsic and extrinsic pathways causes conformational changes in and around the active sites of fX molecules to render the entire complex as a pro-coagulant. FX cleavage between Arg15 and Ile16 by the fVIIIa/fIXa complex is important in its activation. This site near the N-terminus of the fX substrate forms a salt bridge at the catalytic head (27, 33, 50). FXa has both a light chain and a heavy chain held together by a single disulfide bond. The light chain contains the N-terminal glutamate domain (called the Gla domain) conserved across many coagulation factors in addition to two epidermal growth factor-like domains. This Gla domain contains 11 γ-carboxyglutamic acid residues which mediate electrostatic binding to the negatively charged phospholipid membrane in the presence of divalent calcium ions. The heavy chain contains its trypsin-like serine protease domain. At the point of activation, the BCC has left the extrinsic and intrinsic pathways respectively and entered
the common pathway. The common pathway is where pro-thrombin is converted through a series of steps to thrombin. Pro-thrombin contains 10 Gla residues. The prothrombinase complex includes fXa, fVa, phospholipids, and Ca\(^{2+}\). Refer to Figure III for a depiction of the prothrombinase complex.
Figure VIII. General overview of fX Activation
FXa Topography

While the shape and genetic organization of FXa was mentioned briefly throughout this report, it is important to go into further detail as it pertains to this study. Fluorescent Resonance Energy Transfer (FRET) has been used for many years to study biochemical agents on a molecular level such as FX (51-53). FRET is defined in short as a technique used to measure the interaction between two different molecules (53, 54). These molecules are labeled with fluorophores which are also known as either a donor or an acceptor. The donor is said to be the molecule in an excited state and the acceptor is the molecule nearest to it. It was first discovered by Förster et al (55).

There are different types of FRET including but not limited to emission measurement or acceptor photo-bleaching. In either case, FRET techniques monitor whether or not the donor molecule emitted energy and whether the acceptor molecule receives it (53). Qureshi et al measured distances within FXa using FRET, between donor fluorescein dye in the active sites of FI-FPR-inhibited FXa and its N-terminal EGF deletion mutant, FXa-desEGF1 (56). FI-FPR stands for fluorescein-D-Phe-Pro-Arg-chloromethane. The Qureshi results indicated that an EGF spacer functions to hold the FXa active site above phospholipid membrane surfaces. Not only this, this study found that FVa alters this orientation in an unknown but significant conformation (56). This information is integral to understanding more about FXa itself as well as its topography.

FXa inhibition can be measured via enzymatic kinetics as seen in (57-60). Competitive inhibition is measured using solution-based chromogenic substrate competition assays. Chromogenic substrates are colorless chemical compounds which
react within enzymatic reactions by generating a colored product (61, 62). This product is usually generated because the substrate has been cleaved during the reaction. The product of the chromogenic substrate is known based on which substrate is used. The absorbance of the product is noted and is a point of reference during data analysis. This technique has been used many times to characterize protease functioning (61, 62). By measuring the rate of substrate hydrolysis (i.e. consumption). Additionally, Budzynski et al. explains that chromogenic substrates have also been very useful in determining the zymogen form of these proteases as well (62). With the appropriate activator, controlled chromogenic assays can be used to monitor activated enzyme amount to the inactivated enzymes in the area.

By measuring the rates of chromogenic substrate cleavage, other factors involved in these mathematical calculations include inhibitor concentration, Michaelis’ constant for substrate usage ($K_m$), steady-rate velocity without the inhibitor ($K_i$), steady rate velocity with the inhibitor, experimental substrate concentration, and the asymptote or remaining saturating inhibitor present (58). The Michaelis Menten constant represents the substrate concentration at which 50% of maximal enzymatic activity in an experiment is measured. The $K_i$ represents the point at which 50% of the enzymatic activity is inhibited. To calculate this one would measure inhibition of the desired activity takes place until 100% inhibition is reached. Then, calculate the slope of each experiment and use that information to determine the $K_i$. The slopes of these experiments will be useful to plot a linear graph which can indicate the relationship of the enzymatic inhibition over time. The enzymatic inhibition time is directly linked in
determining fX topography as it relates to the distance at which fX can be inhibited using this construct and others constructed after it.

Studies report that the enhancement in the catalytic activity of fXₐ in the prothrombinase complex appears to arise from two orders of magnitude improvement in the \( K_m \) or the concentration of substrate to activate prothrombin. This is because of the \( Ca^{2+} \) dependent interactions between the protease/substrate complex via their Gla domains on the same negatively charged membrane surfaces. There are a total of 5 orders of magnitude increases. Thus, the remaining three orders of magnitude improvement in the catalytic efficiency of fXₐ is attributed to the co-factor function of fVₐ which improves the \( k_{cat} \) (or catalytic rate) of the activation reaction by interacting with the protease and/or the substrate on the membrane surface (56).
Figure IX. A ribbon diagram of fX. Adapted from (115) and PDB ID: 2BOK.
**FX Regulation**

**Serpins**

While regulation of coagulation factors was discussed briefly already, it is necessary to understand the level of importance in relation to fX specifically. Clotting factors are inhibited in a number of ways. Serpins are a group of serine protease inhibitors, hence the breakdown of the word *ser* for serine, *p* for proteases, and *ins* for inhibitors. They are produced mainly in the liver and comprise almost 10% of plasma proteins. Similarly to the clotting factors, any number of serpins float freely in the blood as needed to control hemostasis. KPIs or Kunitz-type protease inhibitors are classified as serpins. This group includes anti-thrombin III (ATIII) and anti-trypsin as well. ATIII is a protease inhibitor produced by the liver which targets serine proteases including fIXa, fXa, and thrombin. ATIII is a small glycoprotein that contains an arginine within its active center that reacts irreversibly with a catalytic-site serine in thrombin; this creates an inactive complex (63).

**Heparin**

Heparin, a known anti-coagulant polymer, potentiates ATIII activity (64-69). A more specific definition of heparin would be that it is a sulfated polysaccharide. Because heparin works well and is very efficient at completing its task, it is often coupled with warfarin in clinical settings. Surgeons often employ it to decrease incidence of clotting during procedures. Enoxaparin is a pentasaccharide form of heparin (70). It is classified as a low molecular weight heparin used to measure its own potentiation characteristics as compared with heparin. One of the benefits of this particular heparin is it has
specificity for fX. Heparin and its derivatives is a powerful anti-coagulant that works specifically by binding to a basic amino group in the ATIII active site such as arginine. When complexed with ATIII, the inactivation rate of thrombin increases significantly (71). Therefore, thrombin remains deactivated for a longer period of time than under normal circumstances, which leads to a decrease in coagulation. Once thrombin is bound to fibrin it is resistant to the inactivating capabilities of ATIII or the ATIII/heparin complex. Thus, ATIII can directly or indirectly inhibit fXa. ATIII blocks thrombin via conformational change from forming a clot in the blood. In addition to its interactions with ATIII, heparin serves as a regulation mechanism for fX vide infra. Because warfarin can take as long as 72 hours to act, heparin is often used to accompany it as an additional treatment. It is said to have the highest negative charge of any natural polymer in the system and is very closely related to another polymer known as heparan sulfate located on epithelial surfaces of blood vessels because it contains structural similarities including repeating disaccharide units.

As discussed, heparin has many benefits, but it has some negative characteristics as well. Not only does high molecular weight heparin lack specificity, it can also cause clinical issues such as heparin-induced thrombocytopenia (HIT). HIT is characterized by excessive clotting within the patient along with a dramatic decrease in circulating platelets (i.e. thrombocytopenia) associated with the presence of excess heparin (20). Heparin has the ability to bind platelet factor 4 (PF4) which triggers an immune response within the body. This immune response causes the body to classify its own cells as foreign resulting in an attempt to remove them. To reverse these effects,
doctors remove heparin from the blood by filtration or allowing the body to do so via metabolic mechanisms and use another anti-coagulant.

Many studies including Deadmond et al found heparin to be a powerful modulator of activity of coagulation factors as well. In an effort to establish the relationship between fIXa and heparin, the Deadmond study used slow-binding kinetics and serine protease inhibitors including basic pancreatic trypsin inhibitor (BPTI), tissue factor pathway inhibitor, and nexin to determine how heparin is increasing fIXa reactivity in their presence. Each of these substances are serpins capable of inhibiting a specific portion of the coagulation process. BPTI targets fXa, TFPI targets the extrinsic pathway via tissue factor and factor VIIa. Nexin targets thrombin and has been used in recent studies to be useful with regard to central nervous system circulation (72). Results indicated that heparin modulates a specific loop at active site 99 within fIXa that allows for better inhibition by BPTI (57).

The Role of Kunitz Inhibitors

The initial discovery of Kunitz-type protease inhibitors (KPI) described them as globular soybean proteins, which act to inhibit trypsin much like the ones that regulate fX. Since then, it has been established that not all KPIs are soybean proteins. Some are human proteins; thus, KPIs are conserved across phyla structurally and functionally. KPIs operate to inhibit a number of processes within the body including digestion, blood coagulation, and inflammation. In digestion, they serve to stop or slow trypsin and chymotrypsin (48). Their influence on chymotrypsin is significantly less than its actions on trypsin. In inflammation, they work to decrease the activity of the inflammatory
enzyme, elastase allosterically (73). In blood coagulation, KPIs inhibit various factors such as kallikrein and fXa (74).

Studies published by M. Kunitz in 1946 established the chemical properties of these inhibitors and were therefore named for him (48, 74). KPIs were characterized as stable globular proteins with estimated molecular weights of up to 24,000 Daltons (Da) and the ability to inhibit the actions of trypsin. It was also reported that the inhibitor is active only in its native or unaltered state (48). The interactions of these inhibitors in combination with their respective substrates take place on the lipid membrane to obtain full enzymatic activity. There have been so many types of KPIs isolated and reported since that time that it is commonly referred to as a superfamily. Some examples of KPIs include basic pancreatic trypsin inhibitor, substilisin inhibitors, and anti-thrombin III.

**Inhibitor Structure**

The molecular structure of these inhibitors contains varying combinations of ≤ 200 amino acid residues. Many KPIs contain conserved regions of disulfide bonds consisting of four cysteine residues within a single or double polypeptide chain (74). Two distinct characteristics in the KPI structure are its secondary and tertiary make-up. The secondary KPI structure is described as devoid of an α-helix; rather, it has 12 anti-parallel β strands connected together via long loops instead. Thus, the tertiary structure is that of tri-folds of these β strands. The active site on these inhibitors is usually located near the lysine or arginine residue within the structure.
Basic Pancreatic Trypsin Inhibitor

Basic pancreatic trypsin inhibitor (BPTI) is a kunitz-protein inhibitor which is of particular interest in this study. BPTI is approximately 58 amino acid residues in length. It is a single-chain polypeptide, which has a molecular weight of about 6,512 Da and a size of 30Å (73, 75). It is string-like in structure and folds upon itself twice and is held together by 3 disulfide bonds. It is one of the smallest and simplest of the globular proteins (76).
Figure X. BPTI structure including the Lys15 region, N-terminus, C-terminus, and three disulfide bonds denoted by the black double arrows. Adapted from (74) and PDB ID: 9PTI.
It functions to inhibit a broad spectrum of digestive enzymes. It works in particular to suppress the breakdown of proteins into its building blocks (i.e. amino acids) by inhibiting trypsin produced by the bovine pancreas (73). This serves to carefully control trypsin and assure that important proteins are not degraded arbitrarily. BPTI has been heavily studied which means that its structure and mechanisms are well-characterized. It is stabilized by a small hydrophobic core formed by an internal packing of non-polar amino acid side chains. Its 3 disulfide bonds add further stability to the molecule.

Understanding how BPTI works has been extremely instrumental in understand protein-protein interaction and molecular recognition on a much clearer level. This interaction and the information it has provided prompted this study regarding the actions of fX more closely. The normal inhibition rate for fXₐ with non-native BPTI ranges depending on the system and the co-factors involved and works well as a tool for comparative purposes. However, previous inhibition studies have suggested that it takes place in a two-step inhibition reaction involving a reversible binding step with a $K_i$ of about 1.13µm/s as well as an irreversible alkylation step with a rate of $k_i$ of about 0.65µm/s. This information is based upon fXₐ interaction with fVₐ and BPTI in solution or on membranes (34).

**Research Design**

*Current Information and Fluorescence Studies*
Fluorescence studies indicate that *distance of closest approach* to the membrane of the active site of several of various clotting proteases is highly important. In other words, the topology of these pro-coagulant complexes is an important concept to grasp especially because it could provide insight into their specificities (*see Future Aims for more information*). However, an understanding of topology and reactivity of these complexes is limited and, therefore, a critical gap that needs to be filled. In multiple instances, substrate binding has been demonstrated to alter the distance of closest approach. X-ray crystallography shows the distance of closest approach to be similar to the length of these coagulation proteins. Thus, these enzymes (i.e. *clotting factors*) appear to orient themselves in a vertical manner on the membrane surface with little or no bending. This inference has not, however, been confirmed using fluorescence resonance energy transfer-based studies on actual distance for optimal pro-coagulant activity. It does not provide any information on the elasticity (ability to change) of these pro-coagulant complexes or its ability to stretch in the vertical direction. Specifically, we believe that the fXₐ pro-coagulant complex may achieve a maximally-effective pro-coagulant capacity when held at a specific orientation above the phospholipid membrane in its cofactor/substrate complex. Ultimately, **this study will examine and compare the range of reactive heights of the fXₐ alone and fXₐ/fVₐ pro-coagulant complex above the membrane surface by using an inhibitor substrate (recombinant BPTI with a linker) that is anchored at specific heights above the phospholipid surface.** The length of the anchor, which gives maximal inhibition will indicate the distance of the maximally reactive catalytic site above the membrane.
Previous Studies and Study Goals

The goal of this study is to gain understanding as to the topography of $fX_\alpha$ in the presence of BPTI. The following are studies done by other laboratories which provide insight regarding the reactivities of the pro-coagulant complex using proven methods planned for use in this project.

Proof of concept using lipid-tethered inhibitors.

The viability of anchoring Kunitz-based inhibitors to lipid membranes for analysis was tested using Surface Plasmon Resonance (SPR) (77). The SPR experiments measure the binding of any two molecular partners in real-time without the requirement of labels via changes in the refractive index (57). In particular, researchers generated a phosphatidylcholine (PC) lipid monolayer containing nickel-chelating head groups (used for neutralization purposes) and a 6-histidine tagged BPTI was successfully attached. Soluble proteases were then flowed over the surface, and their capture by the inhibitor was monitored in real time. The results of these studies indicated that BPTI is capable of binding target proteases under these conditions (78). Though lipid-tethered KPIs can potentially retain their function with these proteases, functioning is optimal when the proteases are in a soluble state. In addition to those results, the on-rates of binding were much lower than expected based on solution-phase studies done prior (79). Negative steric effects derived from holding the KPI close to the lipid surface may be the explanation for this phenomenon as well as preference of enzymes for maintaining a vertical orientation on the membrane. Results from previous studies such as this
indicate that slow binding is predicted to increase substantially when elevated from the membrane.

Expression and purification of an active inhibitor

BPTI and other known protease inhibitors were directionally cloned into pET11a DNA system using techniques outlined in Anderer et al (80). Recombinant inhibitor DNA was confirmed using sequencing. Next, each was expressed as inclusion bodies in BL21(DE3), an expression strain of Escherichia coli (81-83). The methods used in the Deadmond study are outlined here as a template for use in this study. These cells were grown in terrific broth (TB) and 50 µg/ml Carbenicillin at 37°C until log phase was reached. Carbenicillin is a common substitute for ampicillin if unavailable or the need for a more stable antibiotic is necessary (84). Terrific broth is a mixture of peptone (a soluble protein), yeast extract, and glycerol which is rich in nutrients for bacterial growth (85). After log phase was reached, 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was used to induce protein expression over a 4-hour incubation period at 37°C. Using techniques as outlined by Steinle et al, the inclusion bodies were collected and solubilized (86). Next, the solution was clarified using centrifugation. Then, a protein preparation of the inhibitor was exposed to oxidative refolding. Oxidative refolding is a process by which disulfide bonds are forged in the desired protein. Techniques as outlined by Clark et al were used. The 6-hour process was allowed to take place with a coordinated stirring step until the protein was properly folded. Exhaustive dialysis of the folded protein into buffer fluid was prepared for ion-
exchange chromatography. Column chromatography was used for protein expression and purification purposes. Columns designed specifically for BPTI inhibitor affinity were used to elute the protein. First, a column was designed to contain bound trypsin. It was used followed by a 0-1M NaCl gradient wash. Then, the inhibitor was eluted by deactivating bound trypsin with a very low pH elution buffer with Ca\(^{2+}\). Then, the eluate (expected inhibitor) was neutralized immediately using 2M Tris buffer. SDS-PAGE was used to determine the protein concentration at 95% purification. Factors involved in the clotting cascade including fIX\(_a\) and fX\(_a\) were activated using Russell’s Viper Venom (RVV) (87, 88). RVV activates these factors as they would if exposed to extrinsic components. Then, standard single-stage clotting assays with a coagulometer were performed. The reactive sites of recombinant BPTI were then titrated. This method described the inhibitory activity of the inhibitory protein (79). fIX\(_a\) enzymes were incubated in 150 µM biotin-EGR-ck in tricine, NaCl, CaCl\(_2\), and ethylene glycol. The active sites of these biotin-EGR-fIX\(_a\) enzymes were collected as described by Mann et al. The enzyme-linked immunosorbent assay was used to quantify these enzymes with goat anti-fIX polyclonal capture antibody as well as alkaline phosphatase-conjugated Streptavidin. Streptavidin is a commercially available protein with a very high affinity for biotin. Once both the reactive sites of the BPTI inhibitor and the active sites of the fIX\(_a\) were collected, equilibrium enzyme inhibition assays were completed. Specifically, equilibrium of fIX\(_a\) amidolytic activity (cleavage of an amide) was measured using reversible competitive inhibition. The equation for calculating the equilibrium constant or \(K_{ieq}\) was as follows:
\[ v_s = \frac{v_0 K_{ieq} (1 + S/K_m)}{1 + K_{ieq} (1 + S/K_m)} \]

\( v_0 \) describes the steady state rate obtained without inhibitor present. \( v_s \) represents the steady state rate at each concentration of the inhibitor. \( S \) represents the experimental substrate concentration, and the \( K_m \) is Michaelis’ constant for substrate hydrolysis. The equilibrium rates were used to determine the activity rates based on the slow-binding enzyme inhibition assays that followed. Kinetic parameters for these assays were obtained from Morrison et al and Copeland et al (89, 90). The conditions of these experiments were very similar to that of the equilibrium assays except that the \( \text{fIX}_a \) enzymes were incubated with or without enoxaparin (low molecular weight heparin mentioned previously) before they were used in the reaction mixture. Varied concentrations of the inhibitor were co-incubated with a pre-determined quantity of trypsin. At equilibrium, a chromogenic substrate was added to measure absorbance at 405 nm for 30 minutes. These measurements indicated substrate hydrolysis over time. The results were tallied from the lowered rates of color and compared to one another to generate a curve used to calculate the amount of viable inhibitor. The lowered rates indicated trypsin inhibition. A computer software called KINEMAX was used to generate data curves for the analysis of slow inhibition. The series of equations used in this study allowed for the calculation of reactivity using variables including the time, initial absorbance, initial rate of substrate hydrolysis, and so on. This information allowed researchers to generate data plots, use the slopes of each \( k_{obs} \) calculated, and measure how close \( k_{obs} \) is to the equilibrium data.
Construction of a base vector

LAGC Consensus Sequence

An expression plasmid was constructed to include the BPTI inhibitor as well as a bacterial lipophilic tag located after the consensus sequence at the C-terminal called LAGC. This is the common sequence for lipid-anchored bacterial proteins named for [LVI][ASTVI][GAS]C amino acid sequences (83). Biological systems including Staphylococcus aureus and E. coli bacteria have developed this machinery as a means of survival. Nutrient uptake, signal transduction, growth, and development are all processes dependent upon many membrane-bound proteins (83). Additionally, ELISA or enzyme-linked immunosorbent assays and other biosensors use membrane-bound proteins in order to successfully complete tasks such as diagnostics and substance identification. The Kamalakkannan study demonstrated that cloning a target gene sequence behind the LAGC sequence led to lipid-anchored expression of otherwise normally secreted proteins (83). These researchers used two respective strategies to genetically alter a non-lipoprotein into a functional lipoprotein. This motif is recognized by bacterial machinery, more particularly an enzyme called diacylglycerol transferase. This enzyme modifies the moiety sequence in a two-step reaction leading to the cleavage between the Gly and Cys residues. Then, there is an addition of an N-acyl, s-diacylglycerol moiety to the N-terminal Cys (91-93). The moiety is highly hydrophobic and incorporates in the lipid membrane, and its following peptide sequence is expressed in the periplasmic space. Figure XI is a depiction of the chemical structure of this moiety.
Figure XI. N-acyl, s-diacylglyceryl moiety group at the N-terminal Cys group associated with the LAGC consensus sequence. PR1 and 3 are the palmitoyl residues. PR2 may be a palmitoyl or oleoyl residue. Adapted from (83).
Inhibitor Ruler Proof-of-Principle

The Deadmond et al. plasmid developed was modeled exactly as the Kamalakkanan vector with a pET11a backbone modified to contain a ribosome binding site, a 10 base-pair spacer, the sequence coding for the 23 amino acid leader sequence, and followed by sequences coding for Leu, Ala, Gly, and Cys. The leader sequence is of significant importance as it was taken from the apyrase gene (83). *Shigella* apyrase is a model protein used in the Kamalakkanan study for a number of reasons. It is easy-to-use and assists in the release of phosphate groups from organic and inorganic pyrophosphates (93). It guides the protein into the periplasmic space, which supplies the oxidative environment needed for the inhibitor to spontaneously fold. Finally, the construct contained a restriction enzyme site along with the sequence for BPTI. Verification to confirm this construct was built optimally was done via sequencing.

Relipidation of recombinant proteins

Recombinant BPTI with the LAGC-tag was relipidated with 100% phosphatidylcholine lipid vesicles. The relipidation process is completed by incubating the recombinant DNA in the presence of phosphatidlycholine as well as detergent composed of phospholipids and glycopyranoside. It is necessary in order to ensure that the recombinant proteins will work as expected to that of the body. More specifically, in experiments performed on prothrombotic surfaces attempting to mimic *in vivo* injuries, it is necessary that modified factors be relipidated for attachment to surface bound collagen fibers as seen in (94). After that, the mixture is combined with a solution of chloroform and methanol and dried onto a thin film in liquid nitrogen. It is then placed
under a vacuum for a minimum of 2 hours to remove any undissolved organic solvents. Next, the film is dissolved in a mixture of glycopyranoside, Tris-HCl, and apoprotein. Upon incubation for at least an hour, the detergent is removed by dialysis. The relipidated protein is treated for up to 2 days with buffer until it is ready for use. Protein confirmation and concentration was verified by gel filtration chromatography. In this experiment, a large portion of recombinant BPTI co-eluted with a lipid fraction. Whereas, native (non-lipid-anchored) BPTI elution profiles eluted after the lipid fraction using similar conditions.

**EA₃K Linker Sequence**

The EA₃K linker sequence is the portion of the recombinant plasmid that serves as the molecular ruler. Its sequence codes for a string of repeated amino acid residues that read (using single letter coding) as E-A-A-A-K. They are rigid and helical in structure. The structure is important as they allow for better control regarding the length in which the inhibitor will be raised off of the membrane surface. Each linker is approximately 10Å in length. The structural height of the linker is about 7.5Å as they attach to one another in a unique diagonal manner (refer to Figure XII). Arai et al used the EA₃K linker to enhance protein fusion experiments (95). The linkers were an important tool in bridging α and β domains between each protein to create a fully functional chimeric protein. The oligonucleotide sequence designed for this study contains ten of these linkers. This means that the recombinant BPTI would be raised 75Å from the membrane surface.
In addition to the E-A-A-A-K repeats, the sequence also contains a G₄S flexible linker. This linker as suggested by its name codes for peptide sequence, G-G-G-G-S. One important reason these linkers are expected to work well in the recombinant DNA is because they have been used successfully in the past and do not typically alter protein activity. Su et al used this linker to allow for recombinant erythropoietin designed for the study to enable the moiety of the protein the ability to form its native three-dimensional structure properly as well as function in its environment as naturally as possible (96). Its hydrophilic and neutral chemical composition allows these processes to take place. Ultimately, the G₄S linker provides the protein with the ability to float in a flexible manner as it would in the environment and increase its chances of attachment to serine proteases in the area (97).
Figure XII. EA$_3$K Linker Structure. This is a theoretical depiction of what the EA$_3$K linker sequence resembles. The length is demarcated here as well.
**Overall Study Aim**

This study proposes that as the inhibitor is raised from the membrane to an optimal level binding is increased; therefore, its functioning is improved. In an attempt to confirm this hypothesis the following steps were taken.

**Specific Aims:**

**Specific Aim 1: Construct a plasmid using a pET11d backbone containing the LAGC and EA\_3K sequences respectively**

Preliminary results obtained from Neuenschwander et al. demonstrate proof-of-principle for generation of lipid-anchored BPTI (57, 79). The construct will generate sequences encoding a bacterial lipid-anchor consensus site separated by a set number of linker sequences. More specifically, a segment coding for an LAGC leader sequence and EA\_3K linker sequences will be directionally cloned into pET11d E. coli DNA. Below is a simplified image of what the generated construct will look like:
Figure XIII. Projected graphic map of p-LAGC-EAK10-BPTI. LAGC sequence is denoted in the color orange, EA₃K in green, and BPTI in yellow. Restriction enzyme sites are noted in the black outlined blue boxes. The ampicillin promoter (Amp prom) and repressor (AmpR) region is noted. The lacI region is a part of the pET11d is a mutated promoter region that controls the lac operon. ColE region of this bacterial plasmid that aids in the survival of the plasmid.
Specific Aim 2: Use the p-LAGC-EAK10 plasmid to incorporate BPTI inhibitor creating the lipid-anchored “inhibitor ruler”. Previous research in topography and analysis of serine proteases indicate that their range of attachment lies within this distance from the membrane surface (56, 69, 98, 99). The engineered constructs will be sent for sequencing for verification. Once there is confirmation that this construct has been properly cloned, it will be transfected into bacterial cells for expression. Purification will be performed using affinity chromatography as was done in the Neuenschwander study. Affinity chromatography is a technique in which biochemical agents are separated based on specific interaction between antigen and antibody or enzyme and substrate, for example. Specifically, we will immobilize the enzyme trypsin to the solid support, and use this to separate, purify, and elute our newly generated inhibitor product from the remainder of the mixture. The solution-phase inhibitory activity of each of these $\text{fX}_\alpha$ inhibitors will be characterized and compared with the unmodified inhibitor. Once this is complete, it will be used in the next phase of the study.

Materials, Methods, and Results:

Specific Aim 1: Construction of a plasmid using a pET11d bacterial backbone containing the LAGC and EA$_3$K sequences respectively (p-LAGC-EAK10). To generate a lipid-tethered inhibitor construct as a tool to study distances of fully pro-coagulant complex active sites, standard molecular cloning techniques were used (57, 78, 79, 83, 100). Standard experiments in cloning usually require at least seven steps. These steps are as follows and include but are not limited to: 1) choosing a host organism and cloning vector, 2)
vector DNA purification and preparation, 3) design, preparation, and insertion of the desired DNA to be cloned, 4) confirmation that recombinant DNA has been created, 5) introduction of new DNA into host organism, 6) selection of colonies possibly containing new DNA, and finally, 7) screening of said clones for the appropriate biological properties (57, 78, 79, 83, 100).

Choosing a Host Organism and Cloning Vector

For this study, *Escherichia coli* was used as the host organism. *E. coli* is a bacteria used commonly in molecular cloning because of its versatility, availability, and ability to grow quickly (101). It is these same reasons that make it a great system to use for these experiments.

Upon choosing this bacterial system, the pET11d vector system was selected as the cloning vector (102). The pET11d system contains an ampicillin-resistance gene that allows selection for transformants. Other studies have used this system successfully not only to create recombinant DNA but for protein expression as well (101). In addition to antibiotic resistance, pET11d also contains unique restriction endonuclease sites, in particular, *Hind*III and *NcoI* sites which allows directional cloning of the LAGC and EA3K linkers into the vector system respectively. In Figure XIV below, a depiction of the pET11d vector system has been provided. Note the many cleavage sites available for manipulation particularly those at the *Hind*III, *NcoI*, *BamHI*, and *XbaI* sites. The design of each DNA insert (i.e. LAGC and EA3K) was based on these areas within pET11d (see next page).
Figure XIV. Graphic DNA map of pET11d vector. Depicted in this map of the 5,674 base pair plasmid are the ampicillin promoter and repressor region, lacI promoter, and termination sites. www.snapgene.com/pet11d
Preparation of Vector DNA

Competent Cell Generation

Competent cells were generated using frozen glycerol stocks of DH5α cells (82). While still frozen, the cells are scraped onto a metal loop using sterile technique. The cells are then streaked onto an LB plate and incubated overnight at 37°C. The next day, a single well-isolated colony was selected and inoculated into 5 ml of LB in a 10-ml Falcon© tube. The inoculated colony was allowed to grow overnight while shaking in a 37°C incubator at 125 rpm. Then, 1 ml of the overnight culture media was inoculated into 100 ml of fresh LB media. This 250-ml Erlenmeyer flask was shaken for 3 hours at 37°C and 125 rpm to ensure the cells reached OD_{600nm} of 0.4. Next, the cells were placed on ice for 10 minutes. The cells were then transferred into pre-chilled 50 ml conical tubes. Cells were centrifuged at 3000 x g in a 4°C centrifuge for 10 minutes. The supernatant was removed and the pellet resuspended in 1.6 ml of ice cold 100 mM CaCl₂. The cells were incubated on ice for 20 minutes. After the cells are washed twice, 0.5 ml of cold 80% glycerol was added to the suspension. Finally, 500 µl aliquots of cells were prepared in 1-ml cryogenic vials and placed into a -80°C freezer for use.

Plate Preparation

To prepare LB + antibiotic plates, 800 ml of deionized water was placed in a large flask on a stir plate. Then, 25 g of LB mix including 10 g of tryptone, 10 g of NaCl, and 5 g of yeast were weighed and added to the flask (85). Next, 15 mg of agar was added to the flask. All components were stirred until they appeared evenly mixed. Then, about 200 ml of deionized water was added to the mixture and allowed to stir. Once
that is completed, the LB/Agar mixture was placed in an autoclave for one hour for sterilization. After sterilization, the mixture was allowed to cool until 60°C or less is reached. Then, 10 ml of ampicillin (100 mg/ml) was added to the flask. Finally, about 25 ml of LB agar + Ampicillin was poured into each plate. The recipe makes up to 40 plates.

**DNA Extraction and Purification**

pET11d DNA was obtained from BL21(DE3) expression strain DNA gold stocks in the laboratory provided generously by Dr. Francis Jenney, Philadelphia College of Osteopathic Medicine—Georgia Campus. The strain was inoculated into 5 ml of Luria broth (LB) containing a 1:10 dilution of ampicillin (10 mg/ml) and shaken overnight at 37°C (85). Using the Strataprep™ Plasmid Miniprep kit (Agilent Technologies, Santa Clara, CA), the plasmid DNA was purified using the manufacturer protocol (103).

This purified plasmid DNA was transformed into DH5α growth strain competent cells. First, previously cultured DH5α competent cells were obtained from the -80°C freezer and thawed on ice. While the cells thawed, super optimal broth with catabolise repression (SOC) media was prepared using sterile super optimal broth (SOB), 1M MgCl₂, and 40% glucose solution. Media such as this is an optimized version of LB used by researchers attempting to grow and express DNA plasmids in *E. coli* and other bacteria (82). SOB broth containing a mixture of bacto-tryptone, bacto-yeast extract, NaCl, KCl, MgCl₂, MgSO₄, and deionized water (85). Then, 50 µl of thawed competent cells were pipetted into a 1.5 ml eppendorf tube on ice. Next, 10 ng of pET11d DNA [20 ng/µl] was pipetted into the tube and incubated on ice for 30 minutes. Once this
 incubation was complete, the cells were heat shocked at 42°C in a water bath for 30 seconds. Then, the cells were incubated on ice for 2 minutes. Next, 1 ml of the prepared SOC medium was added to the tube. The tube was then placed into a 37°C incubator for 1 hour. After incubation, 100 µl of the cell solution was spread onto an LB + Ampicillin agar plate. The plate was placed into the 37°C incubator to incubate overnight.

The pET11d DNA was extracted from these DH5α colonies in the laboratory using the PureYield™ Plasmid Midiprep System (103, 104). It was found that the DNA recovery yielded using this kit was less than ideal for this study. Thus, the Strataprep EF Plasmid Midiprep Kit (Cat#400721) from Stratagene was used for the remainder of the study (105). The protocol associated with this kit was followed using the steps described here. First, a single colony of DH5α cells was inoculated into 200 ml of LB culture media and vigorously shaking overnight at 37°C. The next day, the bacterial culture was pelleted by centrifugation in 50-ml conical tubes at 4750 x g for 1 hour. The clarified supernatant was drained and discarded. Next, the pellets were vortexed for resuspension in the small amount of culture medium remaining in the tubes. After that, the resuspended pellets were combined into one tube. Then, 7 ml of Solution 1 was added to the tube and mixed vigorously with the vortex until the brown mixture turned blue in color. Solution 1 is a mixture of 50 mM Tris HCl, 10 mM EDTA, 50 ug/ml of RNase A, and 0.5% (w/v) SDS. This solution contains agents which degrade any RNA present in cells. Next, 7 ml of Solution 2 was added to the tube. Contents were mixed by inverting the tube several times until the contents uniformly turned purple in color. This solution contains 0.2 M NaOH and 1% (w/v) SDS. Solution 2 is responsible for degrading all protein in the
cells. The contents in the solution have been used in other studies to degrade proteins (106). After this, 2 ml of Solution 3 was added to the tube. The contents were once again mixed by inversion until the color changed to blue. Solution 3 is a proprietary reagent. Therefore, exact components are not available for public information. Once all 3 solutions were added, the tube was centrifuged at 4750 x g for 15 minutes. This centrifugation resulted in a compact, light blue pellet at the bottom of the tube, a thin blue film on the surface of the supernatant. The supernatant was pipetted into a fresh 50-ml conical tube taking care not to collect any of the pellet of thin film.

Next, 10 ml of Solution 4 was added to the tubes and the contents mixed by inversion. Solution 4 is also proprietary, but it is a chaotropic salt which assists in precipitating all DNA present within the cells. After the contents were mixed properly, the mixture was added to a midi-spin cup seated in a 50-ml conical tube with the cap tightened loosely. Sample was then centrifuged at 1500 x g for 10 minutes. The midi-spin cup containing the plasmid DNA was removed from the 50-ml tube and liquid discarded. The midi-spin cup was then placed back into the 50-ml conical tube. Next, 20 ml of wash buffer was added to the receptacle. The wash buffer contains 10 mM Tris HCl, 100 mM NaCl, 2.5 mM EDTA, and 10 mM Ethanol. As indicated by its name, it serves as a way to cleanse the DNA of any impurities that may have been caught in the fiber matrix of the midi-spin cup. The sample was spun at 1500 x g for 3 minutes. The liquid was discarded in the same manner as mentioned, and the tube was spun once more to ensure all wash buffer and any impurities it may contain was removed. The midi-spin cup was then transferred to a fresh 50-ml conical tube. Next, 500 ml of elution
buffer (i.e. nuclease free water) was added directly onto the fiber matrix at the bottom of the midi-spin cup. This was incubated at room temperature for 5 minutes. After the time elapsed, the tube was centrifuged at 1500 x g for 3 minutes. The purified plasmid DNA eluted into the receptacle was transferred to a 1.5 ml eppendorf tube.

To determine the concentration of this DNA, gel electrophoresis was used. A 0.8% agarose gel was used to run the negatively charged DNA toward the positive electrode on the electrophoresis apparatus. Agarose gels are commonly used as they are composed mainly of agarose, water, and 1M Tris and EDTA buffer. Agarose is a polysaccharide found in algae. This composition makes it easy for DNA to travel through the hole-filled gel. The percentage of this gel was used as it was recommended as the ideal percentage size of the DNA being separated (roughly 5000-6000 base pairs in size). About 10 µl of DNA and bromophenol blue stain (0.0025 mg/ml) were loaded onto the gel and run at 100V (constant voltage?) for 30 minutes. The bromophenol blue stain solution is composed of bromophenol sodium salt and deionized water. New England Biolabs Quick-load 1kb ladder used to determine the DNA size and concentration. Figure XV below is the DNA extracted using the aforementioned Stratagene kit.
Figure XV. 0.8% Agarose Gel used to determine the concentration of pET11d DNA extracted from Stratagene DNA Purification Kit. Lane 1 contains 10 ul of New England Biolabs Quick-load 1kb DNA Ladder. Lanes 2, 4, and 6 contain DNA collected from 3 separate purification experiments. It was determined that the DNA in lanes 2 and 6 are pET11d.
Restriction Digests

Restriction endonucleases were used to prepare the purified vector DNA for insertion of the custom LAGC and EA3K sequences ordered from Integrated DNA Technologies. These sequences were introduced at the same time and were designed to insert into the vector DNA in a certain order. This is known as directional cloning. Therefore, two sites were selected to accommodate for the sticky ends that correlate with these recognition sequences. For directional cloning, Ncol and HindIII sites were selected as the areas to be digested for insertion. Cloning in this manner has been completed successfully by a number of studies over the last few decades (107).

Restriction enzymes generate sticky ends within a vector which allow for the insertion of foreign DNA. These sites were selected because they are unique within pET11d and, therefore, should cut at its only respective cleavage recognition site. The recognition site for Ncol from 5’ to 3’ is C/CATGG (107). For this reason, the EA3K sequence contains a sticky ends coding for the complementary site to Ncol. The LAGC sequence was designed with sticky ends that correlate with the HindIII recognition site A/AGCTT. To ensure that both sequences attached well with one another sticky ends for BamHI were included in each. The BamHI recognition site is G/GATCC (108). This site is important not only to incorporate these 2 sequences, but also because this BamHI site was already within the pET11d vector. Cleaving the DNA with both Ncol and HindIII should have removed that site. Replacing this region allowed for screening of the final product to ensure that the foreign DNA cloned in properly. Restriction digests involve the following components mixed together in one tube: DNA, 10X NEB buffer, bovine
serum albumin (BSA), ≥ 10 units of desired restriction enzyme with attention to star activity, and deionized water. Bovine serum albumin is used as a reagent used to stabilize enzymes in restriction digests. A restriction digest using purified pET11d DNA was prepared using the reaction conditions listed below in Table I. These components were combined at the volumes listed below into a 1.5 ml eppendorf tube. The restriction enzyme was added last. The tube was then incubated for 1 hour at 37°C. After incubation, 5 µl of 1M Tris/0.1M EDTA (i.e. stop solution) was added to cease the reaction.

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Restriction Digest with NcoI (µl)</th>
<th>Restriction Digest with NcoI (µl)</th>
<th>Restriction Digest with NcoI (-) Control (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.5 100 µg/ml</td>
<td>0.5 100 µg/ml</td>
<td>0.5</td>
</tr>
<tr>
<td>NEBuffer 3 10X</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>ddiH2O</td>
<td>32</td>
<td>32</td>
<td>43.5</td>
</tr>
<tr>
<td>DNA (Final) = 60ng/µl</td>
<td>10 600 ng</td>
<td>10 600 ng</td>
<td>1 60 ng</td>
</tr>
<tr>
<td>Restriction Enzyme</td>
<td>2.5 25 u</td>
<td>2.5 25 u</td>
<td>2.5 25 u</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Table IV. Restriction Digestion with NcoI. This table represents the reaction conditions used to prepare pET11d for DNA insertion.

After the digestion was complete, the digested DNA was loaded onto a 0.8% agarose gel and run on a gel electrophoresis apparatus at 100V for 45 minutes. Upon viewing the gel using the [Alpha Imager], the desired band was excised from the gel using a razor and placed into a 15 ml conical tube. After excision, the Strataprep DNA Gel Extraction Kit (Cat #400766) was used to extract the DNA from the agarose gel. Specifically, 6.36 ml of extraction buffer was added to the 15-ml conical tube. Then, the
tube was incubated in a 50°C water bath for 10 minutes (mixing occasionally). After the
gel was completely dissolved, the mixture was transferred to a 2-ml receptacle with a
microspin cup seated in it. Due to volume, 825 µl of the mixture was transferred at a
time. After the mixture was transferred, the tube is centrifuged in a microcentrifuge at
14,000 x g for 30 seconds. The DNA is retained in the fiber matrix of the microspin cup.
Thus, after centrifugation, the liquid was decanted. This process was repeated until all
the liquid had been centrifuged and decanted. Then, 750 µl of wash buffer (with 100%
ethanol added) was pipetted into the microspin cup. The tube was centrifuged at 14,000
x g for 30 seconds. The wash buffer was decanted, and the tube was centrifuged at
14,000 x g for 30 seconds ensure that all wash buffer was removed. The micro-spin cup
was then transferred to a fresh 2-ml receptacle tube. Then, 50 µl of elution buffer was
added directly on top of the fiber matrix of the micro-spin cup and incubated for 5
minutes. Lastly, the tube is centrifuged at 14,000 x g for 30 seconds. The extracted
pET11d + NcoI DNA was seated in the 2-ml receptacle tube. Figure XVI below is a
depiction of the NcoI digest that was gel extracted.
Figure XVI. Restriction Digest with Ncol. 0.8% agarose gel of pET11d digested with Ncol and gel extracted using Strataprep DNA Gel Extraction kit.
Next, the gel extracted DNA was digested with *Hind*III. This experiment was conducted in the exact manner as the *NcoI* digest. However, a gel electrophoresis experiment and extraction were not necessary as this second digestion would be indistinguishable from the first digestion.

**Dephosphorylation**

Next, the pET11d + *NcoI* + *Hind*III was treated using New England Biolabs Calf Intestinal Alkaline Phosphatase (AP-CIP). This was done as a precautionary measure to ensure that the sticky ends generated by the restriction enzyme digests could not close before any foreign sequences could be introduced. Alkaline phosphatase works by removing the phosphate group (PO$_4$-$^3$) from the 5’ end of DNA strands (109). DNA treatment with AP-CIP is carried out in a similar manner to a restriction enzyme digestion. Therefore, Table V below is a representation of the reaction conditions the DNA was exposed to for dephosphorylation.

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Amount used (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X NEB Buffer 3</td>
<td>11</td>
</tr>
<tr>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>Calf Intestinal Alkaline Phosphatase (10 units/µl)</td>
<td>1</td>
</tr>
<tr>
<td>DNA</td>
<td>96</td>
</tr>
<tr>
<td>5.5 ng/µl</td>
<td>528 ng</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>110</strong></td>
</tr>
</tbody>
</table>

Table V. Dephosphorylation of pET11d + *NcoI* + *Hind*III
Water, NEB buffer 3, AP-CIP, and DNA were combined in 1.5 ml eppendorf tube and incubated for 1 hour in a 37°C incubator. Figure XVII A/B is an agarose gel depicting double digested pET11d after treatment with AP-CIP and gel extracted for use.
Figure XVII A and B. 0.8% agarose gel with alkaline phosphatase treated DNA. Lane 1 is QL 1kb ladder and Lane 3 is dephosphorylated pET11d backbone DNA.
Preparation of Insert DNA

As mentioned previously, the LAGC and EA$_3$K linker oligonucleotide sequences were designed and purchased through Integrated DNA Technologies. Among its services, this company provides custom oligonucleotide synthesis using artificial gene synthesis. Gene synthesis techniques have been used since the 1970s to generate usable, customizable DNA constructs. Nucleoside phosphoramidites are synthetic building blocks used to construct oligonucleotides added one at a time to create a chain. Currently, oligonucleotide sequences can be ordered at a length of up to 200 base pairs each. Goeddel et al generated synthetic insulin using synthetic techniques including PCR and radioimmunoassay (110). These synthetic DNA sequences are generated in forward and reverse sequences which are shipped in lyophilized conditions at a 20 nmole concentration. Thus, it was necessary to re-suspend each one in the appropriate buffer. Figure XVIII is a depiction of oligonucleotide synthesis and how oligonucleotides with sticky ends anneal to each other.
Figure XVIII A and B. A—Oligonucleotide Synthesis. B—General Annealing of Oligonucleotides with Sticky Ends.
Fresh, sterilized 10 mM Tris/1mM EDTA buffer was selected as the re-suspension buffer. This media was recommended by the manufacturers because its composition provides a constant pH environment for the synthetic DNA. First, each oligo tube was centrifuged at 14,000 x g for 30 seconds to ensure none of the lyophilized product is lost when the tube is opened. Then, approximately 200 µl of this buffer was added to each lyophilized oligo pellet for a final concentration of 100 uM respectively. This amount was added based on IDT recommendations to suspend as follows:

\[
\# \text{nmole} \times 10 \, \mu\text{l/nmole} = x \, \mu\text{l TE buffer to be added for [100 uM]}
\]

After re-suspension, the oligonucleotides were placed in the -20°C freezer. For use, each oligo forward and reverse sequence was annealed at a 1:1 molar ratio. This is completed as indicated by Table III below. First, 500 ml of water was heated in a beaker until it reached 95°C. Then, 10 µl of nuclease free water was added to an eppendorf tube. Next, 5 µl of DNA per oligo in µl was placed into the tube. Once these components are combined, the tube containing the combined oligonucleotide sequences was placed into the water beaker which was heated until the temperature of the water was 95°C. Annealing began once that tube was placed into the beaker and the water temperature was allowed to decline until a temperature of 30°C was reached. Confirmation that annealing occurred was verified using gel electrophoresis as depicted below in Figure XIX. A successfully annealed oligonucleotide will display a band at the expected size on the agarose gel. In this case, a band would be visible near the 100 bp rung for the LAGC oligonucleotide and near the 200 bp rung for the EA3K linker. If annealing did not take
place, it is possible that a band may not be visible on the gel at all. Furthermore, an incomplete or incorrect annealing may be denoted by a smear on the gel.

Table VI. This table contains the components used to anneal the oligonucleotides. Components were combined and heated in a 95°C water beaker until the water reached 30°C.

<table>
<thead>
<tr>
<th>LAGC</th>
<th>EAgK Linker</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µM LAGC-Fwd 5 µl</td>
<td>100 µM Linker-Fwd 5 µl</td>
</tr>
<tr>
<td>100 µM LAGC-Rev 5 µl</td>
<td>100 µM Linker-Rev 5 µl</td>
</tr>
<tr>
<td>10 ul ddH2O</td>
<td>10 ul ddH2O</td>
</tr>
<tr>
<td>[Final] = 50 µM 20 µl</td>
<td>[Final] = 50 µM 20 µl</td>
</tr>
</tbody>
</table>
Figure XIX. 0.8% Agarose Gel with Annealed LAGC and EA_{3K} Linker Oligonucleotides.
Insertion of LAGC and EA3K Linker DNA into pET11d

Ligation

The dephosphorylated pET11d + Ncol + HindIII, LAGC, and EA3K Linker DNA were combined in the attempt to create recombinant DNA using T4DNA ligase (New England Biolabs, Ipswich, MA). This reaction was set up such that a 3:1 insert-vector ratio was used. The exact reaction components and volumes are listed below in Table IV. Once all calculations were complete to ensure the insert-vector ratio was as expected, the components were mixed together making sure to pipet each gently into a 0.5 ml strip eppendorf tube. The tubes were then placed into the Thermocycler® PCR machine to be incubated overnight at 16°C.

<table>
<thead>
<tr>
<th>REACTION COMPONENTS</th>
<th>LIGATION #1</th>
<th>LIGATION #2 (CONTROL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 DNA LIGASE BUFFER*</td>
<td>3 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>~NEB#3</td>
<td>[Final] = 1X</td>
<td>[Final] = 1X</td>
</tr>
<tr>
<td>Vector DNA (5.674 kb)</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td></td>
<td>100 ng</td>
<td>100 ng</td>
</tr>
<tr>
<td>LAGC INSERT DNA (75 bp)</td>
<td>0.34 µl</td>
<td>0.34 µl</td>
</tr>
<tr>
<td></td>
<td>300 ng</td>
<td>300 ng</td>
</tr>
<tr>
<td>EA3K LINKER INSERT DNA (171 bp)</td>
<td>0.8 µl</td>
<td>0.8 µl</td>
</tr>
<tr>
<td></td>
<td>300 ng</td>
<td>300 ng</td>
</tr>
<tr>
<td>ddH2O</td>
<td>4.86 µl</td>
<td>5.86 µl</td>
</tr>
<tr>
<td>T4 DNA LIGASE 10 units/µL</td>
<td>1 µl</td>
<td>0 µl</td>
</tr>
<tr>
<td></td>
<td>10 u/ µl</td>
<td>0 u/ µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30 µl</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

Table VII. Ligation of pET11d backbone DNA with LAGC and EA3K Linker
**Introduction of Recombinant DNA into Host Organism**

The tubes were retrieved from the thermocycler and chilled on ice. DH5α competent cells retrieved from the -80°C freezer were thawed on ice for 10 minutes. An aliquot of 50 µl of these thawed cells were placed into 14 ml tubes for ligation 1, ligation 2 (- control), and pUC18 DNA (+ control/ transformation efficiency). This is transformation process is very closely related to the one done for obtaining pET11d DNA as mentioned previously. About 3 µl of DNA (or 10 ng) from ligation 1, ligation 2, and pUC18 were added to its respective tube carefully. The tubes were then incubated on ice for 30 minutes. Then, the cells were heat shocked at 42°C for exactly 30 seconds. Next, tubes were placed on ice for an additional 2 minutes. Then, 250 µl of pre-warmed, pre-prepared SOC without antibiotics was added to each tube. Next, the tubes were placed in a 37°C incubator to be shaken for 1 hour at 225 rotations per minute (RPM). While this shaking was taking place, six LB + Ampicillin plates were labeled and prepared for plating of the cells. When the one hour incubation was complete, a volume of cells were spread on each plate in the as described in Table III below. Once cells were spread on each plate, they were allowed to dry and placed into the 37°C incubator overnight.

<table>
<thead>
<tr>
<th>LB + Ampicillin</th>
<th>Ligation #1</th>
<th>Ligation #2 (- control)</th>
<th>pUC18 (+ control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100 µl cells only</td>
<td>100 µl cells only</td>
<td>100 µl cells only</td>
</tr>
<tr>
<td>2</td>
<td>100 µl LB + 10 µl cells</td>
<td>100 µl LB + 10 µl cells</td>
<td>100 µl LB + 10 µl cells</td>
</tr>
</tbody>
</table>

Table VIII. This table correlates to the six plates total from this transformation experiment.
Selection of Colonies Possibly Containing Recombinant DNA

The next day, the six plates were collected and observed for growth of colonies. A number of colonies was observed on four of the six plates total. These four plates included the set from ligation 1 as well as the pUC18 plate. Figure XX below is one representative plate from each set of plates.
Figure XX. Observation of LB/AMP plates containing colonies with possible recombinant pET11d DNA.
For organizational purposes, ligation plate 1 containing possible recombinant \( \text{pET11d} \) DNA was divided into quadrants A, B, C, and D. After selecting quadrants, each well-isolated colony (WICs) in each quadrant was counted and given a number. The number of WICs per quadrant is listed as follows: A-6, B-5, C-6, and D-4. Table VI below indicates the total number of colonies counted on each plate regardless of whether or not they were well-isolated or not.
Each of these WICs was streaked on LB + Ampicillin agar plate and grown overnight at 37°C. Then, the cells were collected by sterile technique and inoculated into 5 ml of sterilized LB broth with ampicillin (50 ug/ml). These inoculated colonies were allowed to grow overnight in a 37°C shaking incubator. The next day, DNA purification using the Strataprep® EF Plasmid Miniprep kit was performed on each colony. The protocol for this kit was followed as listed in a previous section. Figure XXI has been provided to accurately depict each quadrant on the LB + Ampicillin plate in this experiment.

<table>
<thead>
<tr>
<th>Ligation Plates</th>
<th>Plate #1</th>
<th>Plate #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11d + LAGC + Linker</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pUC18</td>
<td>TMC</td>
<td>TMC</td>
</tr>
</tbody>
</table>

Table IX. Ligation Colony Count. Plates with colonies too numerous to count are listed as TMC.
Figure XXI. Ligation 1 plate 1 quadrants A-D and their associated colony growth.
Screening for Clones with Appropriate Biological Properties

As mentioned previously, the design of this recombinant DNA included a number of unique restriction sites within the plasmid that would allow the identification of plasmids containing the correct sequence. The *Bam*H1 site within recombinant *pET11d* indicates that ligation and transformation efforts were successful.
Figure XXII. Vector Map of Predicted Recombinant DNA including *Bam*HI site.

*p-LAGC-EAK10*
Thus, upon DNA purification, each colony was digested with *BamH*I. The protocol for this restriction digest is nearly exact to that mentioned in the restriction digest section above. Table V below is a depiction of the conditions of which the purified recombinant DNA was treated.

<table>
<thead>
<tr>
<th>Components</th>
<th>A-D purified colonies (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.5</td>
</tr>
<tr>
<td>NEB Buffer 3</td>
<td>5</td>
</tr>
<tr>
<td>DNA [ ] = 40 ng/ul</td>
<td>10</td>
</tr>
<tr>
<td>BamHI</td>
<td>2</td>
</tr>
<tr>
<td>ddiH2O</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
</tr>
</tbody>
</table>

Table X. Restriction Digest with *BamH*I

*Pre-Screening of Recombinant Colonies*

Each colony was successfully digested with *BamH*I. Of the 21 colonies collected and purified, a total of 4 appeared to linearize the DNA at the expected size for the recombinant *pET11d* DNA. These colonies containing possible recombinant DNA will be referred to as *p*-LAGC-LINKER for the remainder of the publication. Figure XV A-D represents the restriction digests correlating to quadrants A, B, C, and D (see Figure XIII).
Figure XV A, B, C, and D. Each of these 0.8% agarose gels correlate with their corresponding letters. A restriction digest with *Bam*HI was performed on each colony. Undigested colonies are listed as *uncut*. The lanes of each gel are denoted in blue below. The colonies expected to contain the appropriate sequence are circled.
Table XI depicts which colonies were expected to contain \( p \)-LAGC-LINKER DNA. They are circled and indicated in the table below. It appeared that quadrant A contained at least two colonies that could possibly be the correct sequence. Quadrant B did not appear to have a single colony digest the DNA at the appropriate size. Both quadrants C and D contained one colony that demonstrated a linearized band at the correct size.

<table>
<thead>
<tr>
<th>Quadrant</th>
<th>Colony 1</th>
<th>Colony 2</th>
<th>Colony 3</th>
<th>Colony 4</th>
<th>Colony 5</th>
<th>Colony 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Table XI. Pre-Screening Colonies with BamHI. + indicates linearized DNA band at expected size. – denotes that DNA did not linearize at the expected size.

After the pre-screening tests were completed, the purified DNA from all of these colonies was sent for DNA sequencing at Retrogen Technologies. Because the \( p \)ET11d vector contains a T7 promoter primer (5’ TAA TAC GAC TCA CTA TAG GG 3’) as well as a T7 terminator sequence primer (5’ GCT AGT TAT TGC TCA GCG G 3’) on either side of the targeted insert site, The data from Retrogen can be found in the Appendix: Sequencing Data section of this publication. The data indicate that colony A1 contained the sequence correlating with what was expected should the LAGC and EA3K Linker sequences have incorporated properly. Figure XXIV below is a depiction of this data.
Figure XXIV. Data Analysis of recombinant DNA received from Retrogen Technologies. A color coded sequence map is included.
Upon confirmation that the colony A1 contained \textit{p-LAGC-LINKER DNA}, the plasmid was given the name \textit{p-LAGC-EAK10}.

\textbf{Specific Aim 2:} Use the \textit{p-LAGC-EAK10} plasmid to incorporate BPTI inhibitor creating the lipid-anchored “inhibitor ruler”. Once it was confirmed that the LAGC and EA\textsubscript{3}K sequences were properly introduced into \textit{pET11d} generating a new plasmid named \textit{p-LAGC-EAK10}, an attempt to introduce the BPTI sequence into the recombinant DNA downstream from the EA\textsubscript{3}K linker region was done. This process is identical to that of techniques performed in \textbf{Specific Aim #1}. A graphic map of the current plasmid can be found in Figure XXV below depicting the unique restriction sites within the vector.
Figure XXV. Graphic map of p-LAGC-EAK10

Serial Cloner 2.6
**p-LAGC-EAK10 DNA Purification**

Using techniques as outlined in Specific Aim #1 in the DNA purification section, DH5α cells containing p-LAGC-EAK10 DNA was grown in 500 ml of LB broth with ampicillin, and DNA harvested as described in *Materials and Methods* section. To confirm this DNA had been properly purified, it was separated on an agarose gel at 100V for 45 minutes. The plasmid DNA was observed on the gel exhibiting two crisp bands below the 8,000 bp rung, which indicated that the DNA was successfully purified. Multiple bands are common with circularized DNA as it is often supercoiled. One band ran very high, while the other was observed much lower on the gel suggesting nicked DNA.

*Sequential Restriction Digests using Ncol and Xbal*

BPTI inhibitor DNA insert oligonucleotides were designed to contain sticky ends correlating to unique cutting sites of Ncol and Xbal within recombinant p-LAGC-EAK10 DNA. Therefore, recombinant p-LAGC-EAK10 was cleaved open beginning with the restriction endonuclease Xbal.

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Restriction Digest with Xbal (µl)</th>
<th>Restriction Digest with Xbal (-) Control (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.5 100 µg/ml</td>
<td>0.5 100 µg/ml</td>
</tr>
<tr>
<td>NEBuffer 3</td>
<td>5 10X</td>
<td>5 1X</td>
</tr>
<tr>
<td>ddH2O</td>
<td>22</td>
<td>43.5</td>
</tr>
<tr>
<td>DNA</td>
<td>20 1000 ng</td>
<td>1 50 ng</td>
</tr>
<tr>
<td>Restriction Enzyme</td>
<td>2.5 25 u</td>
<td>0 0 u</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

**Table XII. p-LAGC-EAK10 Restriction Digest with Xbal**
The DNA and all necessary components as listed above were combined in a 1.5 ml eppendorf tube and incubated at 37°C for 1 hour. Upon digestion, stop solution (containing EDTA) was added to cease enzymatic activity. Then, DNA was loaded onto a 0.8% agarose gel and run at 100V for 45 minutes. After gel separation, the band corresponding to the size of linearized DNA (= 5700 bp) was excised and gel extraction was performed.

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Restriction Digest with Ncol (µl)</th>
<th>Restriction Digest with Ncol (-) Control (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>100 µg/ml</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>NEBuffer 3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>(½ 10X)</td>
<td>1X</td>
<td>1X</td>
</tr>
<tr>
<td>ddiH2O</td>
<td>18</td>
<td>43.5</td>
</tr>
<tr>
<td>DNA</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>500 ng</td>
<td>21 ng</td>
</tr>
<tr>
<td>Restriction Enzyme</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>10 units/µl</td>
<td>25 u</td>
<td>0 u</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Table XIII. \( p\)-LAGC-EAK10/Xbal Restriction Digest with Ncol

Once extracted, the DNA was combined with all necessary components as listed above and digested with Ncol. Once this process was completed, the \( p\)-LAGC-EAK10 DNA was dephosphorylated in a 37°C incubator using AP-CIP as described below.

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Amount used (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X NEB Buffer 3</td>
<td>11</td>
</tr>
<tr>
<td>Calf Intestinal Alkaline Phosphatase (10 units/µl)</td>
<td>10 u</td>
</tr>
<tr>
<td>DNA</td>
<td>96</td>
</tr>
<tr>
<td>5 ng/µl</td>
<td>480 ng</td>
</tr>
<tr>
<td>ddH2O</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
</tr>
</tbody>
</table>

Table XIV. \( p\)-LAGC-EAK10/Xbal/Ncol Restriction Digest with AP-CIP
BPTI insertion into p-LAGC-EAK10 recombinant DNA

As mentioned, the BPTI forward and reverse oligonucleotides were designed to contain sticky ends correlating to the recognition sites for NcoI and XbaI. The recognition site for XbaI is T/CTAGA. Because this sequence was purchased as forward and reverse sequences as the other oligonucleotides were, it was necessary to anneal it using the same technique as listed in Specific Aim #1. The size of the annealed product should be 189 base pairs. Figure XXVI is a 0.8% gel depicting the annealed DNA that correlates to the size of the designed BPTI insert. Smearing around the predominant band can be explained by the overloading of the DNA, misalignment of the annealed product, or potentially degraded DNA.
Figure XXVI. Annealed BPTI product on a 0.8% agarose gel. Lane 1 is QL 1kb ladder. Lane 2 is 100 bp ladder. Lane 3 is annealed BPTI product about 189 bp in size.
The reaction components of this ligation are listed below. This ligation was conducted using the protocol as outlined in the previous sections.

<table>
<thead>
<tr>
<th>REACTION COMPONENTS</th>
<th>3:1 LIGATION</th>
<th>p-LAGC-EAK10/XbaI/NcoI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 DNA LIGASE BUFFER*</td>
<td>3 μl [Final] = 1X</td>
<td>3μl [Final] = 1X</td>
</tr>
<tr>
<td>VECTOR DNA (5.674 kb) (5 ng/μl)</td>
<td>20 μl 100 ng</td>
<td>20μl 100 ng</td>
</tr>
<tr>
<td>BPTI INSERT DNA (189 bp) (177 ng/μl)</td>
<td>1.7 μl 300 ng</td>
<td>1.7 300 ng</td>
</tr>
<tr>
<td>DD,H2O</td>
<td>4.3 μl</td>
<td>4.3 μl</td>
</tr>
<tr>
<td>T4 DNA LIGASE 10 UNITS/μL</td>
<td>1 μl 10 u/μl</td>
<td>0 μl 0 u/μl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30 μl</td>
<td>30 μl</td>
</tr>
</tbody>
</table>

Table XV. Ligation of p-LAGC-EAK10 and BPTI

Introduction of recombinant p-LAGC-EAK10 DNA into Host Organism

After the ligation was complete, a transformation was performed using the techniques outlined in a previous section. DH5α competent cells were used in this experiment as in the transformation prior to this. Once the transformation was finished, five LB + Ampicillin plates were used to spread the ligation and control cells for overnight incubation in a 37°C incubator.

Selection and Screening of recombinant p-LAGC-EAK10 Colonies

The next day, the LB + Ampicillin plates were collected and observed for growth of any colonies. Of all the plates, none of them contained colonies for screening. Ligation
experiments were repeated as described as well as with the addition of troubleshooting steps. Multiple troubleshooting attempts were made to remedy any possible reasons for unsuccessful colony growth.

Troubleshooting

Because the plates from the initial ligation of p-LAGC-EAK10 and BPTI were void of colony growth, it was necessary to evaluate for possible issues that would lead the experiment to be unsuccessful.

Attempt 1

In an effort to increase the chances of insertion, a second ligation experiment using insert-vector ratios of 3:1, 6:1, and 9:1 were completed. All other conditions and components remained the same. The experiment was carried out as described in the Materials and Methods section. To ensure that the issue was not the DH5α competent cells pUC18 DNA was transformed into the cells. In addition to the pUC18 DNA, a ligation using no insert was used as a control. No colonies were observed on those plates. While no colonies were observed on any of the ligation plates including the ligation without insert, no growth was observed on the pUC18 plate either. Photos of this attempt were not recorded.
Because no growth was observed on any of the plates, the viability of the competent cells was in question. A set of competent cells were prepared using the techniques described in the Materials and Methods section. After the cells were prepared, the viability of the competent cells was confirmed by transformation of pUC18 DNA as described previously. Another ligation attempt was made by protocol using the conditions in Attempt 1 previously. Though the pUC18 positive control plate set contained colony growth, ligation colonies were not observed.
Once the viability of the competent cells was re-established but not colonies growing on the ligation plates, it was suspected that there may be an issue with the p-LAGC-EAK10 plasmid DNA itself. So, an experiment was performed using the same components as above with pUC18 DNA as a positive control along with uncut p-LAGC-EAK10 plasmid DNA and p-LAGC-EAK10/XbaI/Ncol with all other components except oligonucleotide inserts.

### Table XVII. Ligation Attempt 2

**Attempt 3**

<table>
<thead>
<tr>
<th>REACTION COMPONENTS</th>
<th>3:1 LIGATION</th>
<th>6:1 LIGATION</th>
<th>9:1 LIGATION</th>
<th>p-LAGC-EAK10/XbaI/Ncol</th>
<th>pUC18</th>
<th>p-LAGC-EAK10</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 DNA LIGASE Buffer*</td>
<td>3 µl [Final] = 1X</td>
<td>3 µl [Final] = 1X</td>
<td>3 µl [Final] = 1X</td>
<td>3 µl [Final] = 1X</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>VECTOR DNA (5.674 kb) (5 ng/µl)</td>
<td>20 µl 100 ng</td>
<td>20 µl 100 ng</td>
<td>20 µl 100 ng</td>
<td>20 µl 100 ng</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>BPTI INSERT DNA (189 bp) (177 ng/µl)</td>
<td>1.7 µl 300 ng</td>
<td>3.4 µl 600 ng</td>
<td>5.1 µl 900 ng</td>
<td>0 0 ng</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ddH2O</td>
<td>4.3 µl</td>
<td>2.6 µl</td>
<td>0.9 µl</td>
<td>5.0 µl</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>T4 DNA LIGASE 10 UNITS/µL</td>
<td>1 µl 10 u/ µl</td>
<td>1 µl 10 u/ µl</td>
<td>1 µl 10 u/ µl</td>
<td>1 µl 10 u/ µl</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30 µl</td>
<td>30 µl</td>
<td>30 µl</td>
<td>30 µl</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
This ligation attempt was made by protocol using the conditions (1 hour, 37°C) previously. Though the pUC18 and uncut p-LAGC-EAK10 positive control plate sets contained colony growth, ligation colonies were not observed on any other plate.

**Attempt 4**

No colonies were observed on the ligation plates including the control plate without insert. This plasmid demonstrated viability in past experiment, but it was possible that there was an issue with any one of the ligation components. The protocol associated with the T4 DNA ligase enzyme states that it contains ATP (adenosine triphosphate) which catalyzes the ligation of the DNA strands. It also states that multiple freezing and thawing of this enzyme is not recommended as ATP becomes deactivated after multiple thaws. For this reason, a set of ligations at 3:1, 6:1, 9:1, and 12:1 insert-

<table>
<thead>
<tr>
<th>VECTOR DNA (5.674 kb) (5 ng/µl)</th>
<th>20 µl 100 ng</th>
<th>20 µl 100 ng</th>
<th>20 µl 100 ng</th>
<th>20 µl 100 ng</th>
<th>N/A</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI INSERT DNA (189 bp) (177 ng/µl)</td>
<td>1.7 µl 300 ng</td>
<td>3.4 µl 600 ng</td>
<td>5.1 µl 900 ng</td>
<td>0 0 ng</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>4.3 µl 2.6 µl</td>
<td>0.9 µl 5.0 µl</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 DNA LIGASE 10 UNITS/µL</td>
<td>1 µl 10 u/µl</td>
<td>1 µl 10 u/µl</td>
<td>1 µl 10 u/µl</td>
<td>1 µl 0 u/µl</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30 µl 30 µl</td>
<td>30 µl 30 µl</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
vector ratios was set up using a fresh vial of T4 DNA Ligase and using LB broth alongside a set cultured in SOC. All other components remained the same.

<table>
<thead>
<tr>
<th>REACTION COMPONENTS</th>
<th>3:1 LIGATION</th>
<th>6:1 LIGATION</th>
<th>9:1 LIGATION</th>
<th>12:1 LIGATION</th>
<th>p-LAGC-EAK10/Xmal/Ncol</th>
<th>pUC18</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEW 10X T4 DNA LIGASE BUFFER*</td>
<td>3 µl [Final] = 1X</td>
<td>3 µl [Final] = 1X</td>
<td>3 µl [Final] = 1X</td>
<td>3 µl [Final] = 1X</td>
<td>3 µl [Final] = 1X</td>
<td>N/A</td>
</tr>
<tr>
<td>VECTOR DNA (5.674 kb) (5 ng/µl)</td>
<td>20 µl 100 ng</td>
<td>20 µl 100 ng</td>
<td>20 µl 100 ng</td>
<td>20 µl 100 ng</td>
<td>20 µl 100 ng</td>
<td>N/A</td>
</tr>
<tr>
<td>BPTI INSERT DNA (189 bp) (177 ng/µl)</td>
<td>1.7 µl 300 ng</td>
<td>3.4 µl 600 ng</td>
<td>5.1 µl 900 ng</td>
<td>6.8 µl 900 ng</td>
<td>0 0 ng</td>
<td>N/A</td>
</tr>
<tr>
<td>ddH2O</td>
<td>4.3 µl</td>
<td>2.6 µl</td>
<td>0.9 µl</td>
<td>0 µl</td>
<td>5.0 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>T4 DNA LIGASE 10 UNITS/µL</td>
<td>1 µl 10 u/µl</td>
<td>1 µl 10 u/µl</td>
<td>1 µl 10 u/µl</td>
<td>1 µl 10 u/µl</td>
<td>1 µl 0 u/µl</td>
<td>N/A</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30 µl</td>
<td>30 µl</td>
<td>30 µl</td>
<td>30.8 µl</td>
<td>30 µl</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table XIX. Ligation Attempt 4

After the overnight incubation at 37°C, colonies cultured in SOC were observed on the 9:1 plates. A lawn of colonies was recorded on these plates. Figure XXVII below is the image of this plate.
Figure XXVII. 9:1 Insert-Vector Ratio Ligation Plate
It was necessary to re-plate these cells at a 1:100 dilution for more well-isolated colonies. The next day, the plate was obtained and observed for colonies. No colonies were present. It can be surmised that the transformed DNA was plated at a concentration that was too high which allowed for a lawn to grow successfully on the plate. It is possible antibiotic concentration played a role as well. Therefore, when plated at a lower concentration no cell growth occurred. Another possibility there may have been confusion between the positive control and ligation sample by technician error. Finally, there is a possibility that a lawn grew because the ampicillin concentration was too high or too low to allow colony growth at the appropriate rate.

**Attempt 5**

Although all these adjustments to the ligation experiment had not induced much successful colony growth, it was determined over the last few experiments that 6:1 and 9:1 ratios were the most effective in colony growth. Also, the fresh T4 DNA ligase appeared to have a small role in the growth of colonies seen in **Attempt 4**. So, a set of ligations using 6:1 and 9:1 insert-vector ratios was set up using T4 DNA ligase and additional ATP from a stock solution concentrated at 10 mg/ml. All other components and conditions remained the same.

<table>
<thead>
<tr>
<th>REACTION COMPONENTS</th>
<th>6:1 LIGATION</th>
<th>9:1 LIGATION</th>
<th>p-LAGC-EAK10/XbaI/NcoI</th>
<th>PUC18</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 DNA LIGASE</td>
<td>3 µl</td>
<td>3 µl</td>
<td>3 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>BUFFER*</td>
<td>[Final] = 1X</td>
<td>[Final] = 1X</td>
<td>[Final] = 1X</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>10 MG/ML</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
<td>0.1 mg/ml</td>
<td></td>
</tr>
</tbody>
</table>
The following day colonies were observed on both 6:1 and 9:1 plates. There was a total of 4 colonies are organized in plates E and F. Plate E contained 3 well-isolated colonies while plate 4 contained a single well-isolated colony.

*Pre-screening and Data Analysis for Possible Recombinant DNA*

All well-isolated colonies were cultured and collected for DNA purification using the Strataprep™ kit (see protocol in *DNA Purification* section). Once the DNA from these colonies was properly extracted, pre-screening the DNA with XhoI was necessary. As indicated in Figure XXVIII, BPTI contains a unique XhoI site within the sequence of the expected vector. The recognition site for XhoI is C/TCGAG. Any colony DNA exhibiting a linearized band between 5 and 6kb could potentially contain the desired recombinant DNA. The results from these experiments are listed below.

<table>
<thead>
<tr>
<th>VECtor DNA (5.674 kb) (5 ng/μl)</th>
<th>20 μl</th>
<th>20 μl</th>
<th>20μl</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 ng</td>
<td>100 ng</td>
<td>100 ng</td>
<td></td>
</tr>
<tr>
<td><strong>BPTI INSERT DNA</strong> (189 bp) (177 ng/μl)</td>
<td>3.4 μl</td>
<td>5.1 μl</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>600 ng</td>
<td>900 ng</td>
<td>0 ng</td>
<td></td>
</tr>
<tr>
<td><strong>DD, H₂O</strong></td>
<td>1.6 μl</td>
<td>0.9 μl</td>
<td>4.0 μl</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>T4 DNA Ligase 10 units/μL</strong></td>
<td>1 μl</td>
<td>1 μl</td>
<td>1 μl</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>30 μl</td>
<td>31 μl</td>
<td>30 μl</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table XX. Ligation Attempt 5
Figure XXVIII. Ligation colonies digested with Xhol.
Screening results suggested that though DNA was successfully ligated, none of the colonies incorporated the BPTI DNA into the vector. Though the results suggested the ligation was unsuccessful, the purified DNA colonies were sent to Retrogen for sequencing. The data collected at Retrogen yielded the same conclusion.

**Attempt 6**

The addition of ATP to this ligation appears to have assisted the reaction successfully. Thus, a final ligation attempt was made using the exact conditions as Attempt 5 in anticipation that the results would be more successful.

<table>
<thead>
<tr>
<th>REACTION COMPONENTS</th>
<th>6:1 LIGATION</th>
<th>9:1 LIGATION</th>
<th>p-LAGC-EAK10/XbaI/NcoI</th>
<th>PUC18</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 DNA LIGASE</td>
<td>3 μl [Final] = 1X</td>
<td>3 μl [Final] = 1X</td>
<td>3 μl [Final] = 1X</td>
<td>N/A</td>
</tr>
<tr>
<td>BUFFER*</td>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>ATP 10 MG/ML</td>
<td>1 μl 0.1 mg/ml</td>
<td>1 μl 0.1 mg/ml</td>
<td>1 μl 0.1 mg/ml</td>
<td>N/A</td>
</tr>
<tr>
<td>VECTOR DNA (5.674 KB) (5 ng/μl)</td>
<td>20 μl 100 ng</td>
<td>20 μl 100 ng</td>
<td>20 μl 100 ng</td>
<td>N/A</td>
</tr>
<tr>
<td>BPTI INSERT DNA (189 bp) (177 ng/μl)</td>
<td>3.4 μl 600 ng</td>
<td>5.1 μl 900 ng</td>
<td>0 0 ng</td>
<td>N/A</td>
</tr>
<tr>
<td>D2O2H2O</td>
<td>1.6 μl</td>
<td>0.9 μl</td>
<td>4.0 μl</td>
<td>N/A</td>
</tr>
<tr>
<td>T4 DNA LIGASE 10 UNITS/μL</td>
<td>1 μl 10 u/ μl</td>
<td>1 μl 10 u/ μl</td>
<td>1 μl 0 u/ μl</td>
<td>N/A</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30 μl</td>
<td>31 μl</td>
<td>30 μl</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table XXI. Ligation Attempt 6
After transformation and overnight growth, the six plates were retrieved and observed for colony growth. Of the six plates, four plates including one of the 6:1 and one of the 9:1 contained colonies. The other two plates were the controls pUC18 and p-LAGC-EAK10/XbaI/NcoI without insert. The plates were divided into plates G and H. Plate G represented the 6:1 ratio colonies. Plate H was the 9:1 ratio plates. Figure XXIX is a depiction of these plates.
Figure XXIX. Ligation Plates G, H, and ρ-LAGC-EAK10 without insert.
These colonies were cultured and DNA purified as described previously. Screening of these colonies by restriction digest with Xhol was performed. Figure XXX A and B is a depiction of the results of this digest.
Figure XXX. Restriction Digest with Xhol. Lanes are denoted in blue.
According to the screening results, H6 appeared to be the only colony that could possibly contain the recombinant DNA as the plasmid compared to the undigested plasmid generated a crisp band at the expected digestion site. All the colonies were sent for sequencing at Retrogen. The data analysis indicates that no recombinant DNA was obtained. One possible reason for this is that the annealed BPTI product contained smears when observed on a 0.8% agarose gel. This suggests that the DNA misaligned and therefore would not allow the sticky ends to associate properly with the linearized p-LAGC-EAK10 vector.

Discussion:

Molecular cloning is a multi-step process involving several that are time consuming and unpredictable. As mentioned previously, generating recombinant DNA involves choosing a host organism and orchestrating conditions in which to linearize the vector, insert a foreign insert, and circularize the DNA. Upon circularization of recombinant DNA, sequencing is necessary to confirm the incorporation of foreign DNA insertions. This project was limited in the time frame allowed to complete experiments. While p-LAGC-EAK10 was generated successfully during the course of this project, several unsuccessful attempts to incorporate BPTI into the p-LAGC-EAK10 vector were made.

Possible issues for this have been listed previously including non-viable competent cells, deactivated ligation enzyme, and a low concentration of ATP necessary for ligation. Other possible issues to address could be the potentially misaligned
oligonucleotide annealed product. Perhaps, adjusting the annealing protocol by temperature or time frame would allow for a more successful annealing of the forward and reverse sequences.

**Future Aims:**

*BPTI Incorporation into p-LAGC-EAK10*

As this study ended with unsuccessful attempts to incorporate BPTI into the p-LAGC-EAK10 plasmid, the following are suggestions regarding the continuation of this research. First, increasing the insert to vector ratio would allow the annealed oligonucleotide more opportunities to incorporate properly into the plasmid.

*Expression & Purification of Inhibitor Ruler*

Next, expression of the BPTI-lipid anchored “inhibitor ruler” in the BL21(DE3) expression strain followed by detection of the protein using Western Blotting-SDS-PAGE with a goat polyclonal antibody that would recognize BPTI.

Deadmond et al (57) optimized the procedure for the induction of protein expression in BL21(DE3) cells. Fresh, sterilized TB broth with carbencillin (50 µl/ml) and glycerol would be used to culture the p-LAGC-EAK10-BPTI DNA shaking at 300 rpm in a 37°C incubator until the $A_{600}$ (absorbance) reaches 1.5-2.0. Next, the temperature is decreased to 25°C and the culture is allowed to continue incubating overnight. The next day, the cultures would be transferred into 1-L bottles and centrifuged at 3000 x $g$ for 25 minutes at 4°C. The supernatant would be decanted off and the cells washed with 250 ml of ice cold TBS/Azide solution. This solution is a Tris saline based azide often used as
blocking buffer in western blotting assays. It is used here to remove any impurities that may be present. It is composed of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.02% NaN₃. The cells are centrifuged again for 25 minutes at 3000 x g at 4°C. Next, after the supernatant is decanted, the cell pellets should be weighed. They are then frozen at -80°C for an hour or more. Next the pellets should be thawed at 37°C. Then, 50 ml of lysis buffer per gram of cell pellet should be added. The lysis buffer is a mixture of 20 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100, 0.02% NaN₃, and 1 mg/ml of lysozyme. The re-suspended cell pellets should be mixed well then incubated on a rocker at 4°C for one hour. Next, poly-ethylamine solution should be added at a final concentration of 0.1% and mixed well. This is allowed to stand at 4°C for 10 minutes. The poly-ethylamine solution is composed of 5% (w/v) poly-ethylenimine in water and NaN₃ concentrated at 0.02%. The cells are centrifuged at 3000 x g for 25 minutes at 4°C. If clarity is not achieved, it is suggested that the cells be treated with 5 µg/ml DNAsel for at least 20 minutes and centrifuged at 14,000 x g for 25 minutes at 4°C. The crude cell lysate yielded from this centrifugation should contain the BPTI protein.

After detection by SDS-PAGE (sodium dodecyl polyacrylamide gel electrophoresis), the next step would be purification of the p-LAGC-EAK10-BPTI inhibitor ruler using affinity chromatography with trypsinized columns. SDS-PAGE works by separating proteins based on molecular weights using gel electrophoresis. Because proteins with differing molecular weights usually have differing charges as well, separating them on an electrical field requires that the charges all be negative such that they migrate at the same time on the gel (111). The SDS detergent is used to do this.
Neuenschwander et al also provided proof-of-principle methods for expression and purification of the membrane-tethered BPTI constructs. Expression and purification can be done by the methods these researchers used in a bacterial system to engineer a construct containing a lipid-tethered inhibitors extending 75 Å above the membrane surface (57). Trypsin is used because as mentioned previously, serine proteases are very similar to trypsin. BPTI and trypsin have a very high affinity for one another. This means that any non-specific proteins in the cell lysate will elute out leaving only BPTI. Trypsin must be covalently bound to the column using techniques adapted from the BioRad™ Activated Immunoaffinity Chromatography kit. Then, the 10-ml column is equilibrated with trypsin. A minimum of 5 column volumes of equilibration buffer (20 mM Tris-HCl, 100 mM NaCl, and 0.1% Triton-X100) is flowed over the column at a flow rate of 1 ml/40 seconds. Then, the tubing containing the crude cell lysate should be loaded and flowed over the column at a rate of 1 ml/ 2.5 minutes. The flow-through should be saved because it should contain the desired product. About 50 ml of wash buffer (1 mM Tris-HCl, 100 mM NaCl) should be loaded into the tubing and run over the column at 1 ml/min. A high-salt wash removes any non-specific proteins that may bind to the column leaving only BPTI theoretically. Next, a fraction collector with 40 tubes should be set up. Each tube should contain 20 µl of 1M Tris-HCl to neutralize eluate upon collection. A fraction collector for 1 ml fractions should also be set up. Next, the tubing should be filled with elution buffer containing 10 mM HCl and 500 mM KCl. This elution buffer should be flowed over the column at a flow rate of 1 ml/min. Eluate should be
collected in the fraction tubes that were set up. These fractions can be read at A$_{280}$ in a quartz cuvette. The relevant fractions should be kept and the irrelevant ones discarded.

Once these steps have been completed, the p-LAGC-EAK10-BPTI inhibitor ruler will be relipidated and verified by gel filtration chromatography as described previously. Then, it should be ready for use to assist in the determination of the optimal reactive height for FX$_a$ alone as well as the prothrombinase complex (including fX$_a$, fV$_a$, and Ca$^{2+}$). Using an equilibrium-based chromogenic enzyme assay, researchers could measure the rates and extent of inhibition for both FX$_a$ and the FX$_a$/pro-coagulant complex. These experiments can be used to determine the reactivity of the complex to each modified inhibitor. As described previously, experiments such as this have been carried out successfully by a number of researchers including Deadmond et al. (57).

Comparing the reactivity for this inhibitor at a height of 70Å off the membrane surface will provide insight into the elasticity (ability to reach the cell surface) of these complexes, as well as suggest potential optimal reactive conformation of the fX$_a$ complex. Reactivity at 70Å will indicate the level at which fX$_a$ is inhibited from the membrane surface because this is the height previously predicted to be the location of the active site (57). As the height of this inhibitor ruler is adjusted, the height at which the inhibitor is the most reactive can be determined. Also, the height that is too short to cause inhibition can be determined. This information is indicative of how close to the simulated vessel surface fX can reach. This information will give confirmation as to the conclusions about fX$_a$ topography and exactly how it carries out its role in fibrin clot deposition.
Enzyme Reactivity Experiments

Rates of inhibition will be measured using solution-phase and surface-based equilibrium inhibition. Solution-phase enzyme inhibition assay and surface-based enzyme inhibition can be used to characterize inhibitor activity (i.e. its $K_i$ and inhibitory potential) as compared its native state. Using both of these experiments will indicate $fX_a$ enzymatic activity as it floats in solution as well as its activity when BPTI is attached to the membrane surface. As the study continues, site-directed mutagenesis can be used to generate $p$-LAGC-EAK-BPTI constructs with one fewer linker at a time to determine the range of optimal reactivities of $fX_a$.

Solution-phase equilibrium inhibition

Purified recombinant BPTI should be incubated with trypsin for 30 minutes or more until equilibrium is reached. Then, a chromogenic substrate can be added to measure enzymatic activity. A similar experiment using membrane-bound BPTI/$fX_a$ and soluble (free-floating) BPTI/$fX_a$ will need to be performed as well. The $K_i$ of BPTI/trypsin, anchored BPTI/$fX_a$, and soluble BPTI/$fX_a$ will be compared to one another. These comparisons will indicate whether or not the predicted reactivity height was accurate.

Rates can be recorded and plotted in comparison to the inhibitor concentration to calculate the $K_i$. Data from the inhibition plots/graphs derived from a series of slope calculations can be used to examine $fX_a$ and prothrombinase complex reactivity. A comparison of the modified and unmodified inhibitor could provide some information on inhibitory activity and the effect of the linker region within the engineered construct.
Solution-phase equilibrium as described above can be used to measure fX$_a$ reactivity in the presence of *un-anchored* BPTI. Using anchored BPTI is a plausible experiment too, as it can be done using only the fX$_a$ catalytic head and measuring activity of BPTI attached to a neutral phospholipid surface to ensure the process is not altered by a negative membrane surface. K$_i$ values obtained from each set of inhibitor rulers can be compared. It is important to ensure that modifications added to BPTI did not interfere or alter factor activity and also to correct any differences recorded between the modified and unmodified versions of the inhibitor.

**Surface-based equilibrium inhibition**

Each isolate of the inhibitor complexes can be incorporated separately into liposomes for the purposes of comparing the inhibition at variable-lengths. These liposomes will contain percentages of phosphatidyl choline (PC) (roughly 90%) and phosphatidylserine (PS) (roughly 10%) necessary for optimal binding of fX$_a$. Smith & Morrissey developed the protocol we will use to construct the liposomes by way of detergent removal from lipid-protein mixture using adsorbent beads (112). The fX$_a$, lipid vesicles containing recombinant BPTI, and pro-thrombinase complex members fV$_a$ and Ca$^{2+}$ can be added and incubated with the membrane. Once this forms the pro-coagulant complex and reaches equilibrium, a chromogenic substrate will be added and absorbance measured at 405 nm. This experiment takes place over a monitored time frame to establish the initial rate of substrate hydrolysis and indicate the remaining enzyme activity when equilibrium is reached. Rates recorded during this experiment can be plotted as described previously. Analysis regarding the ratio of anchored BPTI/fX$_a$ and
soluble BPTI/fX₃ Kᵢ rates will assist in making conclusions about the optimal height of active sites above the membrane. For instance, a Kᵢ ratio of greater than 1 suggests a non-optimal distance separating BPTI and the fX₃ active site. Conversely, a Kᵢ ratio of lesser than or equal to 1 indicates an optimal distance is reached. Any changes noted between these two data sets are also helpful with further data analysis on the proximity of BPTI and the FX₃ active site.

*Clinical Significance*

Venous thromboembolism, deep vein thrombosis, and other thrombotic disorders is the cause of over 100,000 deaths each year (113). Patients with these conditions and prone to atrial fibrillation are at the highest risk of death as a result of the anticoagulant treatment they need to maintain hemostasis. As mentioned, current treatments for thrombotic disorders are limited due to the specificity of drug targets and effective medical techniques. Clinicians are currently prescribing thrombotic patients warfarin, low molecular weight heparin, protease inhibitors, and other vitamin-K inhibitors. However, many of these treatments lack specificity in that each of them target more than one portion of the process. Whereas research indicates direct fX₃ or thrombin anticoagulants are much more effective at inhibiting harmful clotting. These two components are the largest proponents of coagulation. Therefore, specifically targeting one or the other has been shown to greatly reduce clotting without disturbing other biochemical processes. A review published by S. Aditya in *the Journal of Postgraduate Medicine* reports newer anticoagulant treatments are effective at targeting specific parts of the coagulation process but put the patient at risk of excessive
coagulation as well. Though these treatments are an improvement, they are still not as ideal as targeting fXₐ (114).

Information characterizing fXₐ topography more clearly would greatly improve researchers’ ability to generate drugs which can be used to target the two most committed steps to coagulation, activation of fXₐ and thrombin bursts. Data obtained from these experiments will be invaluable in gaining knowledge about the rational design of effective membrane-based inhibitors. In addition, findings could be very useful in clinical medicine as well in that these membrane-based inhibitors could be used for the treatment of thrombosis and other thrombotic disorders. The information will provide some guidance on specificity in regard to effective distances of inhibition on the membrane surface which could lead to a number of clinical applications. Among these applications are drugs designed to work more accurately to prevent adverse responses to thrombosis symptoms.
Acknowledgements

I would like to acknowledge a number of individuals and entities without which this project would have been impossible. I would like to thank CCDA and the Philadelphia College of Osteopathic Medicine-Georgia Campus for resources to carry out this study. I would also like to thank my mentor, Dr. Kimberly J. Baker, for her patience and guidance. I would like to thank both Dr. Francis E. Jenney and Dr. Harold Komiskey who served not only on the review committee for this project but also as great advisors. Each of them have provided guidance and assistance that was above and beyond what they were called to do.

Next, I’d like to acknowledge my sweet friend, Siri L. Chirumamilla. She has been invaluable to this project. She is an angel. She dedicated a great deal of her time to seeing this project through. I am certain that I would not have succeeded without her kind words, time, and experience. I would also like to mention Ms. Trina Bursey who has listened to me and offered me sanctuary every time I needed it. I would be remiss if I didn’t acknowledge Betty Ma for everything she has done toward the completion of this project. Also, I’d like to thank my family, friends, and supporters including but not limited to Jennifer Conley, Amelie Bottex, Stacy D. Arrington, LaChanda D. Wright, Shalisa Sanders, Gerald Holifield, Terra Jones-Steele and a host of others. Let me also extend my gratitude to my GA-PCOM family Mary Keith, Christal Santos, Cynthia Brooke Woods, Kristen Brittni Robertson, Linda Williams, Zicole Browne, Atijah Collins, Anthony Cooper, Austin Russell, Indi Shelton, Simone Pitts, Kimberly Lucier, and Robert Bonds. I want to recognize my family: the Jacksons, Blanks, and Sanders families. They have
provided spiritual, physical, and financial support for all of my endeavors. Their love and support cannot be replaced. Lastly, I would like to acknowledge the GA-PCOM Biomedical Sciences program. This is including and especially Dr. Brian Matayoshi and Linda G. Williams for giving me the opportunity to learn and develop my skills as a scientist, lifelong learner, and researcher. I am one step closer to achieving my dreams and it is because of this program.
Appendix I: Truncated pET11d Vector DNA and Protein Sequence

**HindIII** NcoI BamHI XbaI

Serial Cloner 2.6: Alignment pET11d Sequence

Similarity: 5674/5674 (100.00 %)

<table>
<thead>
<tr>
<th>pET11d</th>
<th>ttctcatgtttgcagcttatcatcgcataagccttaaatgcggtagtttatccagctataa 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11d</td>
<td>ttctcatgtttgcagcttatcatcgcataagccttaaatgcggtagtttatccagctataa 60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pET11d</th>
<th>tgtctaaacgcgctcaggccacctctggtatgaatctaacaatgcgtctctgctctctcg 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11d</td>
<td>tgtctaaacgcgctcaggccacctctggtatgaatctaacaatgcgtctctgctctctcg 120</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pET11d</th>
<th>caaccgcacccctggatgctgtaggcataggcttggttatgccggtactgccgggcctctt 180</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11d</td>
<td>caaccgcacccctggatgctgtaggcataggcttggttatgccggtactgccgggcctctt 180</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pET11d</th>
<th>gccccaaggggttatgctagttattgctcagcggtgagcagcagcagctctctcttttccacaccc 300</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11d</td>
<td>gccccaaggggttatgctagttattgctcagcggtgagcagcagcagctctctcttttccacaccc 300</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pET11d</th>
<th>cgggctttgttagcagccgatccgacccatttgctgtccaccagtcatgctagccatgg 360</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11d</td>
<td>cgggctttgttagcagccgatccgacccatttgctgtccaccagtcatgctagccatgg 360</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pET11d</th>
<th>tatatctcctttcttaaagtttaaaaaattttatactgagggatattgtttatccgctcacc 420</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11d</td>
<td>tatatctcctttcttaaagtttaaaaaattttatactgagggatattgtttatccgctcacc 420</td>
</tr>
</tbody>
</table>
Appendix II: BPTI DNA & Protein Sequence

Serial Cloner 2.6: Alignment BPTI Sequence

<table>
<thead>
<tr>
<th>W M R P D F C L E P Y T G P C K A R I</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI</td>
</tr>
<tr>
<td>BPTI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>I R Y F Y N A K A G L C Q T F V Y G G C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI</td>
</tr>
<tr>
<td>BPTI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R A K R N N F K S A E D C M R T C G G A</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI</td>
</tr>
<tr>
<td>BPTI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>* * X</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI</td>
</tr>
<tr>
<td>BPTI</td>
</tr>
</tbody>
</table>
### Appendix III: Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Base Pairs</th>
<th>Molecular Weight</th>
<th>Forward</th>
<th>Reverse</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LAGC</strong>—75 base pairs</td>
<td></td>
<td>46,366 g/mol</td>
<td>5'/5Phos- AGC TTA TGA AAA CCA AAA ACT TTC TTC TTT GTA TTG CTA CAA ATA TGA TTT TTA TCC CCC TGG CTG GAT GTG-3’</td>
<td>5'/5Phos- GAT CCA CAT CCA GCC AGG GGG ATA AAA ATC ATA TTT GTA GCA ATA CAA AAA AGA AGA AAG TTT TTG GTT TTC ATA-3’</td>
<td>LMKTKNLLFCIATNMIFIPLAGCX</td>
</tr>
</tbody>
</table>
| **LINKER**—171 base pairs | | 105,725 g/mol | 5'/5Phos- GATCCGAAGCGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAA90

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Base Pairs</th>
<th>Molecular Weight</th>
<th>Forward</th>
<th>Reverse</th>
<th>Protein Sequence</th>
</tr>
</thead>
</table>
| **LINKER**—171 base pairs | | 105,725 g/mol | 5'/5Phos- GATCCGAAGCGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAA91

**Protein Sequence:**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Base Pairs</th>
<th>Molecular Weight</th>
<th>Forward</th>
<th>Reverse</th>
<th>Protein Sequence</th>
</tr>
</thead>
</table>
| **LINKER**—171 base pairs | | 105,725 g/mol | 5'/5Phos- GATCCGAAGCGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAA92

**Protein Sequence:**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Base Pairs</th>
<th>Molecular Weight</th>
<th>Forward</th>
<th>Reverse</th>
<th>Protein Sequence</th>
</tr>
</thead>
</table>
| **LINKER**—171 base pairs | | 105,725 g/mol | 5'/5Phos- GATCCGAAGCGCGCGCGAAAGAAGCGCGCGAAAGAAGCGCGCGAA93

**Protein Sequence:**
<table>
<thead>
<tr>
<th>BPTI—189 base pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MolecularWeight—116,819 g/mol</td>
</tr>
</tbody>
</table>

**Forward:**

5'/5Phos-

CATGGATCGTGCCGGACTTCTGCCTGAGCCGCCGTACACGGGTCCCTGCAAAGCGCGCATCATCCGTTACTTCTACAGCAGGCTGTGCCAGACCTTCTTACGGTGGCAGAGCTAAGCGTAACAACTCAATCCGCTGAAGACTGCATGCACCGTACCTGCGGTGGTGCTTTAAT3’

**Reverse:**

5'/5Phos-

CTAGATTATTAAGCAACCACCGCAGGTACCGCATGCACTTCTCAGCGGATTGGTTAGTTACGGCTTAGCTCTGCAAGCCCGTTAAACGAAGGTGTTGCGACAGGGCTGTGCTTCTTAGCGTGGTGAGAGTAACCGGAGATAGCGCGCTTTGCTGGGACCCGTGTACGGCGGCTCGAGGCAGAAGTCCGGACGCATC3’

**ProteinSequence:**

WMRPDFCLEPPYTGPCKARIYFYNAKAGLCQTFVYGGCRAKRNNFSEAEDCMRTCGGA**X**
Appendix IV: p-LAGC-EAK10 Plasmid DNA

Serial Cloner 2.6: Alignment p-LAGC-EAK10 Sequence

Similarity: 5526/5526 (100.00 %)

```
L M K T K N F L L F C I A T N M I F I P
p-LAGC-EAK10 agcttatgaaacaaaaacttttctctttttgattctacaatatatgtttttatcc 60
```

```
L A G C G S E A A A K E A A A K E A A A
p-LAGC-EAK10 cccctggtctgtctgtctgggtcgcaagcgcgggcgaagaagccggcgcaaaagaagccggcg 120
```

```
K E A A A K E A A A K E A A A
p-LAGC-EAK10 cgaaagaagccgacggggaaggaaagccggcgcaaaagaagccggcg 180
```

```
K E A A A A K E A A A K E A A A K E A A A K G G G G
p-LAGC-EAK10 ccctggctggatgtggatccgaagcggcggcgaaagaagcggcggcgaaagaagcggcgg 240
```

```
S P R G E L L S A H N S P I V S R I N F
p-LAGC-EAK10gtagccctagaggggaattgttatccgctcacaattcccctatagtgttgtatttaatt 300
```

```
A G S R S R S S T P D A S W P A S P A P
p-LAGC-EAK10 tcgcgggatcgaatcttggtctggttcctctacctccgctgagcgcgcgttcctctgctcgcgcgttcctctgccggtgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgttcctctgctcgcgcgtt
```
Appendix V: Sequencing Data

Downloaded from www.retrogen.com/downloadresults
References


80. Anderer FA, Hornle S. CHEMICAL STUDIES ON KALLIKREIN INACTIVATOR FROM BOVINE LUNG AND PAROTID GLAND. Ann N Y Acad Sci. 1968; 146(2 Chemistry, Ph):381


