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Topography Study of FIXa: Generation of a Lipid Anchored Molecular Ruler with Attached Inhibitor

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TOPOGRAPHY STUDY OF FIXA: GENERATION OF A LIPID ANCHORED MOLECULAR RULER WITH ATTACHED INHIBITOR

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Abstract

Blood coagulation, an important hemostatic property, is directed through activation of proteolytic enzymes known as serine proteases to generate a fibrin plug. Using multiple proteases allows for localized and precise regulation of blood coagulation within the circulatory system. Factor IX, a serine protease, is a key activator in the coagulation cascade by activating Factor X. For activation of FX to occur, FIXa must be in proper conformation on the platelet surface with cofactor FVIIIa to increase enzymatic abilities of FIXa a billion-fold. Current research looks at structure and function of FIXa yet neglects structural plasticity on the membrane, with function of FIX clearly being related to conformation on the platelet. The role of this investigation was to study the optimal reactive topography of FIXa by generating AMRI, Adjustable height Molecular Ruler with attached Inhibitor. AMRI composed of three primary regions contains an Anchor region, Linker region, and Inhibitor. AMRI was generated using the protein anchoring sequence LAGC to fixate to a lipid surface. A height adjustable linker area was then required between the anchor and inhibitor regions of AMRI to ensure proper vertical height of inhibitor. The linker sequence EAAAK forms a rigid structure with a vertical height of 7.5A above the membrane surface, thus we repeated this sequence to achieve a specific desired height. Completing the

protein's linker region is a flexible linker at end of the rigid set. That flexible linker

ensures the inhibitor will fit in FIXa active site. Finishing the AMRI complex is a

PN2-KPI inhibitor, which has been demonstrated to fit and inhibit the catalytic site

of FIXa. Therefore, AMRI should allow for determination of optimal reactive

height of the FIXa active site without affecting structural conformation. Through

the generation of AMRI, a greater insight into blood coagulation will be gained,

along with potential for site specific anticoagulant clinical applications.

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LIST OF ABBREVIATIONS

Anchored Molecular Ruler with attached Inhibitor	AMRI
Activated Protein C	APC
Activated Protein S	APS
Amyloid β Protein Precursor	AβPP
Antithrombin III	AT
Bovine Pancreatic Trypsin Inhibitor	BPTI
Calf Intestinal Phosphatase	CIP
Cell-Based Model for Coagulation	CBMC
Glutamic Acid, (Alanine)3, Lysine	EAAAK
Epidermal Growth Factor	EGF
Factor I	FI
Factor II	FII
Factor III	FIII
Factor IV	FIV
Factor IX	FIX
Factor V	FV
Factor VI	FVI
Factor VII	FVII
Factor VIII	FVIII
Factor X	FX
Factor XI	FXI
Factor XII	FXII
Factor XIII	FXIII
Fluorescence Resonance Energy Transfer	FRET
Glycoprotein	GP
Kunitz Protease Inhibitor	KPI
Leucine Alanine Glycine Cysteine	LAGC
LAGC-(EAAAK)$_{11}$	AMR
Plasminogen Activator	PA
Polymerase chain reaction	PCR
Phosphatidylcholine	PC
Phosphatidylserine	PS
Protease Nexin2	PN2
Protease Nexin2 - Kunitz Protease Inhibitor	PN2-KPI
Ribosome Binding Site	RBS
Single Chain Urokinase	SCU

Surface Plasmon Resonance	SPR
Tris-Acetate-EDTA Buffer	TAE Buffer
Tenase	Xase
Thrombomodulin	TM
Tissue Plasminogen Activator	tPA
von Willebrand Factor	vWF
Gamma-Carboxyglutamic Acid Domain	Gla

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1   Background and Significance

1.1   Hemostasis

Humans are dependent upon the circulation of blood through a pressurized vascular network to sustain life. Continuous blood flow is vital for the transport of nutrients, hormones, and waste to allow proper cellular function. The body includes five major vessel types christened arteries, arterioles, capillaries, venules, and veins. The arteries and veins contain three different layers termed the tunica intima, tunica media, and tunica adventitia; constructing the inner, middle, and outer layers, respectively. The arterioles and venules contain two layers, tunica intima, and tunica media. The capillaries contain only one layer, the tunica intima. The tunica intima is composed of a single layer of endothelial cells found in all blood vessels. The other two layers of the blood vessels are comprised of an extracellular matrix composed of collagens, elastins, fibrils, and proteoglycans, which provide structure and regulation to blood flow[1, 2].

Hemostasis is the balance of clotting at the site of injury, fibrinolysis as part of healing and preventing the random clotting to allow flow of blood throughout its system. The hemostatic system has the responsibility of maintaining integrity of the closed vascular system. Vascular integrity can be affected by a multitude of things including, a needle used to draw someone's blood, rupture of an atherosclerotic plaque, or a deep laceration during a motor vehicle accident. It is possible that one of these events or others could affect an individual at any given time, significantly affecting blood flow.

Failure of vascular integrity resulting in diminished hemostasis will lead to various clinical pathologies and in extreme circumstances, tissue, and cellular death(3).

1.2 Platelet Activation and Coagulation Cascade

The process of blood changing from a flowing liquid to forming a solid clot is coagulation. The ultimate goal of coagulation is to achieve hemostasis, by pinpointing and obstructing the loss of blood at the site of vascular injury. This happens with the activation and adhesion of platelets providing a surface for clotting enzymes to undergo a series of reactions forming a clot. These series of enzymatic reactions in conjunction with platelets can seal off the area of vascular damage by generation of a non-soluble plasma protein, fibrin(4-6) .

Platelets are found in significant numbers within the blood. In intact blood circulation, platelets are smooth, discoid in shape, and contain an internal canalicular system. Additionally, platelets contain granules containing von Willebrand Factor (vWF), platelet-activating factor, and other platelet activating materials(7, 8) .

Activation of platelets is initiated by a rupture in the vessel endothelial lining, exposing von Willebrand Factor (vWF) and extracellular matrix proteins, such as collagen, to the blood. This contact between surface receptors on the platelet with these proteins causes the conformational changes in platelets by extending their pseudopodia

contained within the canalicular system (8). Translocation of the pseudopodia exposes the anionic phospholipid layer on their outer surface for attachment of other platelets, vWF, and other proteins(8-10) .

The mechanism behind the translocation is caused by a platelet membrane receptor, GPIa, which induces the platelet conformational change (see Figure 1). This change exposes a second platelet membrane receptor GPIb and in the presence of vWF, induces greater conformational change. This greater change exposes the GPIIb/GPIII receptor proteins, allowing for binding of more collagen and vWF(8).

Once platelets are activated, these platelets begin to adhere to one another, in a process called platelet adhesion. Just as the activation cause the extension of the pseudopodia and adhesion, it causes the granules to be released from the canalicular system as well. By releasing the granules, this creates more soluble vWF to activate more platelets (8) .

Now with activated platelets, this self-sealing system employs the combination of the tissue factor pathway and the contact activation pathway; termed extrinsic and intrinsic pathways, respectively. These pathways are a series of sequential reactions, in which the inactive enzyme precursors (zymogens) of a serine protease activates to become an activated serine protease. The
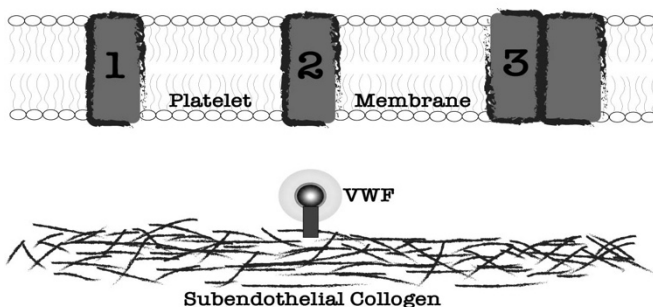
Figure 1. Platelet Adhesion Steps. GpIa binds the exposed subendothelial collagen (Step 1) and allows GpIb to bind vWF (Step 2). This binding with vWF exposes the (Step 3) GpIIb-GpIIIa complex. This image was adapted from: Lieberman M, Marks AD. Marks' basic medical biochemistry

coagulation cascade zymogens are called Factors and identified by Roman numerals (see Table 1 for a list). Once the Factor has been activated its name indicates so by following the name with the letter "a"; i.e. Factor IX (FIX) activated is Factor IXa (FIXa). That serine protease plants on the phospholipid surface provided by the platelet, thus catalyzing the next reaction in the cascade by cleaving the downstream substrate. The intrinsic and extrinsic cascades work in tandem having the goal of activating Factor X (FX), prothrombin (FII), and cleaving fibrinogen (FI) (11-15) .

Table 1 Blood Coagulation Proteins.

Blood Coagulation Proteins with Common Name, Descriptive Name, and Function. An activated form has an "a" added to the common name. An * indicated the proteins that are Vitamin K dependent.

Common Name	Alternative Descriptive Name	Function/Active Form
FI	Fibrinogen	Fibrin
FII*	Prothrombin	Serine protease
FIII	Tissue Factor(TF)	Receptor, Cofactor, and Initiator
FIV	Ca^{2+}	Cofactor
FV	Proaccelerin, labile factor	Cofactor
FVII*	Proconvertin	Serine protease
FVIII	Antihemophilia factor A	Cofactor for FIX activation
FIX*	Antihemophilia factor B, Christmas factor	Serine protease
FX*	Stuart-Prower factor	Serine protease
FXI	Plasma thromboplastin antecedent	Serine protease
FXII	Hageman Factor	Serine Protease
FXIII	Fibrin-stabilizing factor	Ca^{2+}-dependent transglutaminase
Thrombomodulin		Thrombin Cofactor in Protein C Activation, Endothelial Cell Receptor
Protein C*		Serine Protease, Activated by Thrombomodulin-bound thrombin
Protein S*		Cofactor for activated Protein C
Antithrombin III	ATIII	Protease Inhibitor

The Extrinsic pathway, as seen in figure 2, is composed of Tissue Factor (TF) and Factor VII (FVII) to activate FX (16) . TF is expressed on subendothelial tissues and fibroblast cells outside of the vasculature. This is vital for pinpoint identification of a vascular break and formation of the TF-FVIIa complex (3, 17, 18) . The TF-FVIIa complex then activates FX and FIX (19, 20).

The Intrinsic pathway is composed Coagulation Factors IX, VIII, and XI (see Figure 2). Again, the goal is to activate Factor X and ultimately generate a thrombin burst. The pathway starts with the activation of FXII that converts FXI to FXIa. FXIa then cleaves FIX, activating to create FIXa. Then FIXa interacts with FVIIIa to form the tenase complex, whose role is to activate FX into FXa (12).

Figure 2. Blood Coagulation Cascade showing the overall goal of activation of FX to Xa to for the prothrombin complex to form a hard fibrin clot. Not shown are the necessary cofactors that are required for full procoagulant activity. Adapted from: Lieberman M, Marks AD. Marks' basic medical biochemistry: A clinical approach. 4th ed. Lippincott Williams & Wilkins; 2012.
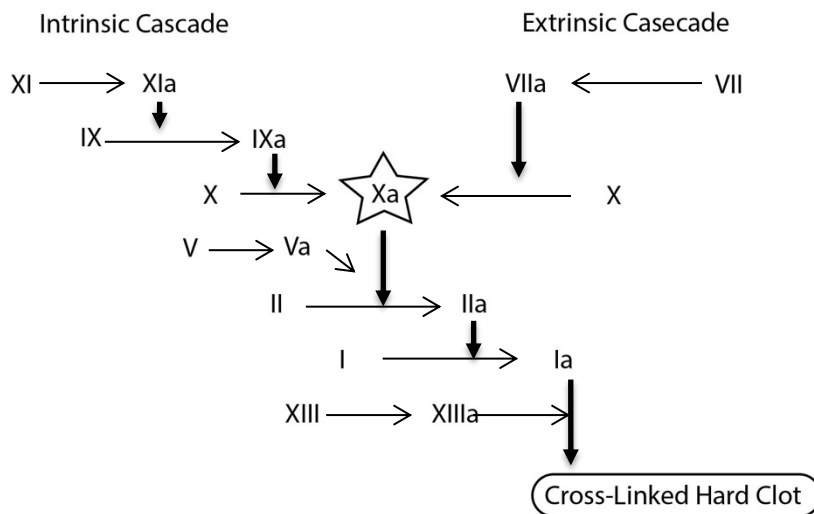
Extrinsic and intrinsic pathway activation results in the activation of the common pathway for a favorable thrombin generation state (see Figure 2). The central objective of the common pathway is to activate FX,

thrombin, and fibrin, inducing clotting. This central objective is best achieved when coagulation is viewed as the Cell-Based Model for Coagulation (CBMC) (21). The CBMC model accounts for the fact that both pathways are necessary to form a fibrin clot and that if one is deficient this leads to clinical issues such as hemophilia or spontaneous clot formation. CBMC is a four-step process for coagulation, composed of Initiation, Amplification, Propagation, and Termination (21).

Initiation occurs when trauma leads to the exposure of TF, extracellular collagen, and von Willebrand Factor (vWF) (13, 17, 22). These indicators allow for injury site detection with pinpoint precision and initiate a response. Platelets, activated by vWF and extracellular collagen, extend their pseudopodia. The activation of platelets and exposure of the TF bearing tissue to circulating blood is important for providing an anionic phospholipid layer for the coagulation factors to sit on (12, 23).

Simultaneous to the platelet activation is the formation of a complex between Coagulation Factor VII (FVII) and Tissue Factor (TF) (23). FVII activation is brought about by auto-activation or activation by trace amounts of FXa (24). Formation of this complex activates a small amount of FIX and FX via proteolytic cleavage, producing FIXa and FXa (10, 25, 26). This small amount of FXa will activate a small amount of thrombin needed to allow for progression to amplification.

Amplification started with the initiation step providing the necessary materials of activated FIX, FX, and importantly thrombin. The small amount of thrombin generated during initiation activates FV, FVIII, and FXI. FXIa is a potent activator of FIX, and FVa is an important cofactor for FXa (27, 28). FVIIIa is an important cofactor for FIXa and

thrombin spurs its release from vWF to bind with FIXa. With these cofactor and factors activated, it amplifies the quantity of pro-coagulation complexes to proceed into the propagation phase (11, 29).

Propagation is a fully primed intrinsic/extrinsic response to generate fibrin. On the platelet surface the tenase (Xase) complex of FVIIIa and FIXa activates FX to FXa. FXa then forms the prothrombinase assembly with FVa to convert prothrombin to thrombin (8). This functional prothrombinase assembly generates the thrombin burst. This thrombin burst catalyzes the cleavage of fibrinogen into fibrin thus forming the clot at the area of injury. The insoluble fibrin monomers aggregate together to form a soft clot. Activation of FXIII to FXIIIa (Transglutaminase) then crosslinks the fibrin clots. This cross-linking forms a rigid network trapping the platelets and erythrocytes inside, forming the hard clot.

Termination, the fourth step of CBMC, is continually ongoing as it employs multiple routes to keep the other three coagulation steps in check. This process ensures healthy vasculature does not form random clots and dissolves the clots that have formed. Formation of spontaneous clots could lead to clinical issues such as thrombus and ultimately result in an embolism (21).

To accomplish this never-ending task, termination employs the Protein C/Protein S/ Thrombomodulin System (21). Protein C is an anticoagulant zymogen, when bound to its cofactor thrombomodulin (TM) (5, 30-33). TM inhibits the clotting abilities of thrombin and activates Protein C. Activated Protein C binds with cofactor Protein S to form the

Protein C-Protein S complex that can terminate thrombin generation on platelets by
destroying FVa and FVIIIa $(34, 35)$.

Fibrinolysis, the process of breaking down a formed clot occurs as part of normal
wound healing. The primary enzyme that breaks down the fibrin clot is plasmin. The
inactive pro-enzyme, plasminogen, flows throughout the blood and activates on contact
with the clot surface to form plasmin. Activation occurs by activator proteins called
tissue plasminogen activator (tPA) and single chain urokinase (scu-PA). Plasmin begins
to break down the fibrin resulting in full dissolution of the clot once the wound has fully
healed.

1.3 Serine Proteases

Hemostatic regulation occurs through the employment of coagulation factors known
as serine proteases. Serine protease production occurs in the liver's hepatocytes and
then they circulate as zymogens in the blood. Proteolytic cleavage turns these inactive
enzyme precursors into active serine proteases. Minute differences allow them to
operate with the high degree of specificity to provide precision with regulation.

All serine proteases share a great deal of similarity in gene sequence (29, 36, 37). Sharing a high degree of DNA and amino acid similarity, protein folding delivers similar structure patterns throughout the serine proteases. As demonstrated in the general clotting factor structure shown in Figure 3. The clotting proteases are composed of a catalytic head, two Epidermal Growth Factor (EGF)-like domains, and a Gla domain (29, 38, 39). This can be best imagined if thought about as a Venus Flytrap.

The catalytic head resembles the digestive enzyme trypsin and forms the "fly catching portion" of the flytrap. The catalytic head contains the protease domain to hydrolyze peptide bonds by utilizing a "catalytic triad" of Serine, Histidine, and Asparagine (40-43). This "catalytic triad" is responsible for the activation of the next sequential protease.

Figure 3 Structure of coagulation factor. The EGF1 and EGF2 domains are the "stem" portion holding up the catalytic head. The "root" of the structure is the Gla domain, that anchors the coagulation factor to the phospholipid surface.

Holding up the catalytic head like a stem, are the two EGF-like domains. The primary role of EGF-like domains is to hold the catalytic head off the phospholipid surface and into the space above (41, 42, 44). This area also contains some binding sites for regulators and co-factors of the enzyme. A flexible linker attached to the two EGF-like domains holds the catalytic head (41, 42).

The Gla domain refers to the conserved feature of amino-terminal carboxylated glutamic acid residues that serve to anchor the entire structure (41). These modified residues serve to anchor the entire structure via calcium bridging to the phospholipid surface (38, 39, 44). The interaction of the Gla domain is possible by the Vitamin K dependent, γ-carboxylase modification to the glutamate residues during synthesis in hepatocytes of the liver (24).

The γ-carboxylation occurs via a quinone reductase that reduces Vitamin K to KH_2. Carboxylase enzyme cofactor Vitamin KH_2 adds the carboxyl group to the Gla domain. Calcium with a positive charge chelates via electrostatic interaction the negative charged Gla domain and the anionic head group of phospholipids (25, 41).

Coagulation factors employ cofactors to assist in the protease activity. These cofactors are comprised of factor specific cofactors in addition to the universal cofactors. This relationship allows for pivotal coagulation cascade reactions to occur through improved posturing of the factor/substrate orientation.

Universal Cofactors that are required by all serine proteases in the coagulation cascade are calcium and an anionic phospholipid membrane. Radcliffe *et al.* found that adding phospholipids and $CaCl_2$ caused an 11-fold increase in activation of FVII (22). Calcium serving as a cofactor with a positive charge chelates via electrostatic interaction the negative charged Gla domain and the anionic head group of phospholipids (25, 41). The interaction between the Gla domain and EGF-like domains is also highly dependent upon calcium for orientation on the membrane in the vertical position of the serine proteases, as described by Bode *et al.* 1997 (32). Krishnaswamy et al. also

demonstrated evidence that substrate-membrane interaction considerably enhances

activation of FX by the extrinsic Xase complex $(14, 45, 46)$. Without proper vertical

orientation and attachment to the phospholipid membrane the serine proteases exhibit

very reduced activity (45).

Though all serine proteases share a great deal of similarity in DNA and amino acid

sequence they are highly specific for activation, cofactor, and substrate $(29, 36, 37)$.

Important cofactor/factor relationships that greatly increase protease activity are: FVIIa

to TF, FVIIIa to FIXa, and FVa to FXa. These specific relationships are what give way

for an optimal K_m and K_{cat}.

As previously mentioned, the key role of FXa is to activate FII. FX activates

prothrombin via two methods and speeds: FXa alone slowly or at exponentially greater

rate with the cofactors in the prothrombinase complex. The prothrombinase complex

forms when there is vascular injury, and the platelet provides a phospholipid surface for

FXa, with cofactor FVa, acting on the substrate, Prothrombin. Specifically, FXa performs

activation of FII via proteolytic cleavage of two peptide bonds on the heavy chain, turning

FII into FIIa (46).

To best understand the role of serine proteases and their respective cofactors it is

important to look at Protein C, a serine protease whose function is to oppose

coagulation. Activated Protein C (APC) is crucial to the regulation of the clotting

cascade by acting on procoagulant serine proteases. Protein C shares similar structure

and generation to the Vitamin K dependent, pro-coagulant serine proteases synthesized

in the liver (30). APC in complex with cofactor Activated Protein S (APS), forms the

APC/APS complex that is responsible for degradation of FVa and FVIIIa. Protein C is activated by thrombin, and this activation is enhanced when thrombin is attached to thrombomodulin. Thrombomodulin is a cofactor for thrombin found on the surface of endothelial cells (34, 35). Walker *et al*. found that Protein C has a high degree of specification and once activated cleaves both chains of the activated clotting enzymes, FVa and FVIIIa (14, 30). Just as with FXa alone can activate a small amount of prothrombin, it was identified that Protein C can inactivate FVa and FVIIIa independently, but the K_m is substantially decreased by the cofactors (30). Interestingly it was discovered that the complex is not able to inactivate FVIIIa when, FVIIIa is coupled with cofactor vWF (35).

FVIIa bound to TF in association with calcium on phospholipid surface forms the extrinsic tenase complex and is the preliminary activator of FX (17, 22, 47). TF presiding as the cofactor for FVIIa embraces the protease in optimal vertical orientation off the membrane surface (48). McCallum *et al*. demonstrated the ability of the cofactor to be so necessary for orientation it allows for prime positioning even if the Gla domain of FVIIa becomes detached (18, 26, 49).

Kovalenki *et al*. investigated the activation of FX to FXa by the extrinsic tenase complex and found that the size of the vesicle affected the rate of the reaction (50, 51). This is important because it draws attention to the topographical and geometrical limits of the factors while sitting vertically on the phospholipid membrane

1.4 Factor IX
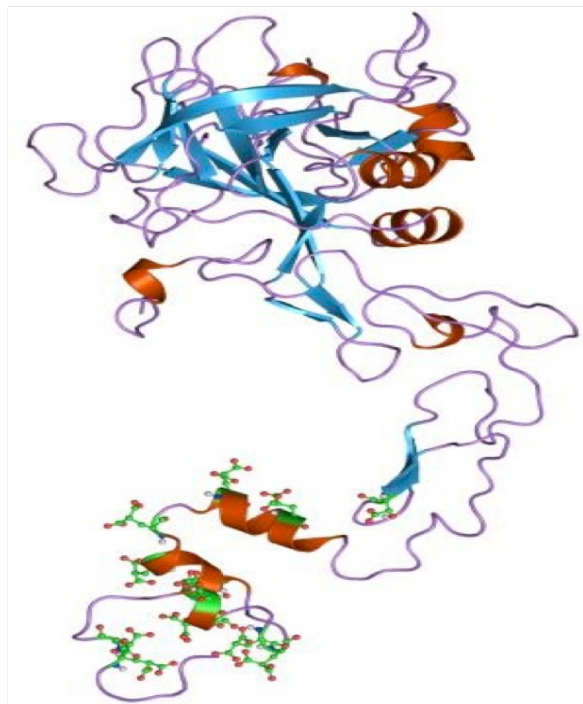
Figure 4 Structure of Factor IX in Ribbon View with protein structure. Photo by Jawahar Swaminathan and MSD staff at the European Bioinformatics Institute / Public domain

Factor IX (FIX) shares similar homology with other vitamin K dependent clotting factors produced in the liver. It is an essential clotting factor and a deficiency in FIX can lead to clinical issues such as hemophilia B, also known as Christmas Disease (52, 53). Analyzing the DNA sequence data of the proband patient who had a bleeding disorder identified the disease as being a unique form of hemophilia. This mutation resulted in a cysteine changed into a serine on the X chromosome within what is now identified as the FIX gene (52-55).

The general structure of FIX shares a similar homology with the other serine proteases (see figure 4). More specifically FIX is composed of two chains equaling 415 amino acids, a C-terminal heavy and N-terminal light chain (52). The C-terminal Heavy chain of 235 amino acids forms from residues 181 to 415 (44). The N-terminal light

chain consists of 145 amino acids (39). Together light and heavy chains are linked with a disulfide bond at Cys-132 and Cys-189 (40-43).

This heavy chain contains the FIX catalytic head region as previously described in the general description of serine proteases. The importance of the FIX catalytic head is that it specifically contains the "Catalytic Triad" composed of Histidine-236, Serine-365, and Asparagine-269 (40-43). This catalytic triad is responsible for the activation of FX.

The N-terminal light chain includes the Gla domain and two EGF-like domains (39). The Gla domain of FIX is composed of the amino acid residues 1-38, which anchors the enzyme with the calcium bridge to the phospholipid membrane of the platelet or other phospholipid surface (39, 44). EGF-like domains are residues 47 to 84 and 85 to 127 (42). There is also a hydrophobic helix at residues 39 to 46, which acts as a support for the EGF-like domains (41). Both EGF-like domains are stacked and joined with a convex hydrophobic loop of EGF1 and a concave hydrophobic socket of EGF2 (41). A flexible linker with residues 128 to 145 holds the catalytic head (41, 42).

Activation of FIX occurs by the intrinsic and extrinsic pathways. FXIa is the activator in the intrinsic pathway model and FVIIa in the extrinsic pathway. FIX activation is in two cleavage events performed by FVIIa/TF or FXIa (56). The first cleavage generates FIXaα with a single cleavage removing Arg-180 to Val-181, followed by a second cleavage removing Ala-146 to Arg-180 to generate FIXaβ (42, 57).

Fully activated FIXaβ forms the Xase complex, with specific cofactor FVIIIa and universal cofactors. The role of FIX catalytic head is activation of FX by hydrolysis of peptide bonds at Arg-194 to Ile-195 (40-43). This complex has been demonstrated to

have 10^9-fold increase in the procoagulant activity compared to active, non-complexed FIXa, as reviewed by Neuenschwander *et al.* (56, 58).

The anticipated height of FIXa is a distance between 50 to 100 Å above the membrane. A review and understanding of the FIXa structural dependency on cofactors is fundamental. X-ray crystal structures have demonstrated the ability of cofactors and ions to change the conformation of factors in the clotting cascade. Length changes of the serine protease, specifically FIX have been demonstrated with cofactors. Bode et al. and McCallum et al. determined how the interaction between the EGF1 domain and Gla domain is highly dependent upon calcium (41, 59). When there is a lack of calcium the protease is no longer able to chelate to the phospholipid surface. Also, through small angle x-ray and NMR studies, a change in orientation based on calcium has been detected (41, 44). The EGF-like domains' binding sites for cofactor FVIIIa act upon the final structure of Xase complex. When bound, they promote activation of FIX by holding in the vertical orientation (41). While all of these studies point to the orientation of the complex, none are capable of addressing the specific optimal distance and orientation of the catalytic head with regard to stretch and flexibility of the factor (60).

1.5 Anti-Coagulation and Inhibitor Structure

Anticoagulants are chemicals used in the prevention of blood clotting by acting on clotting factors within the Coagulation Cascade. Anticoagulants have a long history of uses throughout evolution by animals such as mosquitos to obtain blood from the host animal (61). Currently, anticoagulants are used as therapies in patients, for example with atrial fibrillation to prolong an individual's survival. Most of the current anticoagulant therapies used in medical practice involve coumarins and heparins (62).

Looking at the mechanism by which coumarins work it is important to look specifically at the drug Warfarin. Warfarin first used by man as a rat poison was later approved for use as an anticoagulant by acting as a Vitamin K analog (63).   Vitamin K activity is impeded by Warfarin interacting with Vitamin K epoxide reductase. Without enough active Vitamin K to carboxylate the Gla residues on Vitamin K dependent serine proteases they are no longer able to chelate the phospholipid surface (21, 44). This is a common way to prevent blood coagulation but has negatives to its uses such as a drug loading time, taking around 72 hours before effects are seen in a patient.

The polymer, heparin is another common anticoagulant used by medical professionals in things such as blood filtration procedures and surgeries. Heparin is naturally produced in the body by basophils and mast cells and can be extracted for pharmaceutical uses from mammals such as cattle and pork. Heparin works by binding and increasing activity of Antithrombin III (AT) (15, 32, 61, 64-66).

Antithrombin (AT) belongs to a class of inhibitors known as serine protease inhibitors or serpins. Serpins contain a serine residue in their catalytic triad to help regulate the proteolytic clotting cascade. A principal class of serpins is Kunitz Protease Inhibitors, which includes AT. AT is a small glycoprotein produced in the liver, which binds to the serine residue in the active catalytic triad of thrombin, leaving it inactive. Other common serpins of FXa and FIXa are Basic Pancreatic Trypsin Inhibitor (BPTI) and Protease Nexin-2, respectively, via their Kunitz Protease Inhibitor domains (58, 67). To tie this together heparin has also been shown to increase the rate of AT and FIXa inhibition in the presence of Ca^{2+} (58).

2 Research Design

As discussed, blood coagulation requires the use of enzymes orchestrated in a series of highly specific events. For these events to occur at a tremendous exponential rate, the enzymes require their respective cofactors and a phospholipid membrane. It has also been determined that the topographical characteristics of the activation complex on the phospholipid membrane are also essential. Fluorescence studies have delved into the distance of closest approach to the membrane of the active site for several of the coagulation serine proteases (42-44, 49, 59, 60, 68, 69). In several instances, it was documented that the non-enzymatic cofactor binding altered the distance. With distance above the membrane correlated to the overall length of the serine protease as determined by x-ray crystal structures, it is reasoned that proteases orient vertically on the membrane with little to no bending (Figure 5). This reasoning has not been proven using fluorescence resonance energy transfer (FRET)-based studies as these are only capable of showing the distance of closest approach. FRET cannot determine the optimal height/distance needed for maximum coagulation activity. Also, lacking in the gap of knowledge is the potential for elasticity and their ability to stretch off the membrane, proving to be a significant gap in knowledge. **The goal of this study to gain insight into the optimal reactive height of coagulation factor IXa above the phospholipid surface by the generation of a lipid <u>A</u>nchored <u>M</u>olecular <u>R</u>uler with attached <u>I</u>nhibitor (AMRI).**

The enzymatic characterization of coagulation factors has been frequently studied through use of small peptide inhibitors such as serpins, as they sit directly in the catalytic site of the enzyme (38, 70-73) . Hence, we are using it in the AMRI. To generate AMRI, a protein anchoring sequence of Leucine, Alanine,
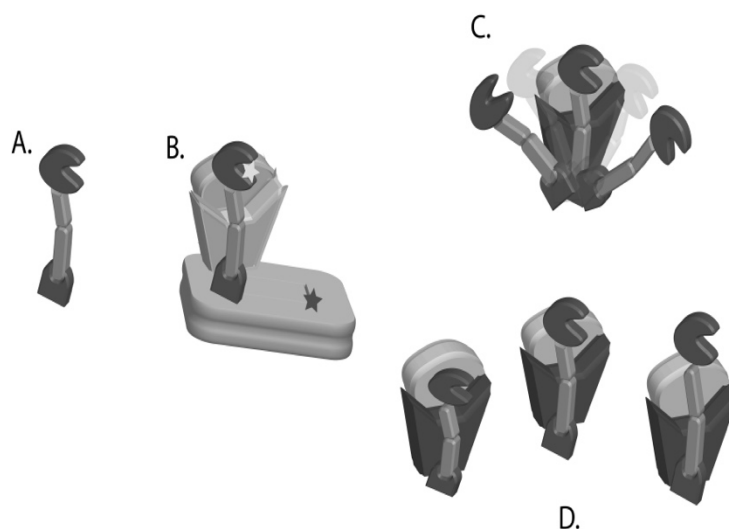
Figure 5 Demonstration of knowledge lacking in the optimal reactive height of FIXa. This looks at Crystal Structure Data (A) and FRET (B). The Crystal structure gives us the height at a moment frozen in time. FRET data gives the distant of closest approach. Lacking is the information on the wobble (C) and the stretch (D) to understand how the Factor moves in a fluid environment for optimal reactivity.

Glycine, and Cysteine (LAGC) is used to fixate the entire structure to a lipid surface. To direct the lipid-bound protein for anchoring, LAGC will be coupled with a leader sequence for shuffling into periplasmic space, originally from the Apyrase gene of Shigella, for protein collection. A height adjustable linker area is then required between the anchor and inhibitor regions of AMRI to ensure the proper height of inhibitor. Employing the linker sequence EAAAK, with a rigid slantwise fixed height of 7.5 Å per sequence, will allow for the creation of a specific height for the engineered protein (74). Based on the Crystal structure data the hypothesized reactive height is a distance between 50 to 100Å above the membrane (6, 42, 43, 69). Completing the protein's linker

region will be a flexible linker at the end of a rigid set to ensure the inhibitor properly fits in FIXa active site. Finalizing the AMRI complex will be a PN2-KPI inhibitor, which has previously been demonstrated to inactivate FIXa.

2.1 Protease Nexin2 - Kunitz Protease Inhibitor (PN2-KPI)

Anchored Kunitz-based inhibitors bound to lipid membranes have been successfully utilized for analysis of serine protease activity using Surface Plasmon Resonance (SPR). SPR allows for measurement in a real-time binding of two molecule companions without the need of labels by detecting the changes in refractive index (GE Healthcare, Technology Note 23: Label-free interaction analysis in real-time using surface plasmon resonance. (2007) BR-9004-63 28-9214-39 AA.). The researchers generated a phosphatidylcholine (PC) lipid monolayer containing nickel chelating head groups and successfully attached 6-histidine-tagged bovine pancreatic trypsin inhibitor (BPTI). Over the attached BPTI lipid layer, soluble proteases flowed over and were bound, establishing proof that a bound Kunitz inhibitor is capable of remaining functional after attachment. However, these studies found that binding rates were drastically lower than in other studies using solution-phase testing (56, 58). One explanation of this is the desired orientation of proteases on the membrane and the close position of the inhibitor on the membrane. This negative steric interaction is the reasoning for wanting the Kunitz inhibitor attached at a set distance above the membrane and allowing more favorable binding to the protease domain.

The Kunitz Protease Inhibitor domain of Protease Nexin 2 (PN2) was employed to bind to the active site of FIXa (56, 71). The precursor form of this protein, PN2-Amyloid β Protein Precursor (AβPP) was found to have a role in the regulation of specific

proteases in extracellular environments; specifically, with plaque deposits of those affected with Alzheimer's disease and Down syndrome (72). Schmaier *et al*. found that PN2-AβPP was 71-fold more effective in ability to inhibit FIXa than antithrombin III (70, 71). Neuenschwander *et al*. experimentally determined that using just the inhibitor domain of PN2 (PN2-KPI) displayed an increased affinity for FIXa (58). PN2-KPI is a more ideal inhibitor for height determination of the FIXa active site.

2.2 LAGC anchor with Apyrase leader sequence

Hydrophobic anchoring of a protein to a lipid membrane is not a novel idea, but an evolved mechanism. In unicellular organisms many membrane proteins utilize the Lipobox motif to anchor to the phospholipid. Roughly 75% of all lipobox sequences consist of the amino acid sequence, LAGC. The cleavage between the Glycine and Cysteine results in the formation of an N-terminal Cys. The SH group on the new N-terminal Cys is acted on by diacylglycerol transferase, creating the diacylglyceryl moiety. This moiety with Cys incorporated serves as the anchor to the phospholipid surface so that the attached protein does not diffuse away (75).

Bhargava *et al*. identified a 23 amino acid leader sequence for virulent apyrase gene that is responsible for translocation of the protein from the cytosol to the periplasmic space in Gram-negative bacteria. Identification was possible via cloning and expression of *Shigella flexneri 2a* apyrase gene in an *E. coli* plasmid (76). Apyrase was directed to exit the cytosol and travel to the periplasmic space through translocase. The leader sequence on the N-terminal end is recognized and transported to the periplasmic space. The clone was then sequenced against the wildtype for confirmation of the apyrase gene.

Kamalallannan *et al*. demonstrated an engineering process for converting a non-lipoprotein into an anchored lipoprotein (75). Using the *Shigella* apyrase leader sequence for transport, anchoring with LAGC sequence combined with normal protein sequence generated a viable lipid bound protein. With the Cys serving as the anchor on the N-terminus end, it allowed for regular protein expression on the C-terminal end.

2.3 Linkers= (EAAAK)₁₁ (GGGGS)

Linker construct has become an essential tool in the development of protein fusion. The ability to link proteins and not interfere with their reactivity is vital and achieved by taking into consideration flexibility and hydrophilicity (77, 78). The sequence EAAAK, whose parent strand, initially described by Marqusee and Baldwin in 1987, is referred to as a rigid linker, forming alpha helices (78). This helix is stabilized by Glutamic acid and Lysine salt bridges and internal Hydrogen bonds (74, 77-79). Arai *et al.* determined that it was possible to control the distance between two proteins by manipulating the repetition of the EAAAK motif (77, 80). Amet *et al.* described the use of EAAAK linker sequence by varying the number of sequence repeats to optimize reactivity of an expressed protein (81).

As the number of EAAAK motif repeats increases, so does the strength and tightness of the alpha helix structure (80). Through FRET studies and high-resolution models, it also determined that the linkers connect diagonally and not longitudinally creating a vertical increase of 7.5 Å and not the interdomain distance of 10 Å, with (EAAAK)₅ having a distance of 43.86 Å (74, 77, 80). It was concluded that the rigid linkers provided a lesser degree of distance fluctuation between the N and C terminus.

Li *et al*. also confirmed through molecular dynamic simulation that EAAAK in helical form

is in the lowest energy state, indicating conformational stability (74).

The GGGGS sequence is a well-known flexible linker with many applications in
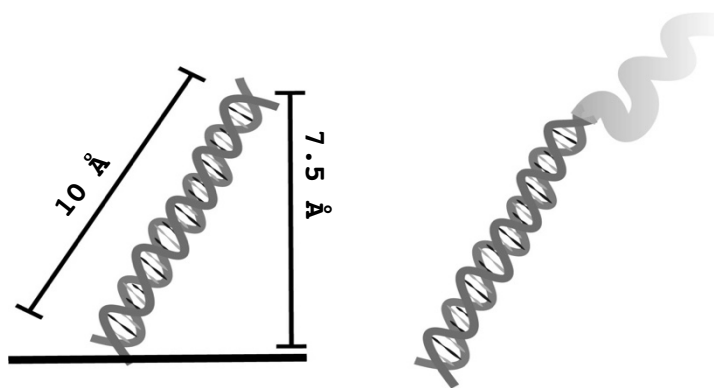
Figure 6 Structure formations of EAAAK and GGGGS. This demonstrates how the proposed protein structure will be rigidly lifted from the phospholipid membrane, while GGGGS provides the flexible conduit for proper insertion of the inhibitor

protein engineering. This flexible nature allows for the mobility of the inhibitor and the

tethered EAAAK rigid sequence. In the Li *et al*. 2015 study on molecular dynamics, it

was found to have no energy change in the 29ns simulation and produced total energy

fluctuation the entire time (74). This is indicative of a protein with no conformation

stability. The flexible linker also produced an increase in variable distances in the FRET

analysis conducted (74, 80). Incorporation of the flexible linker at the end of the rigid

linker sequence supplies a lack of conformation, making it ideal to serve as the

"intermediary" between the rigid linker and inhibitor. This lack of conformity will provide

the inhibitor with freedom of movement to fit within the catalytic head of FIX.

4 Specific Aims

The focus of this study is to generate a lipid **A**nchored **M**olecular **R**uler with attached **I**nhibitor (AMRI) at a fixed height to quantify the optimum reactive height for FIXa. The hypothesized reactive height is a distance between 50 to 100Å above the membrane.

Specific Aim 1: Creation of an AMRI in an expression vector.

Specific Aim 2: Purification of AMRI in an expression vector.

Specific Aim 3: Sequencing of AMRI in an expression vector.

5 Methods

5.1 SnapGene

SnapGene® software (from GSL Biotech; available at snapgene.com) was used for vector mapping and design. The PCR primers and oligonucleotides were designed with the program including the LAGC anchor region to attach to a phospholipid membrane; an EAAAK (EA$_3$K) rigid linker serving as a molecular ruler; a PN2-KPI region serving as the FIXa inhibitor; the PCR primers needed to correct the orientation of the current p-LAGC-EAK10 plasmid.

5.2 Vector Preparation

5.2.1 Description

pET11d plasmid was used for the cloning backbone. The p-LAGC-EAK10 plasmid was a gift from a previous GA-PCOM thesis student. This plasmid contained the *Shigella* apyrase leader sequence, anchor sequence LAGC, and the molecular ruler sequence of EAAAK repeated 10 times inserted between the *Nco*I and *Hind*III restriction sites.

5.2.3 Growth

Streak plates of DH5α *Escherichia coli (E. coli)* were made with LB agar (10 g/L

tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L Difco Granulated agar, pH 7.0)

containing ampicillin with a concentration of 50 μg per ml (82) . The plate was placed in

37°C incubator overnight and removed after growth and placed in 4°C for storage.

Individual isolated colonies were chosen and inoculated into 5 ml culture tubes,

containing LB media. Ampicillin ensures the selection of only *E. coli* containing any

vector with the gene conferring Ampicillin resistance; inhibiting growth of *E. coli* lacking

ampicillin resistance. The culture tube was then placed in a 37°C incubator at 250 rpm

overnight. This growth was to ensure a good cloudy density of bacterial growth. 1 mL of

the initial 5 mL culture was used to inoculate 250 mL and 500 mL LB media cultures.

5.2.4 Transfection

Competent DH5α *E.coli* cells were used for transfections and were a gift from Dr.

Frank Jenney. The DH5α cells were taken from the -80°C freezer and placed to thaw on

ice. The cells were then stirred with the tip of a pipet, before pipetting 70μl into a pre-

chilled 1.5 ml tube. There as one tube per each individual reaction. 10 ng of plasmid

DNA was placed into the 70 μl of competent cells and stirred with a pipet tip. This

mixture was then placed on ice to incubate for 30 minutes, followed by 30 seconds of

incubation at 42°C to heat shock the cells. The tubes were immediately transferred and

chilled on ice for 2 minutes. Next, 300 μl SOC (20 g/L tryptone, 5 g/L Yeast extract, 0.5

g/L NaCl, 10 ml/L 250 mM KCl, 5 ml/L 2 M $MgCl_2$, 20 ml/L 1M glucose, pH=7.0)

prewarmed to 37°C was added to the cell mixture and allowed to shake for 1 hour at 100 RPM. The cells were then plated 100μl and 200μl on LB plates, both with and without the 50 μg/mL ampicillin selection agent. In these experiments, different molar ratios of the digested vector and insert were used to obtain optimum transfection efficiency. Experimental molar ratios of 1:3 and 1:6 vector to insert were used. Controls included vector plus ligase only, and DH5α cells only. Once plated the plates were allowed to dry before placing inverted at 37°C overnight. Isolated colonies on the successful plates were then inoculated into an LB broth with ampicillin as described above.

5.2.5 DNA Miniprep

DNA Miniprep was performed to screen for the intended clone at various stages of

the cloning process. Quantum Prep® Plasmid Miniprep Kit (Bio-Rad Laboratories Inc.,

Hercules, California, Catalog #732-6100) was used in all Miniprep procedures. During

this process, centrifugation was performed at the maximum speed of 13,300 x *g* on a

tabletop centrifuge. Using 1 to 2 ml of the growth culture the protocol was followed

depending on the desired final concentration of pure plasmid. The culture was placed in

a microcentrifuge tube to pellet the cells and ran for 30 seconds. With the cells pelleted

the supernatant was removed by pipetting. Then another quick spin on a small desktop

centrifuge was performed just to ensure removal of supernatant. The pellet was

resuspended using 200 µl of the Cell Resuspension Solution provided by vortexing and

pipetting up and down. Next 250 µl of the Cell Lysis Solution were added and mixed by

gently inverting the capped tube about ten times. The Cell Lysis Solution was followed

by the addition of 250 µl Neutralization Solution then inverted 10 times for complete

mixture. A precipitate formed and was then placed back in the centrifuge for 5 minutes

to collect the cell debris. The plasmid DNA, located in the clear lysate, was transferred

to a spin filter, and combined with 200 µl mixed Quantum Prep Matrix, with the filter

placed in a 2 ml centrifuge tube. The tube with the filter was centrifuged for 39 seconds.

The spin filter was removed, and filtrate discarded, then put back in the centrifuge tube

and washed twice with 500 µl of Wash Buffer. The first wash was centrifuged for 39

seconds and the second was for 2 minutes. An additional spin was performed if a slight

smell of ethanol persisted. The filter was then placed in a 1.5 ml microcentrifuge tube

and eluted for 1 minute with 100 µl of prewarmed 70°C nuclease-free water. The

purified DNA was then either stored at -20°C or used immediately.

5.2.6 DNA Midiprep

Quantum Prep^{TM} Plasmid Midiprep Kit (Bio-Rad Laboratories Inc., Hercules,

California, Catalog Number 732-6120) was used for collection of larger pure plasmid

DNA amounts. All centrifugation was in a swing bucket table-top centrifuge using 250 ml

Oak Ridge tubes.   250 ml growth media was centrifuged at 5,000 × g for 10 minutes to

pellet the cells. The tubes were then drained of supernatant and inverted on a paper

towel to ensure removal of media. The pellet was resuspended with 6 ml Cell

Resuspension Solution and mixed by vortexing until all cells were back in solution. Next,

6 ml of prewarmed 37° Cell Lysis Solution were added and mixed by rolling on the

benchtop. This mixture was allowed to sit for 3 minutes at room temperature before

adding 10 ml Neutralization Solution and mixed by inverting 5 times. After 3 minutes a

white flocculant formed. This solution was then placed in a PureYield^{TM} Clearing

Column and a new 50ml disposable Falcon plastic tube, then allowed to sit for an

additional 2 minutes at room temperature before centrifuging at 2,500 × g for 10

minutes. Then filtered lysate was transferred into a PureYield^{TM} Binding Column and

new 50ml disposable Falcon plastic tube to which 5.0 ml of Endotoxin Removal Wash

Solution were added. This was centrifuged at 1,500 × g for 3 minutes, and the flow

through was discarded. Following the Endotoxin Wash were two washes of 20ml of

Column Wash Solution, discarding the flow through each time. At the completion of the

second wash, the column tip was tapped dry on a paper towel and placed in a new

Falcon tube and spun for 15 minutes at 1,500 × *g*. The column was then allowed to sit

for 5 minutes or until no smell of ethanol remained. Next, the column was placed in a

new Falcon tube, and 600 µl of prewarmed 70°C nuclease-free water was allowed to sit

on the column membrane to rehydrate. After 2 minutes the tube was centrifuged for 5

minutes at 1,500 × *g*. The filtrate was collected and placed in a 1.5 centrifuge tube.

5.3 DNA Analysis

5.3.1 Thermo Nanodrop Spectrophotometer

The ThermoFisher Scientific Nanodrop Spectrophotometer, now referred to as

NanoSpec, was started using the setting for double-stranded DNA and was calibrated

using only 2 µL of nuclease-free water. Then 2 µL of DNA sample was added to the

NanoSpec to determine concentration (ng/µL). The NanoSpec made measurements by

wavelength absorbance at 260 nm for Nucleic Acids and 280 nm for proteins. This

A_{260}/A_{280} ratio reading was calculated to determine DNA purity; a ratio of close to 2.0 is

considered pure for DNA, however anything above a 1.8 was considered acceptable for

use.

5.3.2 Agarose Gel Electrophoresis

A 1% agarose gel (10 mg/mL of Nuclease-free agar in 1X TAE Buffer (Tris-acetate-

EDTA, pH 8.3)) was separated using 0.1 µL/mL GelRed™ Nucleic Acid Gel Stain

(Phenix Research Products, Candler, North Carolina) in 1X TAE running buffer. For

tracking the progression of DNA in the agarose, 5 µL Quick-Load 2-Log DNA Ladder

(New England BioLabs, Ipswich, Massachusetts, Catalog # N0550S) was employed for

size analysis and each loaded sample had 5 µl of Gel Loading Dye, Purple (6X), no SDS

(Catalog # B7025S) per 25 µl reaction volume. The DNA and loading dye mixture were

mixed well on Parafilm before being loaded into the desired well in the agarose gel.

Once the desired amount of DNA was loaded into the agarose gel wells, including the

ladder, the current was started at 100 Volts and ran for approximately 45 to 95 minutes

depending on the tracking of the red band generated by the purple load dye. The red

band tracks through the 1% agarose gel as a piece of DNA with approximate length of

300 base pairs. This was important for direction, speed of travel, and to determine time

run time for proper separation of different sized bands. This same method was

employed for DNA band isolation for determining digested and undigested plasmid DNA.

To determine the DNA amounts gel quantification was applied to either verify the

concentration determined by the NanoSpec or used as a stand-alone result.

5.3.3 Sequencing

Sequencing performed in this project was conducted by Retrogen Incorporated in

San Diego, California. The primer used in sequencing was against the T7 tag vector

sequence. The data generated by Retrogen was then imported into the SnapGene®

software for alignment analysis.

5.4 Resuspension of Lyophilized PN2 oligonucleotide

Lyophilized samples of the forward and reverse PN2 oligonucleotides were ordered from Integrated DNA Technologies. When received the samples appeared translucent and were virtually undetectable to the naked eye. The samples were first centrifuged at maximum speed for 1 minute before opening the tops of their centrifuge tubes. Then concentrated stock for each were resuspended at a concentration of 100 µM and a working stock concentration of 10 µM; both made with TE Buffer (10mM Tris; 0.1 mM EDTA; pH 8.0). The following oligonucleotides were generated by Integrated DNA Technologies, Inc. (Skokie, Illinois):

5.5 Design of PN2

The forward and reverse oligonucleotides were ordered with the plan to anneal them together. Thus, the annealed double stranded product would constitute the sequence for to the PN2 inhibitor with overhangs matching the *Hind*III and *Eco*RI restriction sites.

5.5.1 PN2 forward

Sequence - PN2 forward 20 nmole Ultramer® DNA Oligo, 186 Bases

5- /5Phos/AAT TAA GCA GAA CCA CAA ACA GCC ATA CAG TAC TCC TCA GTG TCG AAG TTG TTT CTG TTA CCA CCA CAA CCA CCG TAG AAG AAT GGA GCA CAC TTA CCC TCA GTA ACG TCG AAG TAC CAT CTA GAA ATC ATA GCT CTA CAT GGA CCA GTC TCA GCT TGC TCA GAA CAA ACC TCT CTA ACA ACC TCC -3

Properties:

Tm (50 nM naCl): 70.7 °C

GC Content: 45.2%

Molecular Weight: 57016.9

nmoles/OD260: 0.6

ug/OD260: 31.6

Ext. Coefficient: 1803400 L/(mole·cm)

5.5.2 PN2 reverse

Sequence - Pn2 reverse 20 nmole Ultramer® DNA Oligo, 186 Bases

5- /5Phos/AGC TGG AGG TTG TTA GAG AGG TTT GTT CTG AGC AAG CTG AGA CTG GTC CAT GTA GAG CTA TGA TTT CTA GAT GGT ACT TCG ACG TTA CTG AGG GTA AGT GTG CTC CAT TCT TCT ACG GTG GTT GTG GTG GTA ACA GAA ACA ACT TCG ACA CTG AGG AGT ACT GTA TGG CTG TTT GTG GTT CTG CTT -3

Properties:

Tm (50 nM naCl): 70.8 °C

GC Content: 46.2%

Molecular Weight: 57940.3

nmoles/OD260: 0.6

ug/OD260: 32.3

Ext. Coefficient: 1793300 L/(mole·cm)

Annealing of forward and reverse PN2-KPI primers results in following structure:
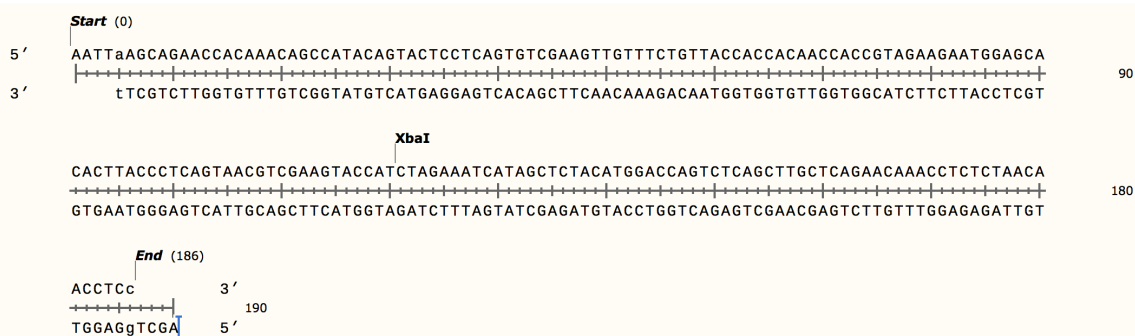
5.6 Restriction Digest

New England Biolabs restriction enzymes, *XbaI* (Catalog #R0145S), *NcoI-HF* (Catalog #R3193L), *HindIII-HF* (Catalog #R3104L), *EcoRI-HF* (Catalog #R3101L), and *Alkaline Phosphatase, Calf Intestinal (CIP)* (Catalog #M0290L)*,* had incubation temperatures of 37°C in NEBuffer CutSmart buffer. All restriction enzymes could be heat inactivated at temperatures of 80°C, except for Calf Intestinal Phosphatase (CIP). Therefore, all reactions were performed in a ThermoFisher Applied Biosystems™ Veriti™ 96-Well Thermal Cycler under controlled conditions. The heat inactivation of *XbaI* was 65°C, but for the simplicity of programming 80°C was used. The reaction conditions *XbaI* (Catalog #R0145S), *NcoI-HF* (Catalog #R3193L), *HindIII-HF* (Catalog #R3104L), *EcoRI-HF* (Catalog #R3101L) were as follows:

Restriction Enzyme	20 units
DNA	Up to 1 µg
10X NEBuffer CutSmart	5 µl (1X)
Total Reaction Volume	50 µl
Incubation Time	3 hours
Incubation Temperature	37°C
Heat Inactivation Time	20 minutes
Heat Inactivation Temperature	80°C

Once the first restriction digest was completed the DNA was purified by gel electrophoresis and extracted as described. The next restriction was only heat inactivated before with the Calf Intestinal Phosphatase (CIP) treatment

The CIP treatment was performed to remove the 5'-phosphate by the addition of 20 units of CIP to the previously heat inactivated digest. The ThermoFisher Applied Biosystems™ Veriti™ 96-Well Thermal Cycler was programmed to run for 1 hour at 37°C to allow the phosphatase to cleave. Afterwards, the temperature dropped in the ThermoFisher Applied Biosystems™ Veriti™ 96-Well Thermal Cycler to 4°C until removed. After the CIP treatment, a Phenol-Chloroform-Isoamyl Extraction with Ethanol precipitation to stop the phosphatase was performed.

5.7 Purification

5.7.1 Phenol-Chloroform-Isoamyl Extraction

To remove the restriction enzymes and ensure that the DNA is free of proteins and lipids it is essential to perform an extraction. Once the extraction is performed the purified DNA is then able to be used in the next phase of the experiment. The DNA mixture was mixed with one volume of phenol:chloroform:isoamyl alcohol in a ratio of 25:24:1, respectively. The solution was then vortexed thoroughly for approximately 20 to 30 seconds, followed by centrifugation at room temperature for 2 minutes at 13,300 x g. Next, the upper aqueous layer was removed and transferred to a fresh tube, being careful to not get phenol during pipetting. To ensure the removal of all phenol another wash was performed with equal volume of chloroform, then vortexed for approximately 20 to 30 seconds, followed by centrifuging at room temperature for 2 minutes at 13,300 x g. The upper aqueous layer was transferred to a fresh tube for ethanol precipitation.

5.7.2 Ethanol Precipitation

The microcentrifuge tube containing the DNA solution had 3M Sodium Acetate

added at 1/10 volume, then vortexed to ensure mixture of salt with DNA. Next, 2X

volume ice-cold 100% Ethanol was added and vortexed to provide a complete mixture.

The mixture was placed in -80°C for at least 1 hour but typically left overnight. When the

mixture was removed, the mixture was centrifuged at 13,300 x g for 20 minutes at 4°C.

The supernatant was then decanted carefully to not disturb the pellet. Next, the pellet

was washed with 70% ice-cold ethanol and allowed to stand at room temperature for 5

minutes. The tube was then centrifuged again at 13,300 x g for 20 minutes at 4°C, and

the supernatant was removed by pipetting. The pellet was then air dried for

approximately 20 to 30 minutes or until no trace smell of ethanol existed. The dried

pellet was then resuspended with 20-40 µL Nuclease-free water or 1X TE buffer via

gently pipetting. The resuspension was incubated at 37°C for 15 to 20 minutes to

ensure resuspension.

5.8 Ligation

New England Biolabs T4 DNA Ligase (400,000 cohesive end units/ml; Catalog #
M0202L) was used in all ligations. The protocol was set up as follows:

Vector DNA	1.000×10^{-5}-2.000×10^{-5} nmol
Insert DNA	3.000×10^{-5}-5.000×10^{-5} nmol
T4 DNA Ligase	400 Units
T4 DNA 10X Ligase Buffer	2µl
Nuclease-free water	Up to 20 µl
Total Reaction Volume	20µl

Vector DNA to Insert DNA was set up at a 1:3 or 1:6 molar ratio. This reaction was
set up on ice and the T4 ligase added last. The reaction was placed in the
ThermoFisher Applied Biosystems™ Veriti™ 96-Well Thermal Cycler for 14 hours at
16°C. The reaction was stored in the 4°C refrigerator or on ice until used in the
transfection.

5.9 Polymerase Chain Reaction

The following PCR primers generated by Integrated DNA Technologies, Inc. (Skokie,
Illinois) were used for sub-cloning the original insert with the restriction sites in a different
reading frame orientation.

5.9.1 Primer #1: HindIII replacing NcoI-NT

The 34 Bases of Sequence - HindIII replacing NcoI-NT were 5'- ATA CAA AGC TTC ATG GGC TAC CAC CAC CAC CTT T -3'. Properties of the designed primer sequence were:

Tm (50 nM NaCl): 65.2 °C

GC Content: 47.1%

Molecular Weight: 10290.7

nmoles/OD260: 3.1

ug/OD260: 32.4

Ext. Coefficient: 317900 L/(mole·cm)
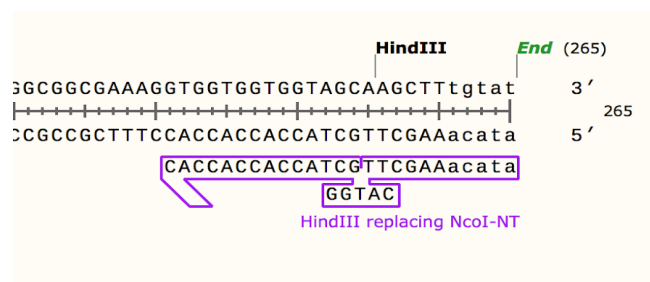
5.9.2 Primer #2: NcoI Replacing HindIII-OT

The 42 bases of Sequence - NcoI Replacing HindIII-OT were 5'- ACA CCA TGG AGC TTA TGA AAA CCA AAA ACT TTC TTC TTT TTT -3'. Properties of the designed primer were:

Tm (50 nM NaCl): 61.9 °C

GC Content: 31%

Molecular Weight: 12805.4

nmoles/OD260: 2.5

ug/OD260: 31.9

Ext. Coefficient: 400800 L/(mole·cm)

```
Start (0)      NcoI

                    NcoI Replacing HindIII-OT
          acaCCATGGAGCTTATGAAAACCAAAAACTTTCTTCTTTTTT

5'    acaCCATGGAGCTTATGAAAACCAAAAACTTTCTTCTTTTTTGTATTGCT
      |++++|++++|++++|++++|++++|++++|++++|++++|++++|++++|
3'    tgtGGTACCTCGAATACTTTTGGTTTTTGAAAGAAGAAAAAACATAACGA
```
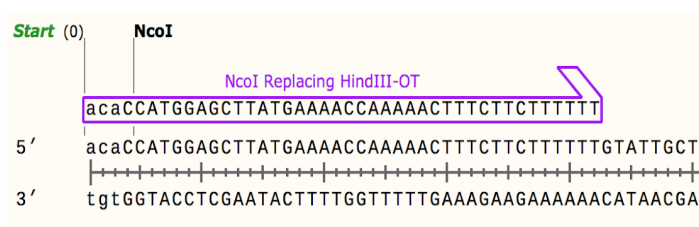
The oligonucleotides were resuspended in TE buffer (10mM Tris; 0.1 mM EDTA; pH 8.0) with a 100 µM concentrated resuspension and a 10 µM working stock working stock concentration

The Polymerase Chain Reaction mixture was set up as follows using the Q5® HF polymerase kit (New England Biolabs; Catalog # M0492S).

	Final Concentration
Q5 HF Master Mix 2X	1X
10 µM of Primer #1: HindIII replace NcoI-NT	0.5 µM
10 µM of Primer #2: NcoI replace HindIII-OT	0.5 µM
PLE10 Template DNA	1 pg
Nuclease-Free Water	Up to 50 µL
Total Volume	50 µL

This mixture was placed in a PCR reaction tube. The ThermoFisher Applied Biosystems™ Veriti™ 96-Well Thermal Cycler ran using the program NPB Q5 HF method 2, which was programmed as follows:

Step	Temperature (°C)	Time (seconds)
Denaturation	98	30
35 cycles	98	10
	72	60
Final Extension	72	120
Hold	4	∞

Once the PCR was completed the PCR tubes were pooled and placed in 4 ℃ refrigerator. Analysis of the PCR product was performed with the NanoSpec to determine DNA concentration of the PCR products.

5.10 DNA extraction from Agarose Gels

QIAEX II® Gel Extraction Kit (Qiagen; Hilden, Germany; Catalog # 20021) was used to harvest DNA from agarose gels. The manufacturer's protocol was followed but modified to fit specific requirements.

DNA product was loaded into the well of a 1% TAE agarose gel that was contained in a Gel Electrophoresis Chamber and electrophoresed at 100 volts for roughly 60 minutes. The 1% TAE agarose gel, containing electrophoresed DNA product, was imaged with an UV gel imager. The individual bands were excised while under UV light to determine precise location using a sharp scalpel blade. If DNA banding patterns were not clearly distinct to separate based on size compared to the NEB Quick-Load 2-Log DNA Ladder and other DNA products in same lane, then the gel was placed back into the Gel Electrophoresis Chamber in 10-minute increments until optimal separation was obtained. To maximize the recovery all agarose lacking DNA was removed from the excised band, so the weight of agarose was approximately 250 mg. The agarose containing DNA was placed in an empty, pre-weighed 2 ml microcentrifuge tube, and weighed to determine mass. Next, 2 volumes of Buffer QX1, 2 volumes of nuclease-free H_2O, and 30 µl of QIAEX II solution were added to 2 ml microcentrifuge tube. The QIAEX II solution was vortexed exceptionally well to ensure proper mixture before

adding to the buffer containing the agarose. After addition of QIAEX II, the solution was

incubated at 65°C for 12 minutes, and vortexed every 2 minutes. Intermittent vortexing

allowed proper binding of QIAEX II to the DNA. Next, the solution was centrifuged for 30

seconds at maximum speed, and the supernatant was removed with a pipet. A pellet

wash was performed with 500 µl of Buffer QX1 and vortexed to resuspend the pellet.

The supernatant was pipetted and discarded after a 30-second centrifuge. Next, the

pellet was washed twice with 500 µl of Buffer PE. For each wash, the pellet was

vortexed to resuspend in solution, then centrifuged to pellet the QIAEX II beads and

supernatant was removed. After final wash was completed, the pellet was air dried for

30 minutes or until the pellet was white. With a dry white pellet, the DNA was eluted with

40 µl of nuclease-free H_2O, vortexed the pellet to resuspend, and incubated at 70°C for

15 minutes. The resuspended pellet was stirred with a pipet tip and incubated an

additional 5 minutes. The DNA yield was increased by a final maximum speed

centrifugation that lasted a minimum of 30 seconds, but less than 45 seconds. This last

step eluted additional DNA into the nuclease-free water. Next the nuclease-free water

that contained eluted DNA was pipetted into a new centrifuge tube.

6 Results

6.1 Dissection of pET11D

E.coli DH5α was chosen as a plasmid maintenance host of pET11D. Figure 7 demonstrates a vector map of the pET11d plasmid isolated from the bacterial cell. The plasmid contains multiple restriction sites that were selected based on location and orientation to the ribosome binding site (RBS) and the open reading frame (ORF).

Figure 7 pET-11d Vector Map with a length of 6,674 base pairs. The restriction sites used are identified with names and locations on the vector. Also noted is the Open Reading Frame direction and location of the Ampicillin Resistance Gene.

The gene encoding LAGC-(EAAAK)$_{11}$, to be named AMR, was previously cloned into the pET11d plasmid and was gifted from a former Georgia Campus – Philadelphia College of Osteopathic Medicine Thesis Student, Robin Conley (83). The Apyrase leader sequence directs translocation of the LAGC sequence from the cytosol to the periplasmic space of the E.coli and is then cleaved by the cellular machinery. The LAGC, a portion of the lipobox protein, serves as a signal sequence that is cleaved, then modified with an N-acetyl moiety. Each EAAAK motif forms a rigid alpha helical structure which will extend

from the N-acyl-S-diacylglycerylcysteine lipid anchor moiety. To achieve the desired

distance between the inhibitor moiety and the phospholipid anchor, the EAAAK motif

was repeated a total of eleven times. Encoded at the terminus of the EAAAK region is a

GGGGS flexible linker sequence. The flexible nature of GGGGS allows for the mobility

of the inhibitor, instead of being directly tethered to the rigid EAAAK sequence

6.2 PCR Reorientation and Purification of AMR
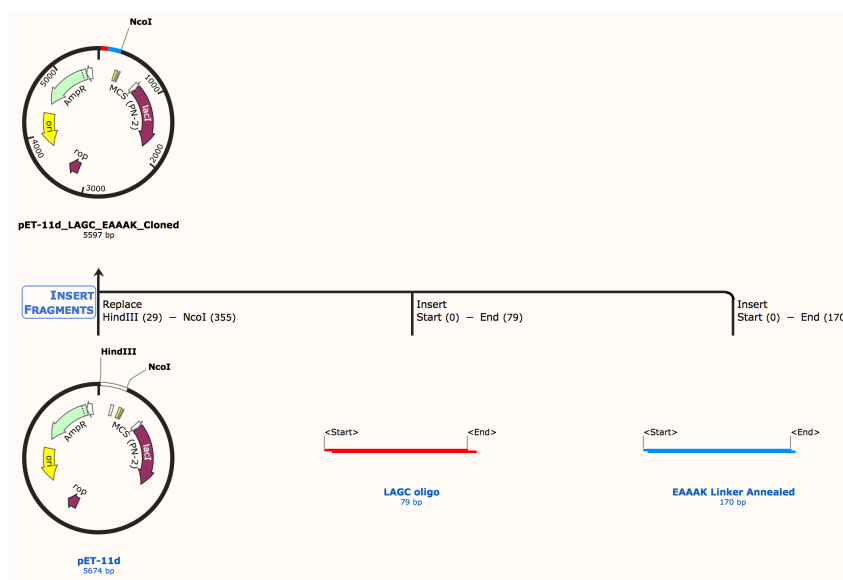
Figure 8 pET-11d Vector Map with the LAGC-EAAAK sequence inherited from a previous student.. The

restriction sites used are identified with names and locations on the vector. Also noted is the Open Reading

Frame direction and location of the Ampicillin Resistance Gene.

The gifted plasmid, p-LAGC-EAK11, contained the *Shigella* apyrase leader

sequence, anchor sequence LAGC, repeated ruler sequence EAAAK and flexible linker

sequence GGGGS inserted between restriction sites *Nco*I and *Hind*III (see Figure 8).

Employing the p-LAGC-EAK11 plasmid as the cloning backbone for the PN2 inhibitor, in-depth analysis determined the reading frame directionality was incorrect. Correct placement of AMR sequence is necessary for not only protein expression but for the addition of the PN2-KPI inhibitor.
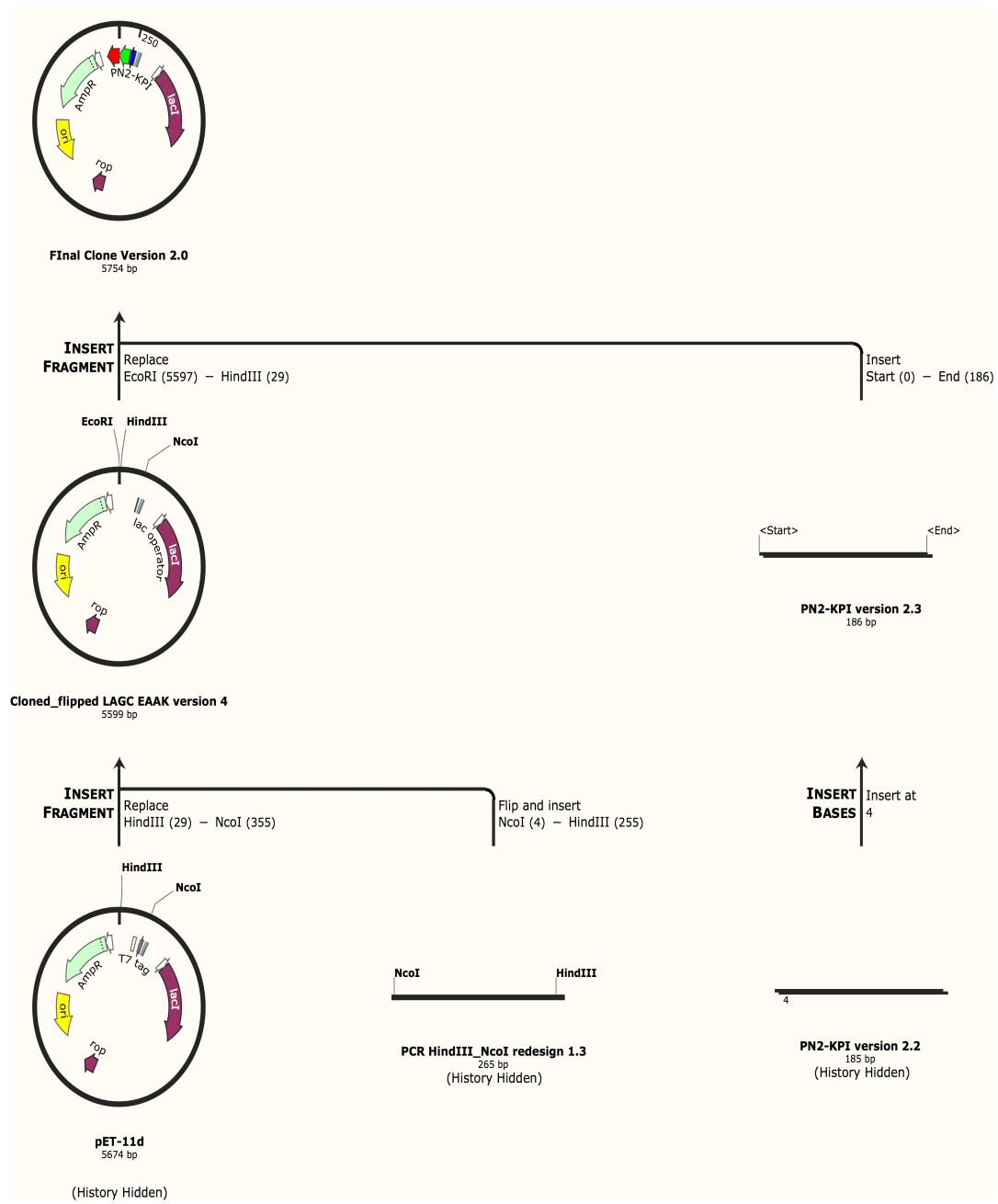
Figure 9 Master Plan Overall View. Correction of pET-11d Vector with the LAGC-EAAAK (AMR) sequence inherited from a previous student. Redesign of the PN2-KPI and insertion into the vector to produce the final product, AMRI. The restriction sites used are identified with names and locations on the vector. Also noted is the Open Reading Frame direction and location of the Ampicillin Resistance Gene.

A master plan (see Figure 9) was developed to use PCR to re-engineer the

restriction sites and correct the AMR reading frame orientation by subcloning out the

previous bits and swapping their orientation before adding the

PN2-KPI sequence. The new reverse strand, named 'HindIII

replacing NcoI-NT', and the new forward strand, named 'NcoI

replacing HindIII-OT' were used to re-engineer the orientation

of the strand. Following the PCR, the entire AMR sequence

underwent a double digest with HindIII and NcoI. Upon

completion of the restriction digest, a Phenol/Chloroform

extraction was performed to purify the AMR product. Gel

electrophoresis (See Figure 10) and was performed on the

AMR product to ensure size of the product. Once confirmed

that the product was of desired size the process was repeated

on a larger scale and gel analysis had a concentration of 725

ng Restricted Flipped Insert used in the gel analysis (see

Figure 10

Confirmation of AMR

PCR product. The

expected band size is

356 base pairs.

Figure 10). The NanoSpec reading on the total collected volume was 6640 ng total of

AMR insert from the PCR reaction.

6.3 Purification of Restricted pET11d Plasmid

Generation of new *E.coli* DH5α containing the pET11d plasmid was performed to

have ample stock for the pET11d-AMR ligation reaction. For the generation of sticky

ends, the pET11d vector was restricted with NcoI in the thermocycler for 3 hours at

37°C, followed by heat inactivation at 80°C for 20 minutes, and held at 4°C until removal

from thermocycler. A 1% agarose gel containing Phenix Red was the standard for all gel purifications with the bands excised under UV light, and plasmid DNA harvested via QIAEX-II gel extraction kit (see Figure 11).

When using the gel extraction kit, following the manufacturer's protocol except for the heat recommendation, a heat setting of 65°C to 70°C was found to be optimal for extraction of DNA from the QIAEX-II beads. The NanoSpec then quantified the NcoI_pET11d plasmid concentrations to ensure both quantity and quality.

pET11d_NcoI plasmid was then restricted with HindIII for 2 hours in the thermocycler, heat inactivated at 80°, and held at 4°C. The pET11d_NcoI_HindIII plasmid was CIP treated at 37°C in the thermocycler for 1 hour. Purified pET11d_NcoI_HindIII_CIP was obtained by two phenol/chloroform extraction and single ethanol precipitation. Quantification was performed using the NanoSpec reading to generate the total 180.4 ng of DNA.
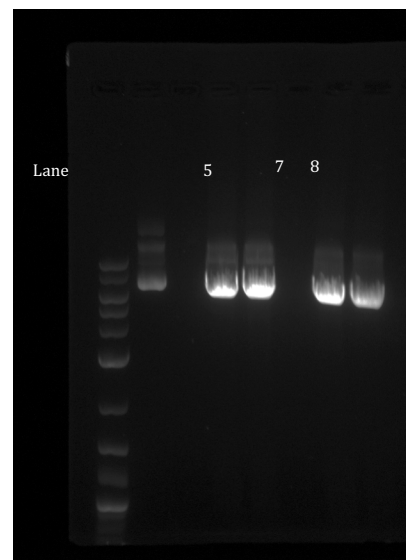
Figure 11 pET-11d Restriction. In lanes 4 and 5 the plasmid has been digested with HindIII, lanes 7 and 8 have been digested with NcoI. Based on the band size an estimated concentration of 600 ng per lane of DNA.

6.4 Insertion and Transformation of AMR Insert

The importance of molar ratios comes into account for ligation reactions to occur between pET11d_NcoI_HindIII_CIP and AMR. The ligation was performed overnight at a 1:3 vector to insert ratio at 16°C in the thermocycler. At the completion of ligation, a transformation was setup using competent *E.coli* DH5α cells, a gift from a former GA-PCOM pharmacy student, Betty Ma. To the competent cells, 10 ng of

Figure 12 Transformation of p_AMR into E.coli DH5α. The colonies were identified and circled so that origin could be traced in detail before Miniprep was performed to send the plasmid DNA for analysis.
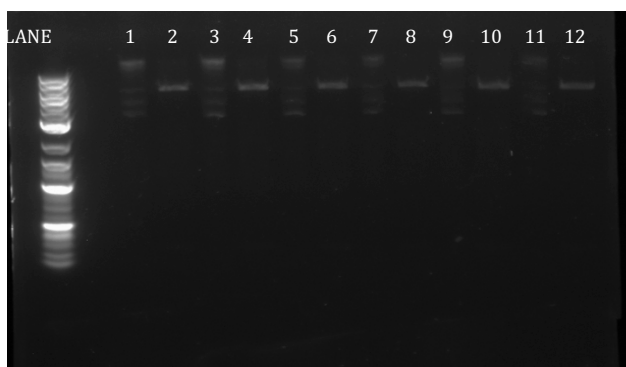
Figure 13 Gel Electrophoresis Image of double digested Transformation Product. The Miniprep harvested plasmid was double digested with NcoI and HindIII as confirmation before sending samples for sequencing analysis. The double digest plasmid has an expected size of 5,348 base pairs. Quick-Load 2-Log DNA Ladder (New England BioLabs, Ipswich, Massachusetts, Catalog # N0550S) was employed for size analysis.

plasmid was pipetted into them, heat shocked at 42°C for 30 seconds, thus allowing the DNA to be transformed into the E. coli cells. The cell cultures were grown overnight at 37°C and colonies were observed (See Figure 12). Selections were made from the chosen colonies and inoculated into 5 ml LB broth with 50 μg/mL ampicillin and grown overnight Midiprep was performed the following day. To

confirm the ligation and transformation a success a double digest with NcoI and HindIII on the harvested P_AMR plasmid was performed with the restricted plasmid being 5,348 bp and the fragment 251 bp (See Figure 13). The harvested plasmids were sent to Retrogen for sequencing from the T7 (See Appendix A). A positive colony, named P1CB1, was chosen as the Gold Stock colony for all future P_AMR plasmids due to its complete sequence.

6.5 Annealing and Purification of PN2-KPI

The PN2-KPI inhibitor was annealed by mixing in equal concentrations of oligo and heating to 94°C for 2 minutes, followed by slowly cooling to room temperature. A small amount was then used to run for analysis and quantification of product.

6.6 Harvest of P_AMR for use in Transformation

P_AMR was then harvested from *E.coli* to be used for a series of restrictions before the ligation of the PN2. The first restriction was with EcoRI as previously performed with HindIII, followed by gel purification, and QIAEX-II gel extraction. NanoSpec quantification and gel verification was used to ensure full linearization before proceeding to the restriction with HindIII. Next, was a restriction of P_AMR_EcoRI with HindIII for 2 hours was performed in the thermocycler at 37°C, followed by heat inactivation at 80°C for 20 minutes. The p_AMR_EcoRI_HindIII was then treated with CIP for 1 hour at 37°C. To purify p_AMR_EcoRI_HindIII_CIP a phenol/chloroform/isoamyl extraction followed by ethanol precipitation, to ensure purity of the restricted DNA. A quantification gel was used to determine the concentration of plasmid DNA after completion of purification.

6.7 Ligation and Transformation of p-AMR with PN2-KPI
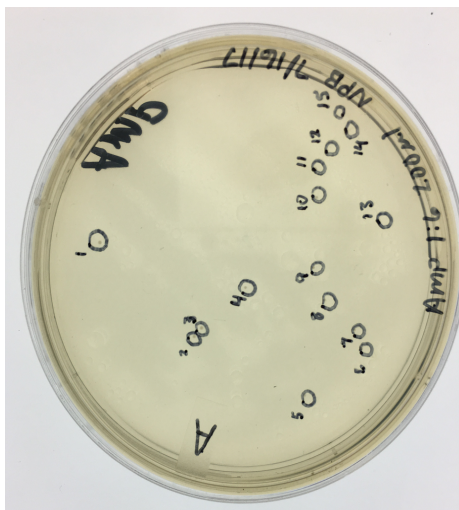
Figure 14 Image of Transformation colony selection of p_AMRI plasmid in E.coli DH5α. Isolated colonies were selected for inoculation to grow culture for sequence confirmation.

A ligation reaction was set up between p_AMR_EcoRI_HindIII_CIP and PN2 insert at a molar ratio of 1:3 and 1:6. This reaction was placed
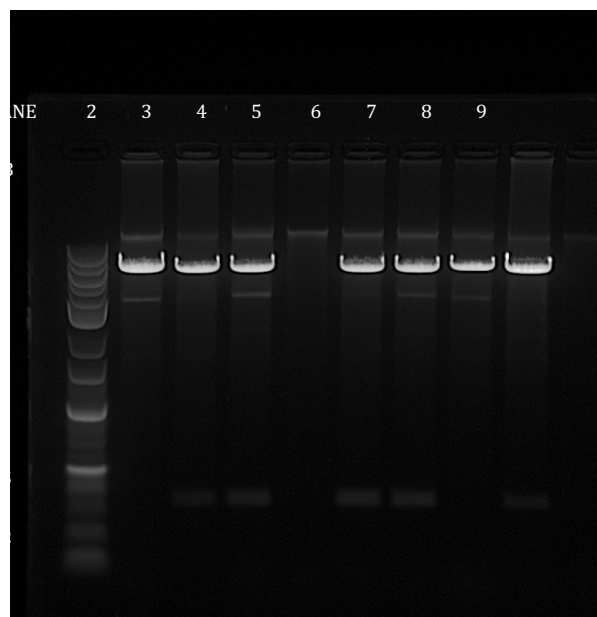
Figure 15 Gel Confirmation of desired p_AMRI product. The plasmid was digested with XbaI. On the engineered p_AMRI XbaI has 2 different restriction sites in the cloned sequence, which ejected a fragment of 356 base pairs in length. Quick-Load 2-Log DNA Ladder (New England BioLabs, Ipswich, Massachusetts, Catalog # N0550S)

in the thermocycler overnight at 16°C and used in a transformation into competent *E.coli* DH5α cells. The chosen colonies (See Figure 14) were grown in 5 ml LB broth with 5 µg/mL ampicillin overnight, and a miniprep was used to harvest the plasmid DNA.

To ensure the confirmation of the P_AMRI product, a restriction with *Xba*I resulted in the expected ejection of a 356 base pair fragment (See Figure 15). After restriction

confirmation, the p_AMRI plasmid was sent to Retrogen for sequencing analysis (See

Appendix B).

7 Discussion

The goal of this study was to design and generate a lipid **A**nchored **M**olecular **R**uler with attached **I**nhibitor (AMRI) at a fixed height to quantify the optimum reactive height for FIXa (see Figure 16). Having a hypothesized reactive height is between 50 to 100Å above the membrane it is important to create AMRI to fluid data and understanding of Serine Proteases, specifically for this study FIXa. This was carried out by creation, purification, and sequencing of an AMRI in an expression vector.
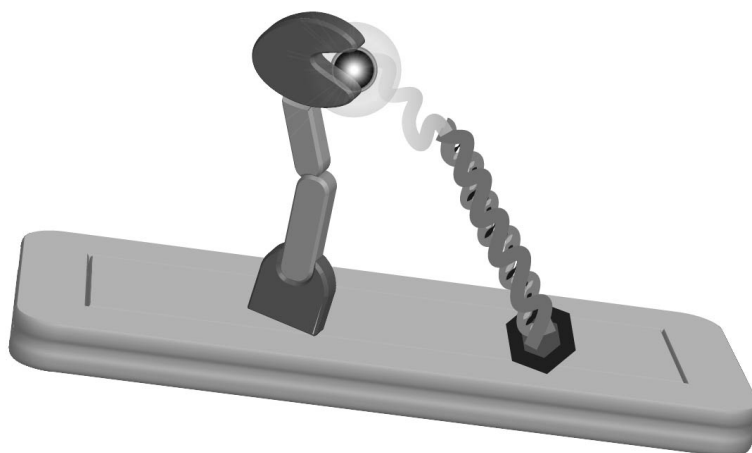
Figure 16. Hypothesized Action of AMRI interaction with FIXa on a phospholipid surface. Here it can be seen how the AMRI (figure on left) gets the right height and the flexible linker allows the inhibitor to fit in the reactive site of FIXa (structure on the left)

Previous studies have demonstrated the use of a variable length peptide linker and a membrane bound inhibitor. The understandings of previous FRET studies delivered great insight into a topographical orientation of the enzymes. Using the FRET data allowed for height estimation of FIXa on a phospholipid surface, but it could not necessarily correlate with the optimal reactive height, nor elasticity of the active site of the enzyme when exhibiting maximum pro-coagulant activity. This study focused on the

generation of a lipid-tethered inhibitor for use as a molecular ruler of FIXa through a successful engineering and multi-step cloning process.

The p-LAGC-EAK10 plasmid underwent a restriction digest with Restriction endonucleases, *Hind*III and *Nco*I, to produce a DNA fragment containing the LAGC and (EAAAK)$_{11}$ coding sequences. Using the molecular biology program, SnapGene, PCR primers were designed to mutagenize the ends, inserting HindIII and NcoI sites opposite to their original design. This "flipped" the sequence and inserted it into the correct reading frame with the Tag7 site, coding for methionine.

Standard cloning techniques were employed to sub-clone the fragment into an empty Pet11d vector, harvested from *E. coli* DH5α. PN2-KPI oligonucleotides were designed on SnapGene and generated by Integrated DNA Technologies. The PN2-KPI oligonucleotides were ordered in forward and reverse reading frames and annealed to create overhangs for alignment with their EcoRI and HindIII restriction sites. For conformation of correct annealing the product was purified using gel electrophoresis; the bands excised were roughly 187 base pairs size.

A digest of EcoRI and HindIII produced the sticky ends for insertion and ligation of annealed oligonucleotides encoding the sequence for PN2-KPI, into the constructed AMR plasmid. The DNA was purified via Gel Electrophoresis and excising the band with a product size of 5754 base pairs. The DNA was for sequencing starting at the Tag 7 promoter to ensure that the final gene product is correct.

The sequencing data (see Appendix B) demonstrate the success of generating a constructed AMRI sequence in a multi-step molecular cloning process. Successful

insertion of 11 EA$_3$K repeats will result in the PN2-KPI inhibitor having a raised height of roughly 83 Å above the membrane surface. Ideally the height of 83 Å will prove ideal for inhibition of FIXa as crystal structures and previous FRET data have indicated that active site is between 61 – 89 Å (59, 60, 68).

8 Future Studies

8.1 Protein expression and purification of AMRI

8.1.1 Expression of LAGC-(EAAAK)$_{11}$-PN2 (AMRI)

The construction of LAGC-(EAAAK)$_{11}$-PN2 in DH5α vector described in Specific

Aim 1 would be expressed in *E. coli* BL21(DE3). Carbenicillin would be used, replacing

Ampicillin, since it would confer more stability with β-lactamases and resistance to

producing satellite colonies in this expression step (82). The plasmid would confirm

correct coding sequence and correct reading frame orientation if *E. coli* BL21(DE3)

produces colonies. The cells would then be lysed, as expressed proteins would be

anchored in *E. coli* periplasmic space, with a zwitterionic detergent and would remain in

detergent. Using the detergent would provide a suitable environment for the protein,

since typically its lipid bound.

8.1.2 Affinity Column Purification of LAGC-(EAAAK)$_{11}$-PN2 (AMRI) Crude Cell Lysate

Purification of LAGC-(EAAAK)$_{11}$-PN2 Crude Cell Lysate would be performed by

Affinity Column Purification in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. The Affinity

column would be generated using Affi-Gel generating an amide bond to Trypsin. The

desired amount of Affi-Gel would be transferred into a funnel to separate and drain the

supernatant. The gel would then be washed three times with gel bed volumes of cold 10

mM Sodium Acetate, pH 4.5. Gel is transferred to a flask and the cold Trypsin solution

is added at 0.5 ml per ml of gel, followed by one hour of gentle agitation at room

temperature. Once completed the gel with trypsin would be transferred to a column and washed with water until column runs clear at O.D.$_{280}$. The column was chosen since Trypsin would bind with PN2-KPI attached at the end of the folded protein. The column would be developed using an Equilibration Buffer of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton-X100 with a flow rate of approximately 1 mL per 2.5 minute. The cell lysate would then be loaded at a rate of approximately 1 mL per 2.5 minute. The cell lysate would then be eluted with 10 mM HCl, 500 mM KCl, 0.1% Triton-X100, pH 2 at a rate of 1 ml/min and fractions collected. Elutions would then be neutralized by adding 20 µl of 1M Tris-HCl, pH 7.5, as collected. Elution fractions would then be analyzed at A$_{280}$ and relevant fractions would be pooled. The column would be stored at 4℃ and in the starting buffer that contains 0.2% Sodium Azide (Unpublished protocol K. Baker).

8.1.3 Re-lipidation of LAGC-(EAAAK)$_{11}$-PN2 (AMRI)

For proper expression of AMRI, re-lipidation must occur on a phospholipid surface such as a liposome, this protocol has been adapted from Smith and Morrissey 2004 (84). These liposomes would form as the detergent is removed from, creating a layer for AMRI to effectively sit along with FIXa. Using the A$_{280}$ readings for estimated protein concentration it would place in a ratio of 300 nM protein with 2.6 µM Phosphatidylcholine (PC) lipid. The mixture would then be dried under Argon gas followed by continued drying under a vacuum. The protein solution still in the elution buffer would be added to solubilize the lipids. Detergent would be removed by adding 50 mg wet SM-2 bio beads to pool the lipid vesicles. As the liposomes are forming, detergent is being removed, and the protein should re-lipidate into the liposome. After two separate 1.5-hour rocking

events the liquid around the beads would be collected; with an additional 350 mg wet

SM-2 bio beads added after first rocking.

8.1.4 Gel Filtration Chromatography

Gel Filtration Chromatography would be employed using a 16 ml sepharose CL 2B

300 column at room temperature. The column would be equilibrated in a High Salt TBS,

composed of 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl with a running flow of 1 ml/min. The

replipidated protein samples would then be added to the column and allowed to settle in

column matrix. The High Salt TBS would then flow over the column at a rate of 1 ml/min

and collecting fractions. The fraction turbidity would be analyzed at A_{600} to determine a

lipid elution profile and proteins would be measured at A_{280}. Then proteins would be

analyzed on 15% SDS-PAGE gels, followed by Silver staining using the Morrissey 1981

protocol (85). This step would confirm the proteins are fixating to the lipid membrane

and not generating protein null liposomes. Proteins that are not bound to the liposome

would elute early as they are small, and the lipids would elute in later fractions. If the

proteins bind with the lipid they would elute in the same fractions.

8.1.5 Anticipated Results

I anticipate the engineering of the protein was correct and would produce a valid

product. Expression of the protein should be accomplished because the DNA sequence

is in the correct reading frame inducible under the IPTG. Isolation of the protein would

be possible as well because of Trypsin and its affinity for binding.

8.1.6 Potential Problems and Alternative Strategies

Potential problems may arise in the extraction method with the detergent. The

detergent is essential in lysing the cells and providing soluble protein environment.

Adjustments would be made at each step and evaluated, since this protocol was

adapted from the purification of BPTI. The previous protocol also lacked the larger rigid

and flexible linker regions, which could affect how the inhibitor folds and behaves.

8.2 Determine ability of AMRI to inhibit FIXa

8.2.1 Protocol

To study reactive height of FIX, AMRI was incorporated into a liposome as described

in specific Aim 2. The liposomes would be constructed of 2 anionic phospholipids,

phosphatidylserine, and phosphatidylcholine (PS and PC). Their concentrations would

be at 10% PS and 90% PC and should be able to promote optimal binding. It has been

demonstrated with their ability to bind Vitamin K-dependent clotting factors, which

contain the N-terminal γ-carboxyglutamic acid (Gla) domain (9, 86). Once the liposome

bound with AMRI is dried to a 96- well plate activated FIXa would be added.   FIXa has

been previously activated by Russell's Viper Venom and stored frozen until needed.

Chromogenic substrate would be added and monitored at 405 nm to determine the

amount of active enzyme that remain unbound by the inhibitor, modified from

Neuenschwander et al. 2006 and Yu et al. 2014 (56, 87). Rates would be plotted

against inhibitor concentration to determine the Ki for AMRI.

8.2.2 Anticipated Results

I anticipate that AMRI would inhibit FIXa at the height hypothesized of using the

(EAAAK)$_{11}$ insert between the LAGC anchor and PN2-KPI.

8.2.3 Potential Problems and Alternative Strategies

A potential problem is the sequence of repeats may alter the binding ability of KPI to

block the catalytic triad, therefore, to test this ability a mixture of inhibitor and substrate

could be placed in solution. If no reaction or weak reaction, this would be conclusive

that AMRI is ineffective in a FIXa topography study. It is also possible the inhibitor could

be raised to an ineffective height; if weak inhibition then alternative repeats of EAAAK

could be used to determine the optimal height of FIX. This would require multiple

cloning experiments to adjust the EAAAK sequence repeats. Also, an issue that may

been encountered is either FIX or inhibitor does not anchor to the phospholipid for

correct positioning. If this is the case, then alternative concentration ratios of PS:PC

could be used to see if a different concentration as a greater anchoring affect.

9 *Clinical Significance*

An abnormal blood clot forms in the blood vessel, called a thrombus. This thrombus is the result of a clinical abnormality causing activation of the clotting cascade. If the resulting thrombus moves in the vasculature, it is termed an embolus. An embolus becomes dangerous as it travels and lodges in the narrowing of the vessel, restricting or terminating blood flow.

Current medical treatment for a patient receiving a stent or angioplasty involves multiple preparation steps to ensure the patient does not develop an embolus. The current medical intervention relies heavily on drugs that interfere with the clotting cascade. Patients going in for stent placement are given a Ca^{2+} channel antagonist and injections of heparin during the procedure. Once the procedure is completed the use of heparin and coumarin (i.e. Warfarin) are continued for 72 hours. Following the 72 hours treatment with the coumarin is done for an indefinite amount of time, depending on the patients clotting times (88) .

The hope of this study of pro-coagulant enzyme/membrane topography is to bridge the knowledge gap by studying the distances of fully pro-coagulant enzyme complexes. The information from this membrane-bound activity study could lead to the generation of inhibitors with an increased specificity. Not only targeting enzyme reactivity but one directed to specific membrane topography of each enzyme. Therefore, the results of a study with AMRI could greatly impact clinical therapies aimed at coagulation reaction

inhibition. Most importantly of the therapies impacted the most would be treatment of

thrombosis, surgical procedures, and the implantation of medical devices (i.e. stents)

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10 Appendices

10.1 Appendix A. SEQUENCE DATA IMAGES OF AMR

Cloned_flipped LAGC EAAK version 4.dna (Circular / 5599 bp)

	Primer	Length	🌈	Binding Sites		⇌	Tm
✓	**HindIII replacing...**	30-mer		35 ..	48	→	50°C

/sequence   = atacaAAGCTTCATGGGCTACCACCACCAC

50% GC / 9089.0 Da

	Primer	Length		Binding Sites			Tm
✓	**NcoI Replacing Hi...**	42-mer		247 ..	286	←	63°C

/sequence   = acaCCATGGAGCTTATGAAAACCAAAAACTTTCTTCTTTTTT

31% GC / 12,805.4 Da

Cloned_flipped LAGC EAAK version 4.dna (Circular / 5599 bp)

```
              10          20          30          40
    |' ' '|' ' '|' ' '|' ' '|' ' '|' ' '|' ' '|' ' '|
```

HindIII

HindIII replacing NcoI-NT

a t a c a A A G C T T C A T G G G C T A C C

5′   T T C T C A T G T T T G A C A G C T T A T C A T C G A T A AGCTT   GCTACC
••• +++++++++++++++++++++++++++++++++++++++   +++++++
3′   A A G A G T A C A A A C T G T C G A A T A G T A G C T A T T C G A A   CGATGG

     Arg Met Asn Ser Leu Lys Asp Asp Ile Leu Lys   Ser Gly
     ◄-------------------- (in frame with T7 tag) --------------------

TTCTCATGTTTGACAGCTTATCATCGATAAGCTT   GCTACC
1 ◄ TTCTCATGTTTGACAGCTTATCATCGATAAGCTT CATGG GCTACC
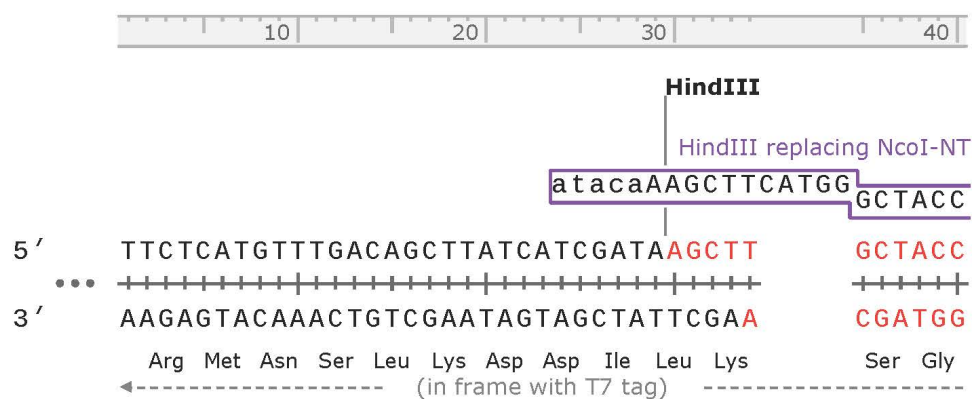
```
              50          60          70          80
    |' ' '|' ' '|' ' '|' ' '|' ' '|' ' '|' ' '|' ' '|
```

HindIII replacing...

A C C A C C A C

A C C A C C A C C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C
+++
T G G T G G T G G A A A G C G G C G G C G A A G A A A G C G G C G G C G A A G A A A G C G

Gly Gly Gly Lys Ala Ala Ala Glu Lys Ala Ala Ala Glu Lys Ala
◄-------------------- (in frame with T7 tag) --------------------

ACCACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGC
1 ◄ ACCACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGC
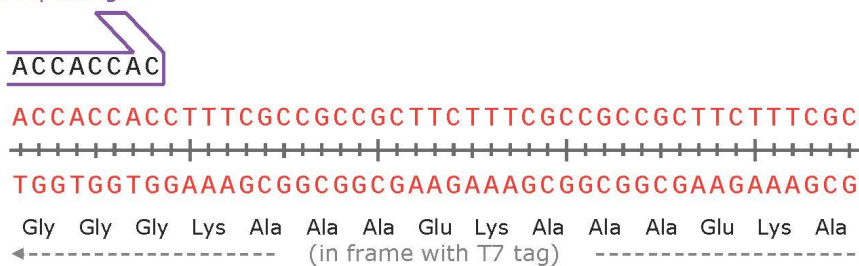
Cloned_flipped LAGC EAAK version 4.dna (Circular / 5599 bp)

| 90 | 100 | 110 | 120 | 130 |

C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C

G C G G C G A A G A A A G C G G C G G C G A A G A A A G C G G C G G C G A A G A A A G C G

Ala Ala Glu Lys Ala Ala Ala Glu Lys Ala Ala Ala Glu Lys Ala
◀- - - - - - - - - - - - - - - - - -   (in frame with T7 tag)   - - - - - - - - - - - - - - - - - -

C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C

1 ◀ C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C

| 140 | 150 | 160 | 170 |

C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C

G C G G C G A A G A A A G C G G C G G C G A A G A A A G C G G C G G C G A A G A A A G C G

Ala Ala Glu Lys Ala Ala Ala Glu Lys Ala Ala Ala Glu Lys Ala
◀- - - - - - - - - - - - - - - - - -   (in frame with T7 tag)   - - - - - - - - - - - - - - - - - -

C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C

1 ◀ C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C

| 180 | 190 | 200 |

BamHI

C G C C G C T T C T T T C G C C G C C G C T T C   G G A T C C

G C G G C G A A G A A A G C G G C G G C G A A G   C C T A G G

Ala Ala Glu Lys Ala Ala Ala Glu   Ser Gly
◀- - - - - - - - - - - - - - - - - -   (in frame with T7 tag)   - - - - - - - - - - - - - - - - - -

C G C C G C T T C T T T C G C C G C C G C T T C   G G A T C C

1 ◀ C G C C G C T T C T T T C G C C G C C G C T T C T T T C G C C G C C G C T T C G G A T C C

Cloned_flipped LAGC EAAK version 4.dna (Circular / 5599 bp)

```
        210      220      230      240      250
```

ACATCCAGCCAGGGGGATAAAAATCATATTTGTAGCAATACAAAA
++
TGTAGGTCGGTCCCCCTATTTTTAGTATAAACATCGTTATGTTTT

Cys Gly Ala Leu Pro Ile Phe Ile Met Asn Thr Ala Ile Cys Phe
◄------------------- (in frame with T7 tag) -------------------

TTTT

NcoI Replacing HindIII-OT

ACATCCAGCCAGGGGGATAAAAATCATATTTGTAGCAATACAAAA
1 ◄ ACATCCAGCCAGGGGGATAAAAATCATATTTGTAGCAATACAAAA

```
        260      270      280      290
```

NcoI

AAGAAGAAAGTTTTTGGTTTTCATAAGCTCCATGGTATATCTCCT
++
TTCTTCTTTCAAAAACCAAAAGTATTCGAGGTACCATATAGAGGA

Leu Leu Phe Asn Lys Thr Lys Met Leu Glu | 1 Met
◄-------- (in frame with T7 tag) -------- ►

T7 tag   RBS

TTCTTCTTTCAAAAACCAAAAGTATTCGAGGTACCa
                                        ca

NcoI Replacing HindIII-OT

AAGAAGAAAGTTTTTGGTTTTCATAAGCTCCATGGTATATCTCCT
1 ◄ AAGAAG**N**AA**NN**TTTTGGTTTTCATAAGCTCCATGGTATATCTCCT

Cloned_flipped LAGC EAAK version 4.dna (Circular / 5599 bp)

|300| |310| |320| |330| |340|

XbaI

TCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG
+++
AGAATTTCAATTTGTTTTAATAAAGATCTCCCCTTAACAATAGGC

RBS

lac operator

TCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG
1 ← TCTTAAAGTTAANCNNAANNNNNNNNNNNNNNNNANNNNNNNNNN

|350| |360| |370| |380|

CTCACAATTCCCCTATAGTGAGTCGTATTAATTTCGCGGGATCGA
+++
GAGTGTTAAGGGGATATCACTCAGCATAATTAAAGCGCCCTAGCT

lac operator   T7 promoter

CTCACAATTCCCCTATAGTGAGTCGTATTAATTTCGCGGGATCGA
1 ← NNN

|390| |400| |410| |420| |430|

GATCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGG
+++
CTAGAGCTAGGAGATGCGGCCTGCGTAGCACCGGCCGTAGTGGCC

GATCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGG

10.2 Appendix B. SEQUENCE DATA IMAGES OF AMRI

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

Original Sequence:

1: P-AMRI-A4_T7 ←
    1258 bases
    1 .. 1252 (54 mismatches, 12 gaps)

2: P-AMRI-A9_T7 ←
    1265 bases
    1 .. 1251 (62 mismatches, 6 gaps)

3: P-AMRI-B2_T7 ←
    1256 bases
    24 .. 1256 (29 mismatches, 3 gaps)

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

10   20   30   40

5′ CGAGGCCCTTTCGTCTTCAAGAATTaAGCAGAACCACAAACAGCC
    •••
3′ GCTCCGGGAAAGCAGAAGTTCTTAAtTCGTCTTGGTGTTTGTCGG

                                60
                        * A S G C V A
                        PN2-KPI

                        * A S G C V A
                        ◄-- (in frame with EAAAK) ·---

CGAGGCCCTTTCGTCTTCAAGAATTaAGCAGAACCACAAACAGCC

1 ◄ CGAGGCCCTTTCGTCTTCAAGAATTAAGCAGAACCACAAACAGCC
2 ◄ CGAGGCCCTTTCGTCTTCAAGAATTAAGCAGAACCACAAACAGCC
3 ◄ CGAGGCCCTTTCGTCTTCAAGAATTAAGCAGAACCACAAACAGCC

50   60   70   80   90

ATACAGTACTCCTCAGTGTCGAAGTTGTTTCTGTTACCACCACAA

TATGTCATGAGGAGTCACAGCTTCAACAAAGACAATGGTGGTGTT

    55   50   45
M C Y E E T D F N N R N G G C
                    PN2-KPI
<
M C Y E E T D F N N R N G G C
◄-------------------- (in frame with EAAAK) --------------------

ATACAGTACTCCTCAGTGTCGAAGTTGTTTCTGTTACCACCACAA

1 ◄ ATACAGTACTCCTCAGTGTCGAAGTTGTTTCTGTTACCACCACAA
2 ◄ ATACAGTACTCCTCAGTGTCGAAGTTGTTTCTGTTACCACCACAA
3 ◄ ATACAGTACTCCTCAGTGTCGAAGTTGTTTCTGTTACCACCACAA

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

```
            100|          110|          120|          130|
  CCACCGTAGAAGAATGGAGCACACTTACCCTCAGTAACGTCGAAG
  +++++++++|++++++++|+++++++|+++++++|+++++++|++++++++|
  GGTGGCATCTTCTTACCTCGTGTGAATGGGAGTCATTGCAGCTTC
       40|              35|              30|
  G  G  Y  F  F  P  A  C  K  G  E  T  V  D  F
  <[            PN2-KPI            ]
  G  G  Y  F  F  P  A  C  K  G  E  T  V  D  F
  ◄-------------------- (in frame with EAAAK) --------------------
```

		CCACCGTAGAAGAATGGAGCACACTTACCCTCAGTAACGTCGAAG
1	◄	CCACCGTAGAAGAATGGAGCACACTTACCCTCAGTAACGTCGAAG
2	◄	CCACCGTAGAAGAATGGAGCACACTTACCCTCAGTAACGTCGAAG
3	◄	CCACCGTAGAAGAATGGAGCACACTTACCCTCAGTAACGTCGAAG

```
  140|          150|          160|          170|          180|
          XbaI
           |
  TACCATCTAGAAATCATAGCTCTACATGGACCAGTCTCAGCTTGC
  +++++++++|++++++++|+++++++|+++++++|+++++++|++++++++|
  ATGGTAGATCTTTAGTATCGAGATGTACCTGGTCAGAGTCGAACG
       25|              20|              15|
  Y  W  R  S  I  M  A  R  C  P  G  T  E  A  Q
  <[            PN2-KPI            ]
  Y  W  R  S  I  M  A  R  C  P  G  T  E  A  Q
  ◄-------------------- (in frame with EAAAK) --------------------
```
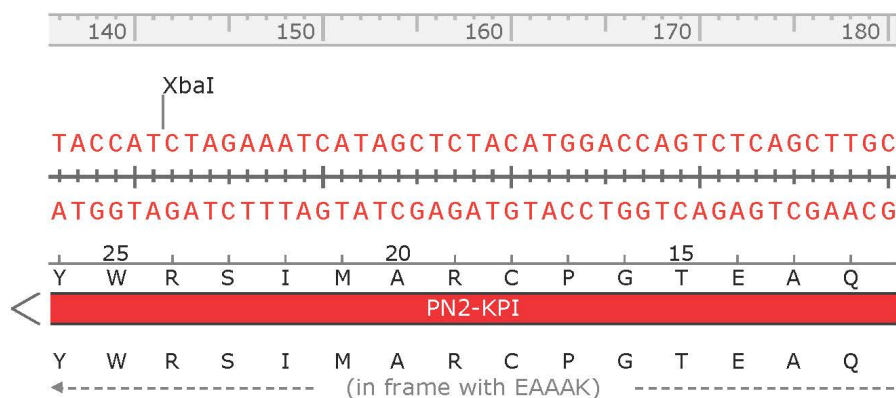
		TACCATCTAGAAATCATAGCTCTACATGGACCAGTCTCAGCTTGC
1	◄	TACCATCTAGAAATCATAGCTCTACATGGACCAGTCTCAGCTTGC
2	◄	TACCATCTAGAAATCATAGCTCTACATGGACCAGTCTCAGCTTGC
3	◄	TACCATCTAGAAATCATAGCTCTACATGGACCAGTCTCAGCTTGC

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

190   200   210   220

HindIII replacing NcoI-NT

atacaAAGCTTCATGG GCTACCAC

TCAGAACAAACCTCTCTAACAACCTCcAGCTT   GCTACCAC

AGTCTTGTTTGGAGAGATTGTTGGAGgTCGAA   CGATGGTG

10   5   1
E   S   C   V   E   R   V   V   E   L   K   S   G   G

< PN2-KPI

E   S   C   V   E   R   V   V   E   L   K   S   G   G   55

(in frame with EAAAK)   **EAAAK**

	TCAGAACAAACCTCTCTAACAACCTCcAGCTT	GCTACCAC
1 ←	TCAGAACAAACCTCTCTAACAACCTCCAGCTT	CATGG GCTACCAC
2 ←	TCAGAACAAACCTCTCTAACAACCTCCAGCTT	CATGG GCTACCAC
3 ←	TCAGAACAAACCTCTCTAACAACCTCCAGCTT	CATGG GCTACCAC

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

```
              230|        240|        250|        260|
```

HindIII replacing NcoI...

```
CACCACCTTT
```

```
CACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
+++++++++|+++++++++|+++++++++|+++++++++|+++++
GTGGTGGAAAGCGGCGGCGAAGAAAGCGGCGGCGAAGAAAGCGGC
```

```
              50|        45|        40
 G   G   K   A   A   A   E   K   A   A   A   E   K   A   A
```

< **EAAAK**

```
 G   G   K   A   A   A   E   K   A   A   A   E   K   A   A
<-------------------- (in frame with LAGC) --------------------
```

		CACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
1	←	CACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
2	←	CACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
3	←	CACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
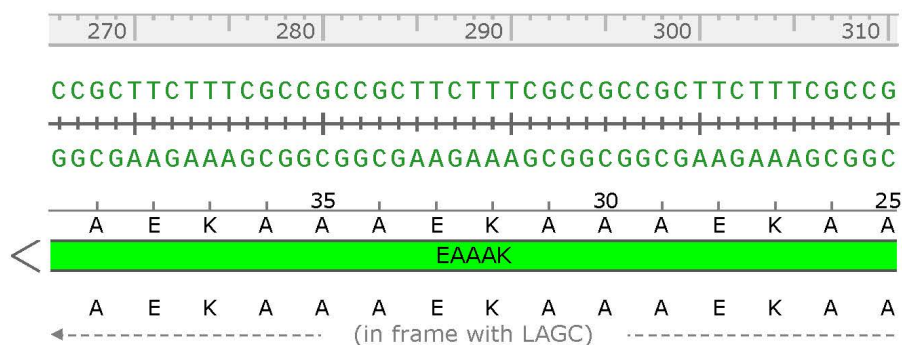
```
        270|        280|        290|        300|        310|
```

```
CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
+++|+++++++++|+++++++++|+++++++++|+++++++++|+
GGCGAAGAAAGCGGCGGCGAAGAAAGCGGCGGCGAAGAAAGCGGC
```

```
              35|        30|        25
 A   E   K   A   A   A   E   K   A   A   A   E   K   A   A
```

< **EAAAK**

```
 A   E   K   A   A   A   E   K   A   A   A   E   K   A   A
<-------------------- (in frame with LAGC) --------------------
```

		CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
1	←	CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
2	←	CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
3	←	CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

320 | 330 | 340 | 350 |

CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
+++++++++|+++++++++|+++++++++|+++++++++|++++++
GGCGAAGAAAGCGGCGGCGAAGAAAGCGGCGGCGAAGAAAGCGGC

20   15   10
A   E   K   A   A   A   E   K   A   A   A   E   K   A   A   A

< **EAAAK**

A   E   K   A   A   A   E   K   A   A   A   E   K   A   A   A
<------------------ (in frame with LAGC) ------------------

CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG

1 ← CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
2 ← CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG
3 ← CCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCG

360 | 370 | 380 |

BamHI

CCGCTTCTTTCGCCGCCGCTTC   GGATCCAC
+++++++++|+++++++++|+++   ++|+++
GGCGAAGAAAGCGGCGGCGAAG   CCTAGGTG

5   1
A   E   K   A   A   A   E   S   G   C

< **EAAAK**

A   E   K   A   A   A   E   S   G   C
<------------------ (in frame with LAGC) ------------------

LAGC

CCGCTTCTTTCGCCGCCGCTTC   GGATCCAC

1 ← CCGCTTCTTTCGCCGCCGCTTC TTTCGCCGCCGCTTC GGATCCAC
2 ← CCGCTTCTTTCGCCGCCGCTTC TTTCGCCGCCGCTTC GGATCCAC
3 ← CCGCTTCTTTCGCCGCCGCTTC TTTCGCCGCCGCTTC GGATCCAC

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

```
      390 |        400 |         410 |        420 |        430
  |    |     |    |    |    |    |    |    |    |    |    |    |

  A T C C A G C C A G G G G G A T A A A A A T C A T A T T T G T A G C A A T A C A A A A A A
  +++++|++++++|++++++|++++++|++++++|++++++|++++++|++++++|++++++|
  T A G G T C G G T C C C C C T A T T T T T A G T A T A A A C A T C G T T A T G T T T T T T

      25                 20                 15
      G   A   L   P   I   F   I   M   N   T   A   I   C   F   L
```

< ██
              LAGC

```
      G   A   L   P   I   F   I   M   N   T   A   I   C   F   L
```

◄------------------- (in frame with T7 tag) -------------------

┌─ T T T T T
└─┐
  └─┐
NcoI Replacing HindIII-OT

	ATCCAGCCAGGGGGGATAAAAATCATATTTGTAGCAATACAAAAAA
1 ←	ATCCAGCCAGGGGGGATAAAAATCATATTTGTAGCAATACAAAAAA
2 ←	ATCCAGCCAGGGGGGATAAAAATCATATTTGTAGCAATACAAAAAA
3 ←	ATCCAGCCAGGGGGGATAAAAATCATATTTGTAGCAATACAAAAAA

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

```
        440        450        460        470
   |    |    |    |    |    |    |    |    |
```

NcoI

GAAGAAAGTTTTTGGTTTTTCATAAGCcCCATGGTATATCTCCTTC
+++
CTTCTTTCAAAAACCAAAAGTATTCGgGGTACCATATAGAGGAAG

```
   10              5              1
   L   F   N   K   T   K   M   L   G   M
```

< [LAGC]   [RBS]

```
   L   F   N   K   T   K   M   L   G   | 1
                                       | M
```

◄------- (in frame with T7 tag) -------► [T7 tag]

CTTCTTTCAAAAACCAAAAGTATTCG⌐GGTACCa⌐ca
                         A

NcoI Replacing HindIII-OT
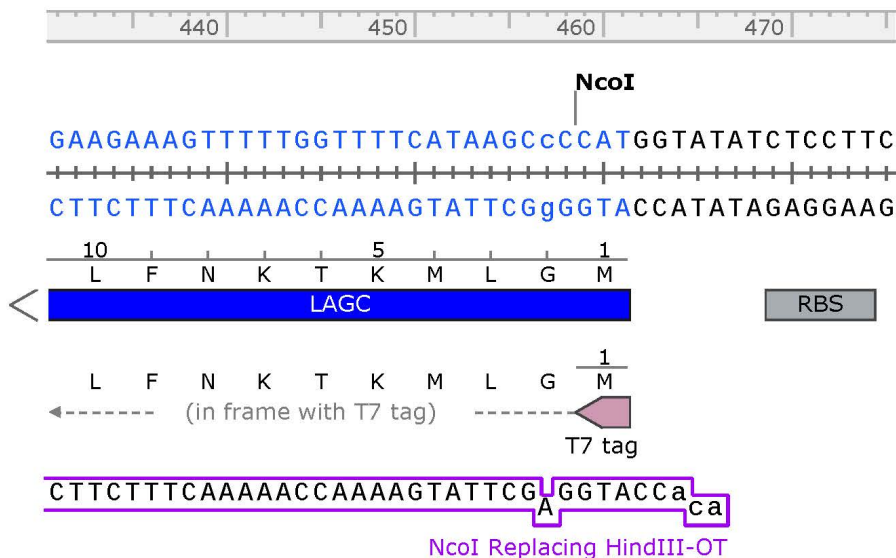
		GAAGAAAGTTTTTGGTTTTTCATAAGCcCCATGGTATATCTCCTTC
1	◄	GAAGAAAGTTTTTGGTTTTTCATAAGC[T]CCATGGTATATCTCCTTC
2	◄	GAAGAAA[N]T[NNN]TGGTTTTTCATAAGC[T]CCATGGTATATCTCCTTC
3	◄	GAAGAAAGTTTTTGGTTTTTCATAAGC[T]CCATGGTAT[N]TCTCCTTC

```
   480        490        500        510        520
   |    |    |    |    |    |    |    |    |    |
```

XbaI

TTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCT
+++
AATTTCAATTTGTTTTAATAAAGATCTCCCCTTAACAATAGGCGA

                                    [lac operator]
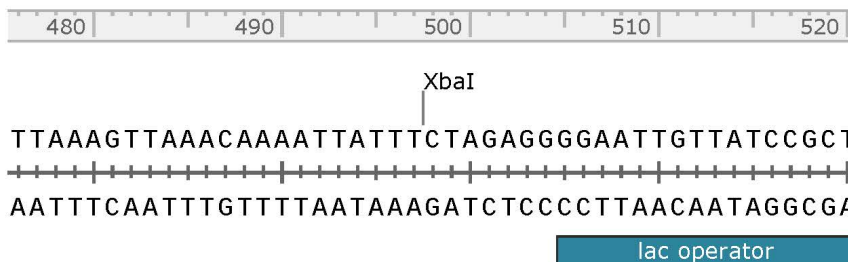
		TTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCT
1	◄	TTAAAGTTAA[NN]AAAATTA[N][T][–]CTAGA[NNNN][NNNNNNNNNNNN]
2	◄	TTAAAGTTAA[NNN]AAAATTA[N][T][–]CTA[N]A[NN]G[N]A[NNNNNNNNNNNNNN]
3	◄	TTAAAGTTAA[N]CAAAATT[N]T

FInal Clone Version 2.1_AMRI_alignment.dna (Circular / 5754 bp)

```
              530           540           550           560
CACAATTCCCCTATAGTGAGTCGTATTAATTTCGCGGGATCGAGA
+++++++++++|+++++++++++|+++++++++++|+++++++++++|++++
GTGTTAAGGGGATATCACTCAGCATAATTAAAGCGCCCTAGCTCT
```

lac operator ◁ T7 promoter

```
CACAATTCCCCTATAGTGAGTCGTATTAATTTCGCGGGATCGAGA
2 ←  NNN
```

```
     570           580           590           600           610
TCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCG
+++++++++++|+++++++++++|+++++++++++|+++++++++++|++++
AGAGCTAGGAGATGCGGCCTGCGTAGCACCGGCCGTAGTGGCCGC
```

```
TCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCG
```

```
     620           630           640           650
CCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATG
+++++++++++|+++++++++++|+++++++++++|+++++++++++|++++
GGTGTCCACGCCAACGACCGCGGATATAGCGGCTGTAGTGGCTAC
```

```
CCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATG
```

```
     660           670           680           690           700
GGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCG
+++++++++++|+++++++++++|+++++++++++|+++++++++++|++++
CCCTTCTAGCCCGAGCGGTGAAGCCCGAGTACTCGCGAACAAAGC
```

```
GGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCG
```