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Characterization of a Fluorescent Protein Reporter System

Sandra J. Dias

CHARACTERIZATION OF A FLUORESCENT PROTEIN REPORTER SYSTEM

THESIS

Sandra J. Dias, Captain, USAF

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DEPARTMENT OF THE AIR FORCE
AIR UNIVERSITY

AIR FORCE INSTITUTE OF TECHNOLOGY

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Sandra J. Dias, BS

Captain, USAF

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Captain, USAF

Approved:

Signed	18 March 2008
Charles A. Bleckmann (Chairman)	Date
Signed	18 March 2008
Nancy Kelley-Loughnane (Member)	Date
Signed	18 March 2008
Sonia E. Leach, Major, USAF (Member)	Date

Abstract

Chemical and biological threats are ever present and attacks have occurred throughout the world in both war and peace-time. Multiple government agencies, academia, and private industry are developing detection capabilities to address such threats. The research presented in this paper supports development of a modular synthetic biology-based system that detects and reports the presence of a threat agent. Synthetic biology builds upon past research in genetic engineering and seeks to combine broad applications within biotechnology in novel ways. This basic research project will help to demonstrate a proof-of-concept design which will guide future studies on the development of a modular sensor platform. In this study, *in vitro* and *in vivo* techniques were used to evaluate a reporter system composed of the Tobacco Etch Virus (TEV) protease and an engineered fluorescent fusion protein for their combined effectiveness as a reporter system. The TEV protease demonstrated the ability to cleave the fusion protein construct to produce a measureable output signal. The coupling of this reporter system with a riboswitch recognition element showed that this modular system can be applied to detecting chemicals. Further studies to modify this recognition element suggest modularity for future biosensors to detect chemicals of military interest.

Acknowledgments

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Sandra J. Dias

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CHARACTERIZATION OF TOBACCO ETCH VIRUS PROTEASE AND FLUORESCENT FUSION PROTEINS IN A REPORTER PATHWAY

I. Introduction

Overview

This thesis addresses a growing need for early detection and reporting of biological and chemical agents that adversely affect the warfighter. This chapter provides a context to familiarize the reader with current and relevant technical background and procedures. The chapter starts with the background of a threat to the warfighter, discussion of synthetic biology, and the top-level research design of this thesis. The remaining sections discuss the problem statement, research objective with investigative questions, thesis methodology, and assumptions and limitations.

Background

Chemical and biological threats are ever present and attacks have occurred throughout the world in both war and peace-time. Military and civilian populations are under an increased threat from biological and chemical agents from terrorists and extremists. In March 1995, the Aun Shinrikyo cult unsuccessfully attempted to deploy botulinum toxin in a Japanese subway station, but succeeded in a second attack at several of the Tokyo subway lines with sarin gas, resulting in 12 deaths and many more were wounded (Center for Disease Control, 1995). In 2001, anthrax spores were mailed to several Unites States (US) media centers and two US senators. These domestic terrorist

actions resulted in five deaths and many individuals infections (Federal Bureau of Investigation, 2001). In order to prevent or deter future events, adequate detection systems are necessary to identify the presence of biological and chemical threat agents.

Multiple government agencies, academia, and private industry are developing threat detection capabilities. According to the 2006 Quadrennial Defense Review (QDR) Report, "the Department [of Defense] has nearly doubled its investments in chemical and biological defenses since the 2001 QDR" (Department of Defense, 2006). The Defense Threat Reduction Agency (DTRA) contracted with the National Research Council to examine "detect to warn" biological sensors for facilities protection (National Research Council, 2004). Within the Department of Defense, the Office of Naval Research, Army Research Laboratories, and Air Force Research Laboratory (AFRL) have departments focusing on biological and chemical detection methods.

Research includes development of computer-based, mechanical, and biological systems. Dr. Stuart Rubin, from the Space and Naval Warfare Center (SPAWAR), has developed an anthrax sensor based on a computer program called Knowledge Amplification by Structured Expert Randomization. This sensor can be modified to detect improvised explosive device (IED)-associated chemicals. Drs. Pamela Boss and Richard Waters of SPAWAR are developing sensors that use micro-electrical mechanical system devices that have the ability to detect multiple chemical and biological agents (Ceruti, 2007).

In addition to examining biological sensors, DTRA is evaluating nanotechnology for applications in detection. Nano-materials are very small, ranging from 1 to 1000 billionths of a meter in size, and behave differently than materials at the macro level

(Peck, 2007). One advantage of nanotechnology is its small size: a higher quantity of engineered nano-detectors will fit on a small surface area. These detectors will be able to detect minute concentrations of the hazard agent, making them much more sensitive than many detectors available today. Research in nanotechnology is adding to both biological and chemical agent detection. Figure 1 provides a relative scale of nano and biological constructs.

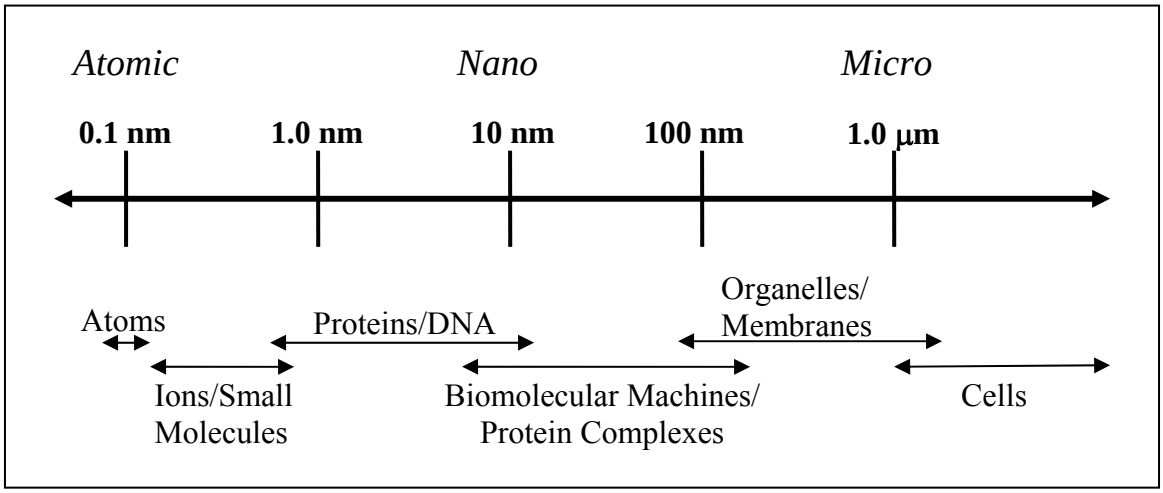


Figure : Relative Scale for Nano and Biological Constructs
(Adapted from Frasier, 2005)

Advancements in the relatively new field of synthetic biology include applications of nano- and biotechnology. The field has matured to the point where the power and flexibility of biology can be harnessed to develop novel threat detection solutions. As the focus of this thesis utilizes the discipline of synthetic biology, it is addressed in greater detail below.

Synthetic Biology

Synthetic biology refers to engineered biological systems, or systems manipulated by man in order to construct materials, produce energy, maintain or enhance human health, and process chemicals. It builds upon past research in genetic engineering and

seeks to combine broad applications within biotechnology, with an emphasis on developing technologies to improve the design and development of biological systems (Endy, 2005). It is a revolutionary science with great potential to impact society, medicine, and military technology.

Synthetic biology follows a strategy of constructing simplified systems in order to understand or manipulate cellular regulatory processes (Simpson, 2006). Typical cellular functions involve retrieving information from deoxyribonucleic acid (DNA), transcribing and translating the information from these genes to synthesize protein, regulating energy metabolism and repair, as well as cellular replication. One method of simplifying such functions is to use cell-free strategies that do not require the entire cellular system. For example, cell-free strategies that replace complex cellular processes with purified enzymes perform limited functions of natural cellular activities (Shimizu *et al.*, 2001). The use of these cell-free strategies minimizes unintended interactions by removing biochemical reactions which support synthetic gene pathways (Simpson, 2006).

Research Design

The Human Effectiveness (RH) directorate at AFRL is conducting related research within the Applied Biotechnology Branch. Within the branch, the Cellular Development and Engineering Program has been tasked to develop a modular synthetic biology-based system that detects and reports the presence of a threat agent. The research is categorized as basic research, resulting in proof-of-concept demonstration. This proof-of-concept research focuses on developing a system which demonstrates detection of a small molecule through the reporting activity within the synthetic system.

The synthetic system is composed of numerous biochemical reactions. An overview of the synthetic system evaluated in this paper is provided in Figure 2. The biochemical pathways are initiated with the binding of a small molecule to a catalytic ribonucleic acid molecule (RNA), called a ribozyme (Thodima *et al.*, 2006). The ribozyme is part of a larger RNA construct, called a riboswitch, which initiates translation of a specific genetic sequence on a plasmid (circular DNA molecule). The gene product in this research design is an enzyme which cleaves a fluorescent protein, resulting in an optical output. The enzyme and fluorescent protein constitute the reporter system. Initial research involved evaluation of the reporter system in cell-free environment using a buffer system and an *in vitro* translation/transcription system with purified enzymes. Additional studies were accomplished *in vivo* to optimize cellular machinery.

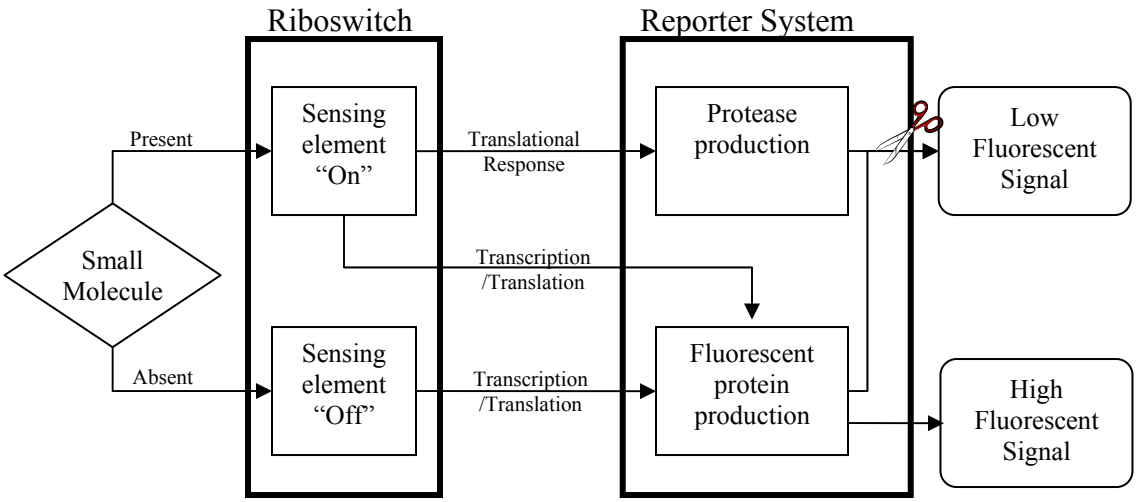


Figure : Research Design Flow Chart

indicates proteolytic cleavage

A future phase of the research plan identifies encapsulation of the synthetic system in order to stabilize and transport the system for use in the field. Many of these steps are being investigated in parallel, a factor that increases the design complexity if some of the biomolecules do not demonstrate the anticipated results.

Problem Statement

This thesis characterizes the reporting activity of an enzyme-substrate reaction in the synthetic system. The experimental design used Tobacco Etch Virus (TEV) protease (enzyme) because of its high specificity to cleave engineered fusion proteins (substrate) containing the TEV protease amino acid linker. Proteases are a class of enzymes that catalyze the cleavage of specific peptide bonds in proteins (Medintz *et al.*; 2006; Zhang, 2004). Fusion proteins are single protein molecules genetically engineered from two separate protein molecules and linked by a peptide sequence of amino acids. For this research, the fusion protein was engineered to include the specific peptide sequence recognized by the TEV protease. The engineered fusion proteins demonstrated different levels of fluorescence at specific wavelengths when whole and cleaved, enabling detection of TEV protease activity. Details regarding TEV protease and the fluorescent fusion protein are addressed in Chapter II: Literature Review.

The problem statement is best described as how to characterize the protease and fluorescent fusion protein interactions in the synthetic system to accurately report the presence of a molecule of interest. Characterization is a critical aspect of the research, because other steps in the research design might introduce factors that affect either the proteases activity or interfere with the fusion protein fluorescence properties. Additional

considerations, such as enzyme reaction time and enzyme/protein concentration, affect optimization. These factors are addressed in more detail in the following sections.

Research Objective/Questions

The objective of this thesis is to characterize TEV protease interactions with a fluorescent fusion protein in order to support the larger research design of a reporter system. To attain the objective, several investigative questions need to be answered:

1. *Does the TEV protease and fluorescent fusion protein interaction result in a robust output signal?*
2. *How will enzyme-substrate interaction behave in different systems?*
    a. *A simple Buffer System, in vitro transcription translation system, and in vivo system*
    b. *What is the time factor for the earliest distinguishable output signal?*
3. *Will introduction of a sensing element (riboswitch) negatively affect the downstream reporter system and output signal?*

Methodology and Data Analysis

This thesis research was based on laboratory analysis of TEV protease and fluorescent fusion proteins. Routine techniques followed published protocols and deviations were documented. Published research papers identified alternative techniques or protocols. Initial experiments were performed as a demonstration of activity, followed by verification that the correct activity occurred. Due to the nature of the multiple demonstration and verification cycles, data for statistical analysis was limited.

In vitro and *in vivo* experiments were conducted to evaluate the enzyme's ability to cleave fluorescent protein. Figure 3 represents the biochemical pathway and

associated analyses performed at each stage. Gene sequencing confirmed the source DNA. Spectroscopy and gel electrophoresis quantified messenger RNA (mRNA) purity and concentration. Complementary DNA (cDNA) was derived from mRNA and evaluated by real-time polymerase chain reaction (qPCR). Polyacrylamide gel electrophoresis and high performance liquid chromatography (HPLC) verified the reaction protein products. Fluorescent spectroscopy evaluated the cell constituents of the reaction products.

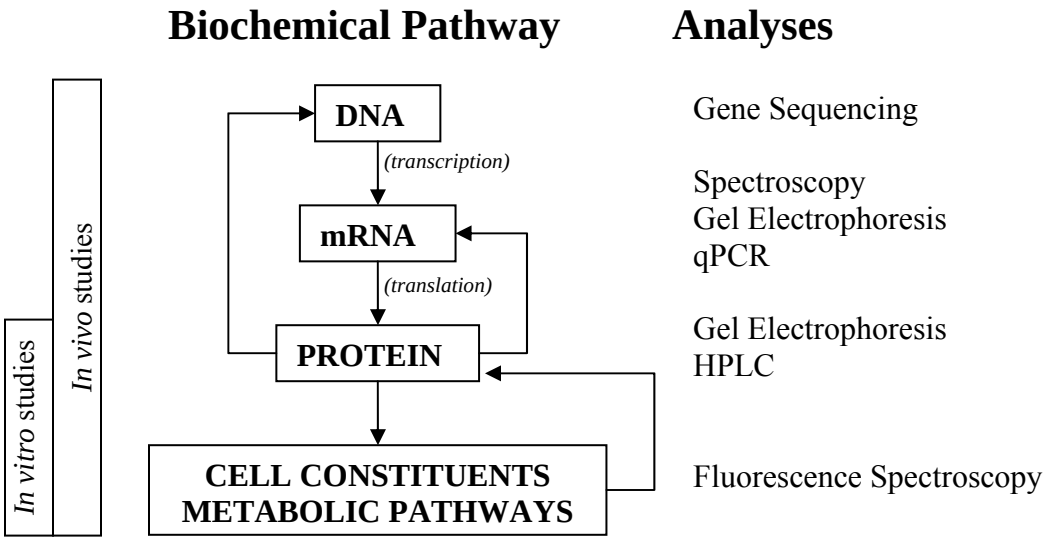


Figure : Biochemical Pathway and Analyses for Experimental Design

Additional analyses on the fluorescent spectroscopy data utilized the student's t-test and Analysis of Variation (ANOVA) to determine if results were statistically significant from the control and each other, respectively. Error bars using standard error of the mean helped gauge the quality of the data collected.

Assumptions and Limitations

Because the research is in its early stages, additional evaluation will be required to design its application for field use. Further studies will be required to refine the concept

for transportability, specificity for specific analytes, and reaction and reporting time. The proof-of-concept design would need to expand evaluation of multiple hazards to address detection of IEDs, biological agent, or chemical compounds.

One limitation of the thesis was the low number of replications for each experiment. *In vitro* experiments were used to provide insight for later *in vivo* analysis of the reporter system. *In vivo* experiments using bacterial cell cultures were repeated using different experimental conditions and required greater time and personnel commitments, so statistical analysis of the data was also limited by a small number of replications. Due to the time limit placed on AFRL/RH to demonstrate the proof-of-concept, concurrent research among multiple scientists was required to develop optimal protocols and investigate the research design under different conditions.

II. Literature Review

Chapter Overview

In an effort to develop a modular sensing platform that identifies molecules that could pose a threat to humans or the environment, scientists have applied synthetic biology techniques to develop sensing and reporter systems. These sensing systems are engineered to detect an analyte or small molecule agent which, through a series of gene expression and downstream interactions, results in a signal that reports the analyte presence (the reporter pathway). Detection might be expressed through optical, chemical, or electrical output from the system.

Programs at the Air Force Research Laboratory are evaluating these types of systems. The development of the sensor and reporter system requires numerous steps. This thesis evaluated the optical reporter system based on the interactions of the Tobacco Etch Virus (TEV) protease with an engineered fluorescent protein construct in both *in vitro* and *in vivo* environments. The *in vivo* evaluation included insertion of the sensor element, the riboswitch, upstream of the TEV protease gene to determine down-stream signal efficiency. In this chapter, previous research on riboswitches, TEV protease, fluorescent proteins, and general concepts of Fluorescence Resonance Energy Transfer (FRET) are addressed. In addition, a review is provided on the molecular biology techniques used throughout this evaluation.

Riboswitches

Riboswitches were used in the experimental design as a small molecule sensing mechanism that moderates the TEV protease expression at the translation level. This

section defines the riboswitch, describes a proposed mechanism of action, and addresses some limitations and benefits from riboswitch research performed to date.

Riboswitches are small segments of messenger RNA (mRNA) that function as genetic "on" or "off" switches in the presence of a small molecule ligand (Lynch *et al*, 2007; Desai & Gallivan, 2004). They are composed of two domains: an aptamer domain which recognizes a metabolite or small molecule ligand, and an expression platform which is a secondary structure formed upon ligand-binding that directly controls gene expression at the transcriptional or translational level (Stoddard & Batey, 2006; Lynch *et al*., 2007; and Buck *et al.*, 2007). The riboswitch consists of non-coding elements located in the 5' untranslated region upstream of regulated genes in prokaryotic and some eukaryotic mRNA (Buck *et al.*, 2007; Lynch *et al*., 2007; and Barrick *et al.,* 2004). Because of the potential applications for riboswitches as genetic screens for small molecules, much research has focused on development of synthetic riboswitches that can be adapted to selectively recognize small molecules and metabolites (Barrick *et al.,* 2004; Desai & Gallivan, 2004; Bayer & Smolke, 2005; Gilbert & Batey, 2005; and Lynch *et al*., 2007).

The riboswitch gene expression is thought to be modulated through allosteric conformational changes affected by ligand-binding to the aptamer domain (Buck *et al*., 2007; Sashital & Butcher, 2006; and Barrick *et al.,* 2004). The aptamer domains can adopt complex structures that precisely recognize the ligand with high binding specificity and a greater affinity than the non-ligand-bound state, resulting in a structural rearrangement in the mRNA tertiary structure (Barrick *et al.,* 2004; Sashital & Butcher, 2006; and Lynch *et al.*, 2007).

Lynch *et al.* (2007) proposed potential mechanisms of action of synthetic riboswitches with their research on the theophylline-sensitive riboswitch in *Escherichia coli*, illustrated in Figure 4. In the absence of the theophylline ligand, hydrogen bonding and electric static forces pair the aptamer sequence (ligand binding site) with the ribosome binding site (RBS). The structure bonds the Start AUG codon to complementary nucleic acids and prevents initiation of mRNA translation. Addition of theophylline causes a conformational change in the secondary structure as the aptamer binding site forms a pocket for the ligand and complementary base-pairing shifts, thus liberating the RBS and Start codon sites. Because the 30S subunit of the ribosome binds most efficiently to the single-stranded regions of RNA, translation of the downstream gene can be initiated. The theophylline-bound state was determined to be thermodynamically favorable over the ligand-free state through free energy calculations.

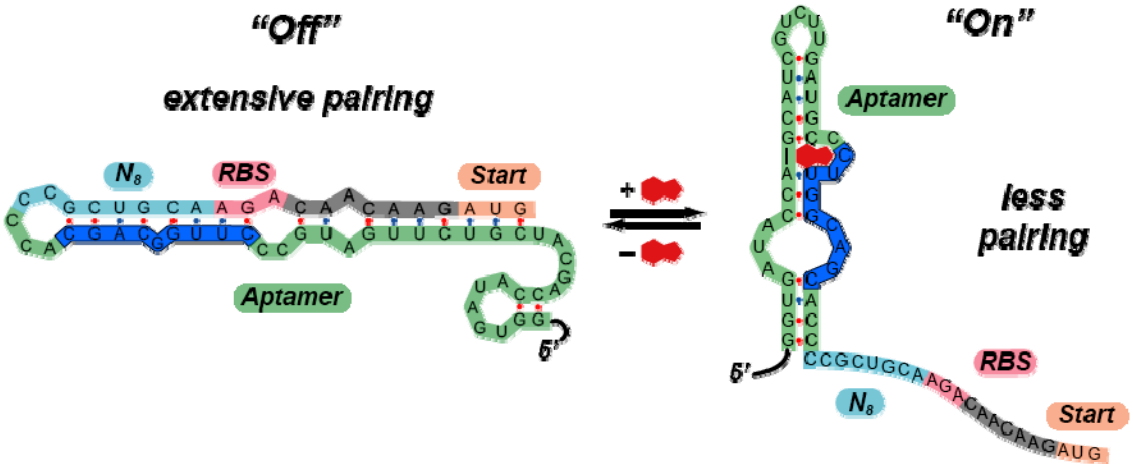


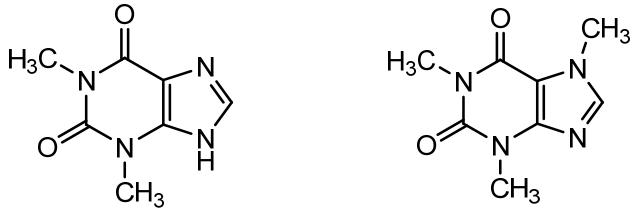
Figure : Predicted Mechanisms of Action of Synthetic Riboswitches
(Lynch *et al.*, 2007)

In 2007, Lynch *et al.* reported that the theophylline-sensitive riboswitch did not completely repress protein translation in the absence of theophylline, resulting in

background noise. They postulated that, in the absence of the ligand, the RBS is paired such that transcription is minimized. Their research reported that the most effective riboswitch showed a signal to background ratio of approximately 8, and that an increase in that ratio is desirable for more demanding screening applications. Buck *et al.* (2007) also reported low levels of protein expression in the ligand-free state, suggesting that the base-pairing interactions "are not sufficient to completely lock the positions of helices in a defined orientation," whereas the ligand-bound state tightens the complex and decreases fluctuations.

Even though the structural conformity of synthetic riboswitches is not static in the ligand-bound and unbound states, riboswitches offer flexible control strategies as small molecule sensors due to their design variability as genetic regulators. A range of riboswitch designs have been cataloged over the last few years. Research performed by Desai & Gallivan (2004), Lynch *et a.l* (2007), and Buck *et al.* (2007) report on synthetic riboswitches in which the bound ligand turns "on" gene expression, while research by Sashital & Butcher (2006) and Barrick *et al.* (2004) describe riboswitch designs in which the bound ligand turns "off" gene expression.

These studies indicate small molecule specificity to the aptamer section of the riboswitch, further enhancing their significance as discriminating sensors. Topp & Gallivan (2007) report that caffeine, a small molecule differing from theophylline only by a methyl group at the N7 position, does not bind to the riboswitch (refer to Figure 5).



Theophylline Caffeine

Figure : Theophylline and Caffeine Molecules

Anderson & Mecozzi (2005) demonstrated through computational modeling and experimental observations that an RNA sequence as short as 13-nucleotides can selectively bind to theophylline. According to Lynch *et al.* (2007), "synthetic riboswitches can be engineered to respond to any non-toxic cell-permeable molecule that is capable of interacting with RNA."

Tobacco Etch Virus Protease

The Tobacco Etch Virus (TEV) protease was chosen for use in the experimental design of this thesis for its well-characterized specificity and its ability to operate in a wide range of conditions. This section describes the physical nature of the TEV protease, the properties that make it desirable for cleaving fusion proteins, and the research accomplished to improve limitations of the TEV protease found in nature.

TEV protease is an efficient, highly specific enzyme, categorized as a nuclear inclusion a (NIa) protease, with a unit mass of 27 kilo Daltons (kDa) (Fang *et al.*, 2007; Nunn *et al.*, 2005). The NIa protease recognizes a standard amino acid sequence recognition site: Glu-X-X-Tyr-X-Gln|Gly, where X represents any residue and Gln|Gly represents the protein cleavage site (Fang *et al.*, 2007; Nayak *et al.*, 2003). Parks *et al.* (1994) indicate that cleavage is also successful with a Gln|Ser mutation. Because the TEV protease recognition sequence can be cloned between fusion proteins, its seven

amino acid standard recognition site makes it valuable when cleaving genetically engineered fusion proteins (Kapust *et al.*, 2001). The short, highly specific cleavage site can be inserted between proteins not found in nature. TEV protease is active over a temperature range from 4^{o}C to 37^{o}C and a pH range from pH 6.0 to pH 8.5 (Nayak *et al.*, 2003; Fang *et al.*, 2007).

For purification and identification, a 6x polyhistidine (His)-tag can be attached to the carbon (C) or nitrogen (N)-terminus of the protease (Nayak *et al.*, 2003). Purification is normally required after *in vivo* protein expression. Cells are lysed (broken apart) to separate the cellular components from the cytoplasm. Cell lysates contains many types of proteins, the His-tag enables single step purification through Nickel (Ni^{2+}) affinity chromatography (Fang *et al.*, 2007).

Using Ni-nitrolacetic acid (NTA) beads, the cell lysate equilibrates with the column beads, allowing the protein with the His-tag to bind to the Ni-NTA column. The column is then washed with buffer containing low concentrations of phosphate and imidazole to elute out other cell lysate proteins that have low affinities for the Ni^{2+} in the column. Imidizole will affect the pH of the buffer, and also has a higher affinity for nickel. Addition of an imidazole and high salt solution results in target protein elution from the Ni-NTA column (Wallert & Provost Lab, 2004; QIAGEN, 2008). The His-tag provides a means to identify the protein in a complex mixture using immunoblotting with an antibody to the His-tag (Western Blot). Figure 6 depicts one variation of TEV protease, mutant S219D, with its catalytic domain and His-tag.

Figure : Ribbon Model of the TEV(S219D) Protease Structure
The residues that compose the catalytic triad and the N-terminal His tag are depicted as ball-and-stick models (carbon, *violet*; nitrogen, *blue*; oxygen, *red*; and sulfur, *yellow*). The peptide product is also colored *blue* to distinguish it from the protein (Phan *et al.*, 2002).

Fang *et al.*, 2007, reported an improved strategy for high level yield of soluble TEV protease in *E. coli* through the use of molecular chaperones and lower culturing temperatures. The molecular chaperones *E. coli* GroES/EL and trigger factor promoted correct protein folding and inhibited incorrect hydrophobic interactions of peptides during translation. The lower temperature appeared to reduce folding errors by slowing the translation speed. Both factors were shown to contribute to a high yield of soluble TEV protease.

The wild-type TEV protease is limited by its low stability which results in auto-digestion. It cleaves itself at a specific site resulting in a truncated protease which exhibits reduced activity, reduced enzyme purification product, and longer processing time to separate the truncated and full-length protease (Kapust *et al.*, 2001). Kapust *et al.* (2001) identified that S219V mutant was impervious to autolysis while maintaining its

catalytic activity, whereas Lucast *et al.* (2001) identified that the S219N mutant inhibited auto-proteolysis and subsequent inactivation.

Another limitation of the wild-type TEV protease, when expressed in *Escherichia coli*, (*E. coli)* is its low solubility. Van den Berg *et al.* (2006) performed directed evolution studies and identified the S219N mutant of TEV protease as demonstrating increased solubility. After analysis of several mutants through purification and activity testing, one mutant, designated as TEV_{SH}, was selected as the top performer due to its five-fold increase in yield after purification. The substitutions at T17S, N68D, and I77V on TEV_{SH} improved solubility and retained proteolytic activity.

In 2007, Fang *et al.* noted that the cleavage efficiency of TEV protease was more dependent upon the substrate than the enzyme itself. The protease efficiency was affected by the substrate's conformal structure and accessibility to the cleavage site; however, comparative studies on TEV protease efficiency with different types of substrates are still being evaluated (Kapust, 2001). Further analysis may be required to identify an enzyme-substrate combination that maximizes cleavage efficiency when designing an optimal reporting system.

For this reporter system design, both an enzyme and substrate are required. The TEV_{SH} protease variant was selected for this research because it demonstrated stability, solubility, and high yield in *E. coli*. The selected substrate must incorporate the TEV protease recognition sequence and display good optical characteristics, which are discussed in the next section.

Fluorescent Proteins, FRET, and FRET Pairs

To be effective as a reporter system, the activity of the optical assay must discretely measurable. The optical signal verifies the TEV protease activity as well as activation of the riboswitch. This section describes the fluorescent protein engineered with the TEV protease linker, the fluorescence resonance energy transfer (FRET) phenomenon that enhances its optical significance and an analysis of different FRET pairs.

Fluorescent Proteins

Green fluorescent protein (GFP) is a fluorescing molecule, called a fluorophore, widely used in synthetic biology for their chromophoric (color-changing) properties. GFP has become well established as a marker in gene expression and protein targeting for *in vitro* and *in vivo* studies (Tsien, 1998). Low amounts of soluble GFP can be detected in a reaction mixture and fluorescence intensity correlates with concentration of soluble protein (van den Berg *et al*., 2006). GFP has five classes of mutants that have been studied in cellular biology: enhanced GFP (eGFP); blue fluorescent protein (BFP), cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and sapphire fluorescent protein (Pollok & Heim, 1999). The fluorophores are excited, or absorb energy, at one wavelength and emit energy, fluorescence or brightness, at a different wavelength (Pollok & Heim, 1999). These values are necessary when evaluating their presence in a biological system. Figure 7 illustrates the different excitation and emission spectra for different GFP mutants.

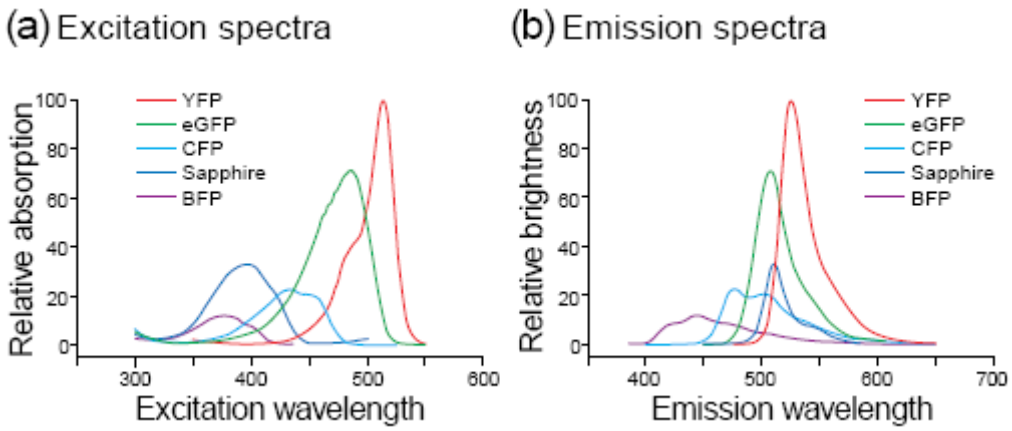


Figure : Relative Excitation and Emission Spectra for GFP Mutant Classes (Pollok & Heim, 1999).

FRET

Fluorescence Resonance Energy Transfer (FRET) has been used in cell biology to analyze gene expression, monitor protein folding, and design fluorescence sensor molecules (Pollok & Heim, 1999; Mitra *et al.*, 1996; and Kikuchi *et al.*, 2004). Some literature has also referred to FRET as Förster resonance energy transfer, due to the Förster equation which describes the rate at which energy is transferred from donor to acceptor (Mitra *et al.*, 1996).

FRET is a quantum-mechanical phenomenon demonstrated in this study by the fluorescent fusion protein. The event occurs when two fluorophores are excited and the donor fluorophore transfers energy to an acceptor fluorophore (Mitra *et al.*, 1996; Pollok & Heim, 1999; and Zhang, 2004). These fluorophores must be in close proximity (10-100 Å) and the emission spectra of the donor must overlap with the excitation of the acceptor fluorophore (Mitra *et al.*, 1996; Eggeling *et al.*, 2005; and Zhang *et al.*, 2002). Because energy is transferred without emission of a photon, FRET is considered a non-radiative process (Kikuchi *et al.*, 2004), as illustrated in Figure 8 .

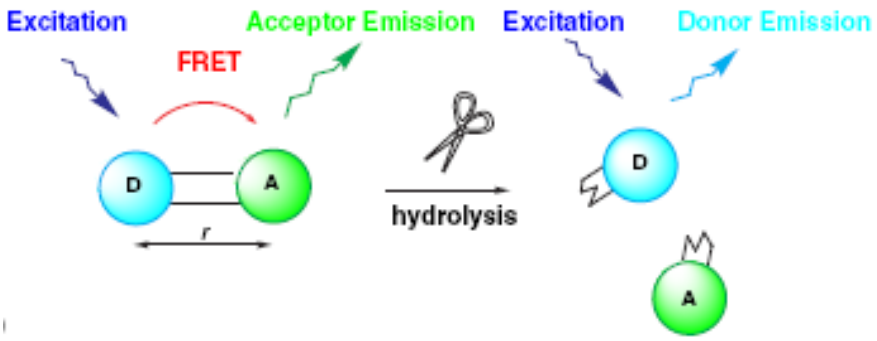


Figure : Detection Principle of FRET Sensor
D represents donor; a represents acceptor; cleavage of bond represented by hydrolysis or enzyme (Kikuchi *et al.*, 2004)

One benefit of FRET is that it enables a ratiometric measurement in which a ratio calculation is obtained from simultaneous recording of the fluorescent intensities at two wavelengths (Kikuchi *et al.*, 2004). This technique provides greater precision and is preferable to a single wavelength measurement or fluorescent intensity measurement (Kikuchi *et al.*, 2004; Zhang *et al.*, 2002).

FRET Pairs

FRET pairs are used to enhance detection and monitoring of target proteins, protease activity, and effects of protease inhibitors in real-time and in living cells (Mitra *et al.*, 1996; Zhang, 2004). Several types of FRET pairs exist, such as pairs with protein-protein interactions, a conformational change region, intra-molecular interactions in the linker region, and fusion proteins (Zhang *et al.*, 2002). FRET-based fusion proteins are the result of recombinant DNA in which two DNA fragments that code for different proteins are ligated together to code for a single protein (Sindelar & Crommelin (ed), 2002). In experimental studies supporting this thesis, a FRET-based fusion protein was constructed as the substrate to monitor TEV protease activity.

The FRET fusion protein is linked by an amino acid sequence that can be recognized and cleaved by an enzyme of interest. Some common FRET pair combinations are BFP-eGFP and CFP-YFP (Pollok & Heim, 1999). In a FRET pair naming convention, the first fluorophore is the donor and the second fluorophore is the acceptor. If the fusion protein has been engineered with a peptide linker for enzyme cleavage, the enzyme name is inserted between the proteins. For example, BFP_{TEV}EGFPHis indicates the BFP-EGFP fusion protein is cleaved by a TEV enzyme. The His-tag at the end of the fusion protein is used during purification as well as a tool for identification.

The FRET pair selection is dependent upon several factors: overlapping excitation and emission spectra, wavelength distance between emission spectra to be able to distinguish between fluorophores, orientation, and physical distance between the fluorophores (Eggeling *et al.*, 2005). In addition to selection of appropriate fluorophores, the length of the polypeptide linker should be optimized to ensure the spatial distance required for FRET is not compromised (Zhang *et al.*, 2002; Zhang, 2004). The amplitude of the *in vitro* FRET signal may be affected by factors such as salt concentration, buffer composition, reducing agents, and mineral concentration (Dong *et al.*, 2004).

The first demonstration of FRET between two GFP derivatives was accomplished by Mitra *et al.* in 1996, using BFP-red shifted GFP. Zhang *et al.* (2002) reference other applications that have used BFP-GFP; however, results indicated dimness and BFP experienced photo-bleaching – an irreversible destruction of a fluorophores being illuminated (Zhang *et al.*, 2002). Research by Zhang in 2004 reported that cleavage of the CFP-YFP fusion protein by thrombin demonstrated an increased emission ratio and

concluded that the pair was a better candidate for design of FRET-based GFP probes than BFP and GFP fluorophores. Though Zhang's experimental design tested the FRET probe by thrombin cleavage, the results indicate the CFP-YFP pair is worth investigating in the experimental design.

This section addressed the use of fluorescent molecules as a fusion protein. The construct and cleavage of the fusion protein demonstrate FRET, an optical property which can be measured to determine enzyme activity.

Techniques

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels are formed from the polymerization of acrylamide, creating a matrix which aids in separating proteins based on size. The size of the protein(s) to be separated dictates the percent acrylamide in the gel to be used. The smaller the protein, the larger the percent acrylamide in the gel required. For example, 10% acrylamide gels are recommended for proteins ranging from 21 to 100 kDa in size, whereas 12% acrylamide gels are recommended for proteins ranging from 10 to 40 kDa. Electrophoresis is the movement of charge molecules in an electric field. Either non-denaturing or denaturing polyacrylamide gel electrophoresis can be used to extract information about the protein of interest.

Non-denaturing gels, also called native gels, are used to determine whole protein size and charge. Most proteins have an inherent electrical charge. Electrophoresis provides the electrical gradient which transports charged protein through the gel while the acrylamide provides the molecular sieving. Larger proteins migrate more slowly and are observed closer to gel wells (Boyer, 1993).

Denaturing gels, also known as SDS-PAGE, are used to determine the molecular mass of proteins. Proteins samples are treated so they have a uniform charge by using a detergent, sodium dodecyl sulfate (SDS), and 2-mercaptoethanol (β-ME), a disulfide reducing agent. These SDS denatures the proteins secondary, tertiary, and quarternary structure to produce a linear polypeptide chain coated with negatively charged SDS molecules. The β-ME assists in protein denaturation by reducing disulfide bonds. The bound SDS molecules mask the native charge of the protein, allowing the protein to move though the polyacrylamide gel based soley on size when electrophotesed. Like non-denaturing gels, larger molecules migrate slower and are found near the top of the gel (Boyer, 1993).

Agarose Gel Electrophoresis

Agarose gels are used primarily for analyzing DNA or RNA because of the pore size of the agarose medium allows movement of lager nucleic acid fragments or intact DNA molecules (up to 50 kilobases (kb) or 1850 kDa in terms of protein). DNA is measured by kb, whereas proteins are measured in terms of kDa, with a conversion of 1 kb = 37 kDa. Agarose is a product extracted from seaweed and sold in powder form. The powder is dissolved in warm eletrophoretic buffer, and prior to cooling, ethidium bromide is added. The ethidium bromide intercolates between nucleic acid bases and the RNA or DNA fluoresces when illuminated with ultraviolet light. Similar to polyacrylamide gels, the molecular size and conformation of the nucleic acid should determine the percent agarose in the gel. Smaller, compact and supercoiled molecules move faster when an electrical current is applied.

Polymerase Chain Reaction (PCR)

In this experimental design, polymerase chain reaction (PCR) was used as a method to assist in plasmid construction as well as analysis of the experimental products. PCR is a technique that amplifies a small amount of DNA without the use of cloning techniques. Developed by Kary Mullis in the 1980's, PCR has been widely used in molecular biology because it requires only a small sample of DNA, it amplifies a specific sequence of interest, and it helps to facilitate site specific mutagenesis using primers to amplify a changed sequence. It has been found useful for DNA diagnostics to check for the presence of a gene, check mutation states of genes, and amplify a specific DNA sequence in a population of mixed DNA (Moran *et al.,* 1994; Griffiths *et al.,* 1999). PCR can be used to verify the previous experimental results by blotting or sequencing the PCR products, or can be used for additional experimentation by cloning the PCR product into other DNA sequences.

With PCR, sequenced information from double stranded DNA is used to design primers that flank the region to be amplified. These primers are complementary to the opposite strand with their 3' ends oriented toward each other. The reaction mixture contains the source DNA, excess primers, DNA polymerase, oligonucleotides, and a buffer solution.

Polymerase chain reaction is broken down into three steps per cycle: denaturation, annealing, and elongation. The time and temperature in each step varies, and is primarily dependent on the melting point of the DNA primer. A temperature and time range is described below for each step; however, protocols will specify optimal conditions that should be followed. The source DNA is denatured by heating the reaction mixture at 94-

98°C for 10 to 30 seconds, disrupting hydrogen bonds between bases to yield single stranded DNA. Upon cooling to 50 to 65°C for 20 to 60 seconds, the primers preferentially anneal to their complementary sites, which border the DNA sequence of interest. Elongation proceeds when a temperature-resistant DNA polymerase, commonly Taq polymerase, catalyzes growth in the 5' to 3' direction from the DNA primers using available oligonucleotides. Elongation process occurs at 72-80°C and lasts for one minute. At the end of the first cycle, the quantity of initial double-stranded DNA has doubled. After the second cycle, the DNA has again doubled. Each cycle result in an exponential increase in the number of DNA segments replicated. These cycles are repeated from 20 to 35 times, depending on the application, until the desired DNA constitutes virtually all the DNA (Moran *et al.,* 1994; Griffiths *et al.,* 1999; McPherson & Moller, 2006).

Many variations of the basic PCR procedures have been developed to aid in biomolecular analysis. Some examples include reverse transcription PRC (RT-PCR) which amplifies RNA, and real-time PCR (qPCR), which quantifies DNA or RNA by measuring the accumulation of DNA product after each round of PCR amplification.

Plasmid Construction

In order to express TEV protease or BFP_{TEV}eGFP protein in a cellular environment, recombinant DNA techniques were used. These techniques insert a specific DNA code into a plasmid vector which will express the protein when transcription and translation are initiated. This section provides a generic description of a plasmid and recombinant DNA techniques used to clone foreign DNA into a plasmid.

A plasmid is an independently replicating circular double stranded DNA molecule (Moran & Scrimgeour, 1994). It carries genes for the inactivation of antibiotics in addition to hosting foreign DNA strands (Figure 9). The origin of replication refers to the site where the entire plasmid DNA is replicated during DNA synthesis. Alberts *et al*. (1994) provided the best definition of a promoter: "the specific DNA sequence that directs RNA polymerase to bind to DNA, to open the DNA double helix, and to begin synthesizing an RNA molecule." Within the promoter lies a short region of regulatory DNA called an operator that, when occupied by a repressor protein, blocks RNA polymerase access to the promoter, thus preventing expression of the downstream gene (Alberts *et al*., 1994). The presence of an inducing ligand either prevents occupancy of the operator or binds directly to the repressor protein, enabling RNA polymerase to bind to the promoter. These inducing ligands are often referred to as inducers.

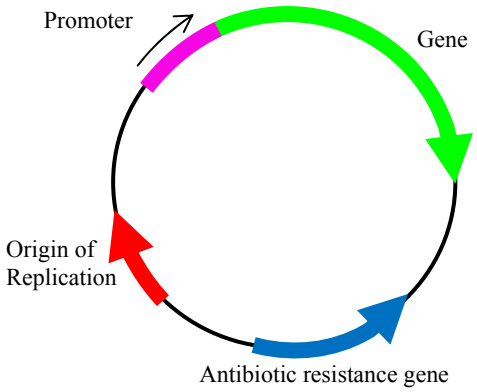


Figure : Plasmid Map

Constructing the recombinant DNA molecule requires a plasmid vector and foreign DNA, such as TEV protease or $BFP_{TEV}eGFP$ protein DNA. The specific plasmid vector is chosen based on experimental design factors and the host cell line to ensure protein synthesis is optimized. Factors to consider when selecting a plasmid include

insert size, incompatibility with host cell machinery, selectable gene markers, cloning sites, promoter sites, and specialized vector functions. Most plasmids contain a gene for antibiotic resistance that is used as a selection tool later in the cloning process (Stryer *et al.,* 1988). The foreign gene can be a PCR product or cut from whole DNA using restriction enzymes, which cleave DNA at specific amino acid sequences. The same restriction enzymes are used to cut both the foreign DNA and plasmid, resulting in an open plasmid vector and foreign DNA molecule with overhanging complementary ends, called "sticky ends". If a PCR product is used, the primers must be designed to ensure the foreign DNA insert contains the appropriate complementary sticky ends. The sticky ends of both the foreign DNA fragment and plasmid are connected through use of DNA ligase. The result is a loop of DNA containing the foreign gene of interest. At this point, the plasmid can be coupled with an *in vitro* transcription/translation (IVTT) system to express the gene product, or further processed for *in vivo* analysis. Figure 10 illustrates the techniques used for plasmid construction and additional manipulations for IVTT and *in vitro* analysis.

In Vitro Transcription/Translation Systems

The *in vitro* transcription/translation (IVTT) system, also referred to as a cell-free protein biosynthesis system, generates proteins by using the transcription and translation components of cell-free extracts of prokaryotic or eukaryotic cells. The IVTT system provides an alternative to *in vivo* protein synthesis which requires additional steps to transform a plasmid into the living cells and grow and culture the transformed cells.

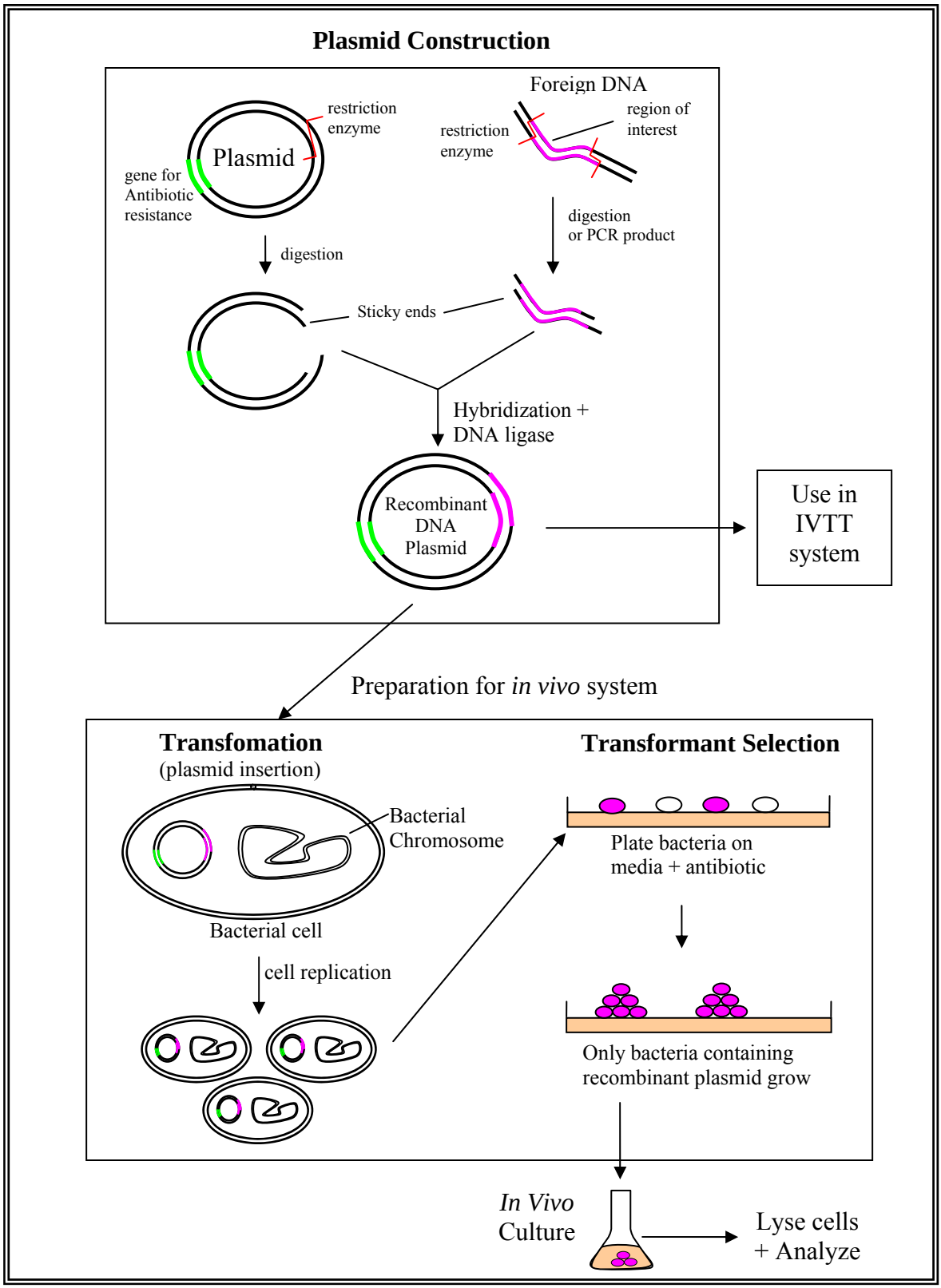


Figure : Plasmid cloning techniques for IVTT and *in vivo* systems

In addition, *in vivo* studies require steps to break the cells and purify the proteins from the whole-cell lysates, which can introduce analysis challenges due to the aggregation or degradation of proteins within the cell. The IVTT system provides time savings, flexibility to adapt the reaction conditions to the synthesized protein requirements, and high protein yield per unit volume (QIAGEN (1), 2005).

In general, the IVTT system is comprised of purified enzymes from a cell's transcription and translation system. These enzymes may be extracted and purified in house or purchased commercially. Each manufacturer producing an IVTT system maintains proprietary rights on the ingredients (including proprietary concentrations and purities) in its cell-free extract products. For example, QIAGEN uses highly productive *E. coli* cell lysates, which contain all translational machinery components such as ribosomes, ribosomal factors, transfer-ribonucleic acid (tRNA), aminoacyl-tRNA, T7 RNA polymerase, and more (QIAGEN (1), 2005). Invitrogen manufactures an IVTT product that that uses optimized *E. coli* extract containing an ATP-regenerating system, amino acids, and T7 RNA polymerase as well as a supplemental Feed Buffer containing a mixture of salts, amino acids, and other substrates that are depleted or degraded during protein synthesis (Invitrogen, 2006). Shimizu *et al.* (2001) describe a protein synthesis using recombinant elements (PURE) system that contains all the necessary translation factors, purified with high specific activity that allows efficient protein production. The choice of IVTT system should be considered during the experimental design, for each manufacturer recommends a specific plasmid series that will be optimized in its IVTT system.

In Vivo Systems using Bacterial Cells

There are three main steps to cloning foreign DNA into a plasmid for *in vivo* analysis: constructing the recombinant DNA molecule (plasmid), introducing the molecule into the host cell (called transformation), and selecting cells that contain the recombinant DNA molecule (Stryer, 1988).

Transformation of the plasmid into the host cell is the process of inserting the plasmid DNA molecule into the host cell. The two components are combined in a reaction tube and heat shocked. As the pores of the host cell expand, the circular plasmid enters into the cell. After the short heat shock treatment, a nutrient-rich medium is added the cooled reaction tube. The bacteria are incubated until cell replication is sufficient for evaluation of the transformation process.

In order to select the cells that contain the plasmid, the cells are evenly spread onto an agar plate containing nutrients and an antibiotic which matches the antibiotic resistant gene on the plasmid. The agar plate is incubated overnight to ensure sufficient cell growth. Excess plasmid from the reaction mixture will not replicate on the agar, and cells that do not contain the plasmid will not grow in the presence of the antibiotic. The resulting cell growth will be colonies from a single transformed bacterial cell containing the inserted plasmid. Verification of the transformation should be performed using PCR with the primers for the foreign gene. The transformed cells can be cultured for *in vivo* experiments or frozen on a bead media for future *in vivo* experimental use.

In this experimental design, the TEV protease was cloned into the pET28a plasmid and the FRET protein was cloned into the pHWG640 plasmid. Both plasmids contain promoters which utilize the T7 RNA polymerase for increased protein

expression. The promoters for each plasmid house different operators and require separate inducing agents, which enables control of specific protein expression when both plasmids have been transformed into the same cell.

All plasmids were transformed into *E. coli* host cells, strain BL21 (DE3). *E. coli* host cells were chosen because they grow quickly, transform efficiently, and is a useful host for the biocatalytic production of small molecules. The BL21 (DE3) strain was chosen for its ease of transformation with plasmid DNA and its high efficiency to express proteins from T7-based plasmids, such as the pET vector series. BL21 (DE3) has been engineered to express T7 RNA polymerase which synthesizes mRNA more rapidly than *E. coli* RNA polymerases (Invitrogen, 2002).

Chapter Summary

This chapter discussed the primary components of the research designs' sensing and reporter system. The experimental design included a riboswitch to initiate translation of TEV protease which catalyzes proteolytic cleavage of a fluorescent fusion protein engineered with the TEV protease cleavage site. The use of fluorophores for the fusion protein confers a FRET signal which can be measured, thereby quantifying TEV protease activity. The optical output is used to demonstrate the presence of the analyte of interest. Techniques, such as electrophoresis, PCR, plasmid construction, IVTT, and *in vitro* systems were discussed to enhance the reader's understanding of the experimental design.

III. Materials and Methods

Materials and methods cover performed procedures and protocols to support the research investigative questions. This chapter addresses general considerations, *in vitro,* and *in vivo* analyses of tobacco etch virus (TEV) protease. Throughout this chapter, the BFP_{TEV}eGFP-His protein will be referred as the fluorescent resonance energy transfer (FRET) protein.

General Considerations

Plasmid Construction

Materials

The pET28a plasmid was procured from Novagen. The TEV protease gene construct was generously donated by Dr. Helena Berglund, from the Department of Medical Biochemistry and Biophysics at the Karolinska Institute in Sweden (van den Berg *et al.*, 2006). Dr. Josef Altenbuchner, from the Institute of Industrial Genetics at the University of Stuttgart, contributed the pHWG640 plasmid (Gröger *et al.,* 2005). The FRET gene was engineered in the laboratory by Dr. Svetlana Harbaugh through standard molecular cloning techniques.

Methods

All plasmid manipulations were performed according to standard cloning techniques. Two different plasmid constructs containing the TEV protease gene were generated by cloning into the pET28a plasmid vector. One plasmid construct harbored the TEV protease gene, denoted as TEV plasmid. The other construct contained a riboswitch upstream of the TEV protease coding sequence, denoted as RS-TEV plasmid.

The plasmid vector pET28a contained a selection marker for kanamycin antibiotic resistance and a T7 promoter requiring isopropyl-beta-D-thiogalactopyranoside (IPTG) for induction of transcription and translation (Novagen, 2006). A third plasmid construct containing the FRET protein gene was generated by cloning into plasmid vector pHWG640, which contained a selection marker for chloroamphenicol resistance and a rhamnose promoter. Separate plasmids were chosen for the TEV protease and FRET protein to tailor co-expression during *in* vivo studies. Table 1 provides a summary of all molecular clones used in the experiments.

Table : Summary of Molecular Clones

Plasmid	Genetic Element	Activating Agent	Inducing Agent	Product
pET28a	TEV protease DNA		IPTG	TEV protease
pET28a	Riboswitch mRNA TEV protease DNA	Theophylline	 IPTG	TEV protease
pHWG640	BFPTEVeGFP DNA		Rhamnose	BFPTEVeGFP-His* (FRET protein)

* The His-tag is used for purification

All plasmids were transformed into the BL21 (DE3) strain of *Escherichia coli (E. coli).* Sequences of all constructs were verified by DNA sequencing at the Plant Microbe Genomics Facility, Ohio State University, Columbus OH.

Transformation

Materials

Invitrogen supplied BL21 (DE3) *E. coli* cells and Super Optimal Catabolite (SOC) medium. Kanamycin and chloroamphenicol were purchased from Sigma. Luria-

Bertani (LB) agar media was purchased from Difco. ProLabs Diagnostics supplied Microrank® beads.

Methods

Transformation procedures followed Invitrogen One Shot® BL21 (DE3) protocols. One vial of One Shot® was used per transformation. Each vial was thawed on ice before addition of 10 nanograms of plasmid, in a volume of 1 to 5 microliters (μl). Vial(s) were incubated on ice for 30 minutes, transferred to a water bath (42^{o}C) for 30 seconds, and then placed on ice. Pre-warmed SOC medium (250 μl) was added to each vial. SOC medium consisted of 0.5% yeast extract, 2.0% tryptone, 10 millimolar (mM) sodium chloride (NaCl), 2.5 mM potassium chloride (KCl), 10 mM magnesium chloride ($MgCl_2$), 20 mM magnesium sulfate ($MgSO_4$), and 20 mM glucose. SOC medium was used as a cell growth medium used to ensure maximum transformation efficiency. Vials were placed on their side in an incubator and shaken at 37^{o}C for one hour at 225 revolutions per minute (rpm). A volume of 20 to 100 μl of the transformation reaction was distributed on LB agar plates containing 25 μg/ml of the appropriate antibiotic(s) to inhibit growth of non-transformed bacteria. For transformations with TEV protease and FRET plasmids, 25 μg/ml of both kanamycin and chloroamphenicol were added to the media. For transformants with the TEV protease plasmid, 25 μg/ml of kanamycin was added. For each transformation, two plates were prepared to ensure appropriate growth and viability.

To maintain colony integrity for future experiments, 0.8 ml of inoculate was aliquoted into a Microrank® Bead vial, and then removed with a sterile pipette tip. The

vial of Microrank® Beads was placed on dry ice, and then stored at -80°C until future use.

Plates were inverted and incubated overnight at 37°C. The number of colonies per plate was counted and the transformation efficiency calculated using the following formula:

$$\left(\frac{\#\,\text{colonies}}{10\ \text{pg transformed DNA}}\right)\times\left(\frac{10^6\ \text{pg}}{\mu\text{g}}\right)\times\left(\frac{300\ \mu\text{l transformed cells}}{\text{X}\ \mu\text{l plated cells}}\right)=\left(\frac{\#\,\text{transformants}}{\mu\text{g plasmid DNA}}\right)$$

Plates with a transformation efficiency of 1×10^8 tranformants per µg plasmid DNA or greater were used. This number indicated the level of viable cells necessary for successful growth in cell culture experiments.

Protein Analysis by Polyacrylamide Gel Electrophoresis

Materials

The following materials were procured from Bio-Rad: trishydroxymethylaminomethane-hydrochloric acid (Tris-HCl) resolving gels (10% and 12%), Tris/Glycine solution (10X), Tris/Glycine sodium dodecyl sulfate (SDS) solution (10X), Native sample buffer, Laemmli SDS reducing buffer, 2-mercaptoethanol (β-ME), Coomassie Blue Stain and Destain solutions, pre-stained molecular weight marker, and pre-stained SDS-polyacrylamide gel electrophoresis (PAGE) standard. Bio Labs supplied a broad range protein marker and 2-log DNA Ladder. Tris-Borate-Ethylene diamine tetraacetic acid (EDTA) (TBE) buffer solution (10X), and Tris-Acetate-EDTA (TAE) solution (50X) were obtained from Fisher Scientific.

Non-denaturing Conditions

Protocols and reagent preparation for electrophoresis were adapted from Mini-PROTEAN® 3 Cell Instruction Manual (Bio-Rad Laboratories, 2001). The Tris-HCl Resolving Gel (10% or 12%) was placed in a 1X Tris/Glycine native running buffer. Samples to be analyzed were mixed with Native sample buffer at a 1:1 ratio prior to placement in wells. Electrophoresis run time was 150 volts for 60 minutes using a Fisher Scientific FB300 electrophoresis power supply. The fluorescent proteins were visualized using a Spectraline TP-2100E UV transilluminator using excitation wavelength of 365 nanometers (nm), or the Clare Chemicals Research DR-88M Dark Reader™, emission range from 400 to 500 nm. Fuji FinePix S20 pro digital camera was used to photograph the gels.

Denaturing Conditions

Protocols and reagent preparation for electrophoresis were adapted from Mini-PROTEAN® 3 Cell Instruction Manual (Bio-Rad Laboratories, 2001). The Tris-HCl resolving gel (12%) was placed in electrode running buffer, (1X, pH 8.3). Samples (10 μl) were mixed with Laemmli SDS reducing buffer (5% β–ME) at a 1:1 ratio and heated at 95^{o}C on an Eppendorf Thermomixer or Fisher Scientific Isotemp 125D thermo block for ten minutes in order to denature the proteins. Samples were centrifuged for one minute at 13000 rpm in the Eppendorf 5417R Micro-centrifuge. The samples were loaded into the wells along with a broad range protein reference marker with established molecular weights to verify size. Samples were electrophoresed for 60 minutes at 150 volts using a Fisher Scientific FB300 electrophoresis power supply. Gels were stained with Coomassie Blue Stain for 10 minutes, rinsed with Coomassie Destain solution for 10

minutes, and placed in water/destain solution over night. Coomassie-stained SDS gels were visualized on a UVP TW-26 white light transilluminator and photographed.

In Vitro Analyses of TEV Protease

Materials

UltraPure™ DNase/RNAse-free distilled water and TEV buffer (20X) were purchased from Invitrogen. Dithiothreitol (DTT) was obtained from Fluka. EDTA and Tris base were supplied by Promega. HCl (36%) was obtained from Sigma. Tris-HCl solution, 1.5 M, pH 8.8, was obtained from Bio-Rad Laboratories. QIAGEN supplied the QIAGEN EasyXpress® Kit for the *in vitro* transcription/translation (IVTT) system experiments. Costar furnished Corning 384-well black plates with clear bottoms.

TEV Protease Activity Using TEV Buffer

Methods

Each reaction consisted of 19.5 µl of Distilled water, DNAse and RNAse-free, 1.25 µl of 20X TEV buffer (1 M Tris-HCl at pH 8.0; 10 mM EDTA), 2.0 µl of FRET protein (in TEV storage buffer, concentration of 1 µg/µl, final concentration of 0.8 µg/µl), 0.25 µl of 0.1 M DTT, and 2 µl of TEV protease (in TEV storage buffer, varied concentrations), for a final volume of 25 µl per reaction. TEV storage buffer consisted of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, and 10% (v/v) glycerol. TEV protease was added to the reaction mixture in the following concentrations: 0, 0.074, 0.148, 0.269, 0.741 and 1.481 µM. The reaction with 0 µM TEV protease was evaluated as the negative control.

All stock solutions were stored on ice prior to mixing. Since the enzymatic reaction starts immediately upon addition of TEV protease, all reaction components were

mixed preceding addition of TEV protease. Each reaction mixture (25 μl final volume) was transfered into Corning 384-well black plates with clear bottoms and placed in the SpecraMax5 fluorescent spectrophotometer (Molecular Devices) with excitation at 365 nanometers (nm) and emission at 445 nm (corresponding to peak for BFP protein) and 510 nm (corresponding to peak for FRET protein). Measurement readings were taken at time points 0, 6, 21, 52, 113, 180 and 360 minutes. The instrument sensitivity, defined as the number of readings taken per well, was set for 19 repeated measures that resulted in a single emission measurement for each time point. It should be noted that time point 0 represents the initial reading on the spectrophotometer, not the addition of TEV protease. Approximately five minutes elapsed between addition of TEV protease and initial emission reading.

TEV Protease Activity Using IVTT System

The protocols for using the IVTT system were the same as above except the reaction mixture consisted of 10 μl QIAGEN Reaction Buffer, 8.75 μl *E. coli* extract, 2.0 μl of FRET protein, 2 μl of TEV protease (in TEV storage buffer, varied concentrations), and an adjusted volume of distilled water (DNAse and RNAse-free) to make a final volume of 25 μl per reaction. TEV protease was added to the reaction mixture in the following concentrations: 0, 0.269, 0.741, 1.481, and 2.962 μM. The reaction with 0 μM TEV protease was evaluated as the negative control. Measurement readings were taken at time points 0, 60, 120, 180, and 360 minutes. Approximately five minutes elapsed between addition of TEV protease and initial emission reading.

In Vivo Analyses of TEV Protease

Two main types of cell cultures were evaluated: cultures that contained both TEV protease and FRET plasmids, denoted as Two Plasmid Assay; and cultures that contained only the TEV protease plasmid, denoted as TEV Plasmid Assay. Experiments using the riboswitch design with specificity to theophylline were based on research performed by Lynch *et al.* (2007)

Cell Culture Growth

Materials

LB-broth media was purchased from Difco. Kanamycin, chloroamphenicol, theophylline, and caffeine were procured from Sigma. Promega supplied IPTG. L-(+)-Rhamnose monohydrate and dimethyl sulfoxide were obtained from Fluka. Aldrich supplied 2, 4-Dinitrotoluene (DNT). RNA Protect was supplied by QIAGEN.

Two Plasmid Assay

Methods

One flask of bacterial cells were cultured overnight from single colonies (fresh transformation or one Mircobank® Bead) of *E. coli* BL21(DE3) containing FRET protein gene on pHWG640 plasmid and TEV protease gene on pET28a plasmid vector in 50 ml LB media supplemented with the appropriate antibiotic (25 μg/ml of kanamycin and 25 μg/ml cloroamphenicol) at 37°C and shaken at 225 rpm. A second flask of *E. coli* BL21(DE3) cells containing the FRET protein gene on pHWG640 plasmid and upstream riboswitch/TEV protease gene (denoted RS-TEV) on pET28a plasmid were cultured under the same conditions. Cultures were performed in triplicate.

Overnight cultures were separated into separate treatment culture flasks, diluted to 1:100 in 500 ml LB media (25 μg/ml kanamycin and 25 ug/ml chloroamphenicol), and grown at 37°C with shaking (225 rpm) for three to four hours until reaching the mid-log phase. The mid-log phase of growth was verified when cultures reached an optical density at 600 nm (OD_{600}) of 0.8, as determined by Cary 300 Bio UV-Visible Spectrophotometer using a 1 cm path length cuvette. Treatment cultures were performed in triplicate.

When $OD_{600} \approx 0.8$, all cultures were inoculated with rhamnose (0.4%) to initiate production of FRET protein. After thirty minutes, appropriate cultures were inoculated with IPTG (0.5 mM) to initiate production of TEV protease and RS-TEV protease. Thirty minutes after IPTG addition, appropriate cultures were treated with theophylline or caffeine (2.5 mM). Since theophylline is not soluble at room temperature, a 100 mM stock solution was prepared in DMSO (20 mg/ml) and heated in 37^{o}C for 30 minutes. Table 2 represents the treatment design. The positive control (TEV protease) represents cells with TEV protease plasmid and FRET protein plasmid.

Table : Two Plasmid Treatment Design

Treatment Culture	Rhamnose	IPTG	Theophylline	Caffeine
Positive Control (TEV + FRET)	X	X		
RS-Neagtive Control (FRET)	X			
Riboswitch (RS) - Off	X	X		
Riboswitch (RS) - On (Theophylline)	X	X	X	
Riboswitch (RS) - On (Caffeine)	X	X		X

Samples were collected for protein analysis (10 ml), RNA analysis (0.5 ml), and cell density (1 ml) over the time course illustrated in Figure 11. Aliquots for protein

analysis were taken after addition of theophylline of caffeine (time point 0), and every 60 minutes until 360 minutes. Samples for RNA purification were aliquoted into 1.5 ml microcentrifuge tubes containing 1 ml RNA Protect Bacteria Reagent and collected at time points 0, and every 15 minutes until 120 minutes. Aliquots for cell density were collected at each protein and RNA collection time point and measured at OD_{600}.

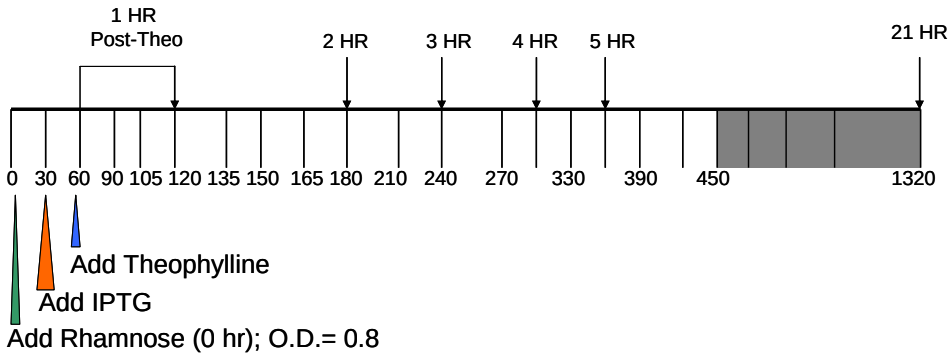


Figure : Time Course Overview for Two Plasmid Assay

Extracted aliquots for protein analysis were harvested in 15 ml conical tubes by centrifugation in a Centra CL3-R (Thermo Electron Corporation) at 4°C for 10 minutes at 3,200 rpm. The supernatant (LB media) was discarded and pellets were placed on dry ice for an immediate quick freeze prior to storage at -80ºC for later processing. Extracted aliquots for RNA analysis were vortexed and allowed to incubate at room temperature for 5 minutes. Samples were centrifuged at 5000 x *g* for 10 minutes. The supernatant was discarded; pellets were placed on dry ice and subsequently stored at -80ºC until processing for protein extraction.

Protein Extraction by Cell Lysis

Materials

UltraPure™ DNase/RNase-free distilled water was purchased from Invitrogen. $MgCl_2$ was obtained from Fluka. EDTA and Tris Base were procured from Promega. Sigma supplied HCL (36%), Benzonase® nuclease, lysozyme lyophilized powder (protein ~95%), and iodoacetamide. NaCl (5M) was obtained from Ambion. Tris-HCl solution, 1.5 M at pH 8.8, was obtained from Bio-Rad.

Methods

The lysis protocol was adapted from QIAGEN Ni-Fast Purification of 6x His-tagged protein under native conditions (Kohl *et al.*, 2002). Lysis buffer was prepared from 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM $MgCl_2$ prior to use. For the Two Plasmid Assay, 8 mM iodoacetimide was added to inhibit protease activity. The lysis buffer was then supplemented with 0.01% Benzonase® nuclease solution and 1% lysozyme solution. The lysozyme solution was prepared by dissolving the contents of a lysozyme powder vial in 600 µl of native lysis buffer.

Cell pellets were thawed for 15 min on ice and resuspended by vortexing in lysis buffer. The volume of lysis buffer added to each pellet was four percent of the original aliquot volume. For the Two Plasmid Assay, 200 µl of lysis buffer was added based on a pellet resulting from 5 ml of culture. The resuspended cells were incubated on ice for 30 minutes, and gently mixed two to three times by gently swirling the cell suspension.

The Two Plasmid Assay cell lysates were centrifuged at 13,200 rpm in the Eppendorf 5417R Micro-centrifuge for 30 minutes at 4°C to pellet the cellular debris. The cell lysate supernatant, containing soluble fraction of recombinant proteins, was

transferred into a new 1.5 ml microcentrifuge tube and stored at -80^{o}C. The pellet was stored at -80^{o}C in case of incomplete lysis.

TEV Plasmid Assay

Cell growth and protein extraction for the TEV plasmid assay followed the same protocols as the two plasmid assay with the following changes: cell cultures contained either TEV protease or RS-TEV protease vector and were grown in 50 ml LB media supplemented with antibiotic (25 μg/ml of kanamycin). Overnight cultures were diluted 1:100 in 100 ml LB media (25 μg/ml kanamycin). Cultures were inoculated with IPTG (0.5 mM) only. Theophylline was added after 30 minutes. Aliquots of 48 ml culture were collected in 50 ml conical tubes after 360 minutes and centrifuged in the Centra CL3-R (Thermo Electron Corporation) at 4^{o}C for 10 minutes at 3,200 rpm. The supernatant was discarded; pellets were placed on dry ice and subsequently stored at -80ºC until processing for protein extraction.

Cell lysis buffer was prepared without iodoacetimide to enable TEV protease activity for kinetic evaluation. Lysis was performed using 1.9 ml lysis buffer based on a pellet resulting from 5 ml of culture. The cell lysates were centrifuged at 14000 x *g* in the Centra CL3-R (Thermo Electron Corporation) for 60 minutes at 4 °C. Two aliquots of 190 μl were transferred into 0.5 ml tubes for purification, bicinchoninic acid (BCA®) protein assay, and polyacrylamide gel electrophoresis. Two aliquots of 750 μl were transferred into 1.5 ml tubes for kinetic analysis.

Kinetic Analysis

UltraPure^{TM} DNase/RNAse-free distilled water and TEV reaction buffer (10X) were purchased from Invitrogen. Dithiothreitol (DTT) was obtained from Fluka. Each

reaction consisted of 50 µl cell lysate supernatant, 15 µl 10X TEV reaction buffer (1 M Tris-HCl at pH 8.0; 10 mM EDTA), 1.5 µl of 0.1 M DTT, 75 µl of 0.5 uM FRET protein (0.25 uM final concentration), and 8.5 µl distilled water.

All stock solutions were stored on ice prior to mixing. Since the enzymatic reaction starts immediately upon addition of TEV protease, all reaction components were mixed preceding addition of cell lysate supernatant. Each reaction mixture (150 µl final volume) was transferred into Corning 96-well black plates and placed in the SpecraMax5 fluorescent spectrophotometer (Molecular Devices) with excitation at 365 nm and emission at 445 nm (corresponding to peak for BFP protein) and 510 nm (corresponding to peak for FRET protein). Measurement readings were taken every minute for 60 minutes. The instrument sensitivity was set for 19. Each reaction was performed in duplicate.

RNA Purification

Materials

Bio-Rad supplied β-ME. Ethanol (EtOH), 200 proof, was purchased from AAPER. Tris-EDTA (TE) Buffer was purchased from Ambion. QIAGEN supplied proteinase K, RNA Protect, RNeasy Bacteria Protect Mini kit, and EZ1 RNA Tissue Mini Kit. The RNease Bacteria Protect kit contained RLT buffer, RW1 buffer, RPE buffer, RNAse-free water, and RNeasy spin columns. Lysozyme and ethidium bromide (EtBr) was otained from Sigma. UltraPure™ DNA-grade agarose, 10X BlueJuice™ gel loading buffer, and high DNA Mass ladder were procured from Invitrogen.

Methods

Protocols were adapted from QIAGEN RNAprotect Bacteria Reagent Handbook (QIAGEN (2), 2005). For each bacterial sample which did not receive the RNA Bacterial Protect Reagent during the initial aliquot from the cell culture, two volumes of reagent were added to one original aliquot volume that yielded a sample pellet. The pellet was resuspended by pipetting, vortexed for 5 seconds, and allowed to incubate at room temperature for 10 minutes. After the RNA was centrifuged for 10 minutes at 5000 x *g*, the supernatant was decanted by inversion.

For each RNA pellet, 300 µl TE + lysozyme (1mg/ml) and 10 µl proteinase K was added and mixed by vortexing for 10 seconds. The mixture was incubated for 15 minutes at room temperature with vortexing every 2 minutes for 10 seconds. Next, 150 µL RPE buffer was added and vortexed vigorously. EtOH (750 µl) was then added to the supernatant and mixed by repeated pipetting.

Up to 700 µl of lysate sample was transferred into an RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm on the IEC MicroMax microcentrifuge. The flow-through was discarded and the remaining lysate was processed in the same manner. RW1 buffer (700 µl) was added to the spin column, incubated at room temperature for 5 minutes and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. DNase (80 µl) was added to the column and incubated for 15 minutes at room temperature. A repeat wash using 620 µl RW1 buffer was accomplished, incubated at room temperature for 5 minutes and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. Using a new collection tube, 500 µl of RLT buffer (with 1% β-ME) was added to the spin column and centrifuged for 15 seconds at 10,000 rpm. After

discarding the flow-through, this step was repeated with centrifugation for 2 minutes at 10,000 rpm. The spin column was placed in a new collection tube and centrifuged for 1 minute at 10,000 rpm to ensure excess buffer was removed. After placing the spin column in a clean 1.5 ml tube, RNase-free water (25 µl) was added to the column, incubated for 1 minute at room temperature, and centrifuged for 1 minute at 10,000 rpm. An additional 25 µl of RNase-free water was added to the column, incubated for 1 minute at room temperature, and centrifuged for 1 minute at 10,000 rpm.

RNA samples (2 µl) were mixed with BlueJuice™ (1X), for a total volume of 10 µl, and loaded on a 1% DNA agarose gel made with TBE buffer (1X), using a TBE running buffer (1X) with EtBr (5ul/ml). The EtBr binds to RNA and DNA, allowing the bands to be viewed on a fluorescent spectrophotometer. A 2-log DNA ladder or a high DNA mass ladder was used as molecular weight standard. Agarose gels were electrophoresed at 120 volts for 60 minutes and photographed on the Syngene Bio Imaging Systems G-Box using the transilluminator setting without filter.

The purity and concentration of the RNA samples (1 µl) were evaluated using the Nano-Drop ND-1000 spectrophotometer. The remaining RNA samples were stored at -20°C for later processing into complementary DNA (cDNA).

Complementary DNA (cDNA)

Materials

Invitrogen's SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green was used to synthesize first strand cDNA from purified RNA. The kit contents used during this procedure were diethylpyrocarbonate (DEPC)-treated water, 2X RT Reaction mixture, RT Enzyme mixture, and *E.coli* RNAse H.

Methods

All materials were initially chilled on ice. In a 0.5 ml microcentrfuge tube, the following components were combined: 10µl of 2x RT Reaction Mix; 2 µl of RT Enzyme Mix; 8 µl RNA (adjusted to a final concentration of 1 µg using DEPC-treated water). For samples with less than 1 µg RNA, 8 µl RNA was used, and the final concentration in cDNA was calculated for later reference. Each tube was gently mixed prior to the next step.

All samples were placed in the ABI GeneAmp® PCR System 9700 Thermocycler, set with the following program: incubate at 25°C for 10 minutes; 42°C for 50 minutes; terminate the reaction at 85°C for 5 minutes, then remove from Thermocycler and chill on ice (4°C) while adding 1 µl (2 U) of *E. coli* RNAse H. Samples were gently mixed then placed back in the Thermocycler to incubate at 37°C for 20 minutes. Upon completion of the program, the samples were removed and stored at -20°C until used for qPCR or other analyses.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism, version 5.01 for Windows. After the completion of each respective assay, the results from the SpectroMax 5 Fluorescent spectrophotometer exported to GraphPad Prism. Statistical analysis for the *in vitro* assays compared each concentrations to the negative control. Statistical significance was determined by using the students *t*-test (one-tailed analysis using Welch's correction by assuming unequal variances) at a 95 percent confidence level. For *in vivo* assays, two-way Analysis of Variance (ANOVA) was used to compare means between several time points and treatments. The Bonferroni post test was

performed to evaluate statistical significance at each experimental time point. Evaluation was performed at 95 percent confidence interval.

IV. Results and Analysis

Overview

All experiments supported evaluation of the tobacco etch virus (TEV) protease and the BFP_{TEV}eGFP-His protein for their effectiveness as a reporter pathway. Throughout this chapter, the BFP_{TEV}eGFP-His protein will be referred to as fluorescent resonance energy transfer (FRET) protein. Results are segregated into two categories, *in vitro* and *in vivo* analyses.

For all experiments, TEV protease activity was evaluated by observing the FRET signal output from each reaction. In the FRET protein construct (BFP_{TEV}eGFP-His), the BFP donor emission peaked at 445 nm, whereas the eGFP acceptor emission peaked at 510 nm when both were excited at 365 nm, as illustrated in Figure 12.

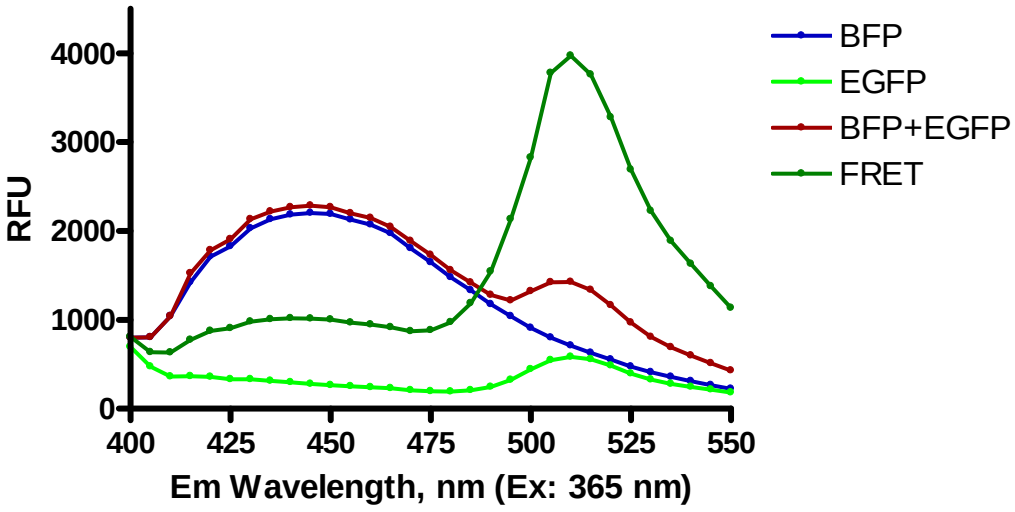


Figure : Spectra for Purified FRET Protein and Subcomponents
Each line represented by 1.3 μM protein. RFU = relative fluorescent units; Em = emission; Ex = excitation. Graph provided by Dr. Svetlana Harbaugh (AFRL/RHPB)

It is important to note that the presence of BFP and eGFP together exhibited a lower FRET signal at 510 nm than the FRET protein.

The ratio of emission of 510:445 nm (eGFP:BFP) was utilized to quantify the FRET protein signal and qualify its cleavage by TEV protease. A higher emission ratio indicates a greater presence of the FRET protein due to the higher FRET signal (approximately 1.8 for the FRET protein in Figure 12). Conversely, a lower ratio suggests greater enzyme activity as TEV protease cleaved the FRET protein into its BFP and eGFP components (approximately 0.7 for BFP +EGFP proteins in Figure 12). Because it eliminates the variability of the relative fluorescent unit measurements, the ratio enables signal comparisons between experiments.

All graphical data are presented using error bars showing standard error of the mean (s.e.m.). Some error bars are relatively small and are not visible beyond the data point symbol. S.e.m. is a measure of the precision of the data, as opposed to the measure of variability when using standard deviation (Motulsky & Christopoulos, 2006). S.e.m. is commonly used experimental biology when comparing samples from two groups to infer if the two groups are different (Cumming *et al.*, 2007).

In Vitro Analyses of TEV Protease

TEV protease activity was examined using varying concentrations of purified TEV protease to cleave 1.38 μM FRET protein in a buffer and an *in vitro* translation and transcription (IVTT) system over time. An additional experiment using the TEV plasmid (circular DNA containing TEV protease gene) was evaluated in the IVTT system. For all experiments, the negative control contained FRET protein (no TEV protease) to establish a baseline FRET signal.

The goals of these experiments were to determine the following: (1) if the TEV protease and FRET protein interaction resulted in a discernable output signal in different systems, and (2) the effect of the IVTT system on that output when compared to an ideal buffer system. Based on the literature review, the FRET protein design was expected to result in a robust output signal when cleaved by TEV protease. The buffer system represented ideal conditions for enzymatic reactions due to the lack of DNA and other cellular machinery which participates in protein synthesis in the *in vivo* system. The IVTT system simulated *in vivo* conditions with minimal background protein synthesis of other gene products due to absence of DNA. The FRET signal from both systems was expected to be similar. The FRET signal from the TEV plasmid in the IVTT system was expected to be similar to the purified proteins in the IVTT system, though the signal at the 0 minute time point was expected to be lower since the TEV protease required manufacturing by the IVTT system components before cleaving FRET protein. The negative control signal was expected to remain the same over the time course since no TEV protease was present to initiate cleavage.

Figure 13 displays TEV protease activity on 1.38 μM FRET protein in a buffer system (1M Tris-HCl at pH 8.0; 10 mM EDTA). As expected, the negative control (0 μM TEV protease) emission ratio remained stable (mean = 4.588, s.e.m. = 0.015) and thus is a valid control condition.

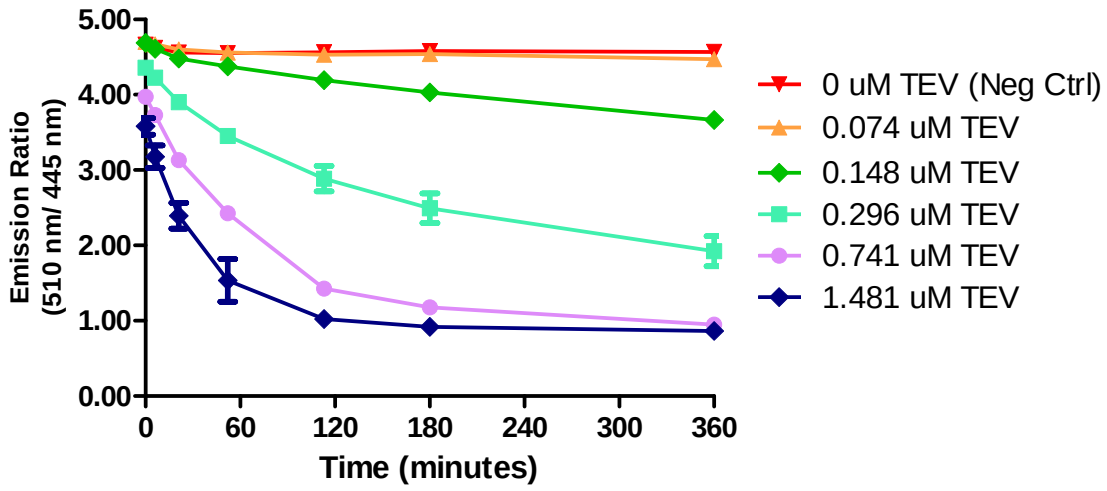


Figure : TEV Protease Cleavage of 1.38 μM FRET Protein in Buffer System
Excitation at 365 nm. n=2; error bars represent s.e.m. Data was analyzed using column statistics of tabulated data on GraphPad Prism version 5.01 for Windows.

Using the student's one-tailed t-tests with Welch's correction (assuming unequal variances), varied concentrations of TEV protease were evaluated against the negative control (0 μM TEV protease) to determine if the difference in enzyme activity was statistical significance. Statistical significance is indicated by an asterisk (*) based on $p < 0.05$ in Table 3 below.

Table : Statistical Analysis for *In Vitro* Buffer System

TEV Protease Concentration Comparisons (uM)			Time (minutes) 0	6	21	52	113	180	360
0	&	0.074	0.320	0.283	0.281	0.457	0.258	0.082	0.136
0	&	0.148	0.373	0.422	0.168	0.069	0.028*	0.009*	0.006*
0	&	0.296	0.105	0.077	0.023*	0.026*	0.032*	0.030*	0.024*
0	&	0.781	0.030*	0.020*	0.010*	0.000*	0.003*	0.001*	0.001*
0	&	1.481	0.037*	0.036*	0.026*	0.030*	0.006*	0.003*	0.001*

Probability values from student's one-tailed t-test using Welch's correction between negative control (0 uM TEV protease) and varied concentrations of TEV protease in a TEV buffer system. Data analyzed on GraphPad Prism version 5.01 for Windows. Legend: * $p < 0.05$.

No statistical difference was observed between 0 μM and 0.074 μM TEV protease. Concentrations of TEV protease at 0.074 μM and 0.148 μM were not used in further experimentation because their activity was significantly lower than the higher concentrations of TEV protease. Statistical significance was observed between 0 uM and both 0.781 μM and 1.148 μM TEV protease concentrations for all time points. Emission measurements and statistical analysis showed that a TEV protease:FRET protein ratio of 0.5 or higher resulted in measurable and predictable signal change. The reporter pathway will produce a robust output signal when concentrations of the protease and its substrate are controlled.

The factor of time was considered in order to evaluate the effectiveness of the reporter system. Based on the 1.481 μM TEV Protease concentration curve, a significant amount of enzyme activity occurred within the first 120 minutes, followed by reduced activity as the FRET protein concentration declined. After 360 minutes, the emission ratio dropped below one, indicating most, if not all, FRET protein had been cleaved. Examination of the change in emission ratio over 360 minutes illustrated that the 1.481 μM TEV protease concentration produced a discernable signal change when compared to the negative control (0 μM TEV protease concentration), as presented in Figure 14. The difference between the emission ratios was statistically significant at $p < 0.001$ using student's t-test (Table 3), further validating the operational value of the reporter assay.

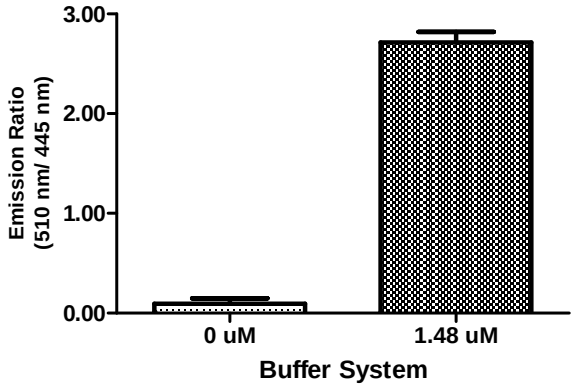


Figure : Emission Ratio Difference (t_0-t_{360}) in Activity for Buffer System
Emission at 365 nm. n=2; error bars represent s.e.m. 0 μM: x = 0.09; 1.48 μM: x =2.71. Data analyzed using column statistics of tabulated data on GraphPad Prism version 5.01 for Windows.

The results for TEV protease activity using the IVTT system were quite different, as illustrated in Figure 15. Although the negative control was expected to maintain a steady ratio, it decreased over the time course with a wide error (mean = 1.856, s.e.m. ± 0.238).

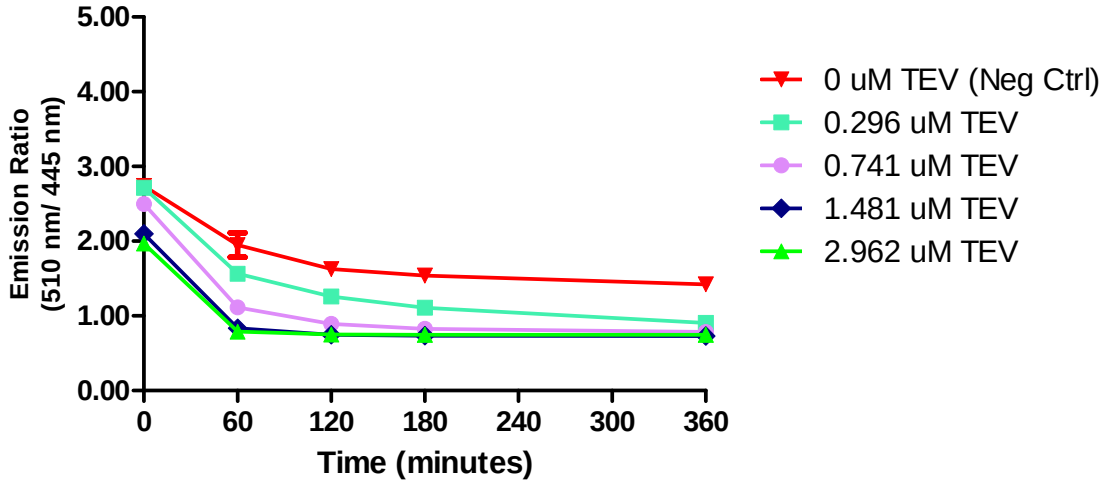


Figure : TEV Protease Cleavage of 1.38 μM FRET Protein in IVTT System
Excitation at 365 nm. n=3; error bars represent s.e.m. Data analyzed using column statistics of tabulated data on GraphPad Prism version 5.01 for Windows.

Parallel research was performed by John Lynch (AFRL/RHPB) on the effectiveness of the IVTT system to express TEV protease from the TEV protease gene cloned into the pET28a plasmid vector. Experimental methods were the same used for the TEV protease in the IVTT system, with two substitutions: purified TEV protease was replaced by pET28a_TEV plasmid and 0.25 µl of water was replaced with the same volume of IPTG (10 mM) to induce transcription. The data, illustrated in Figure 16, was included to compare different parameters between experimental designs.

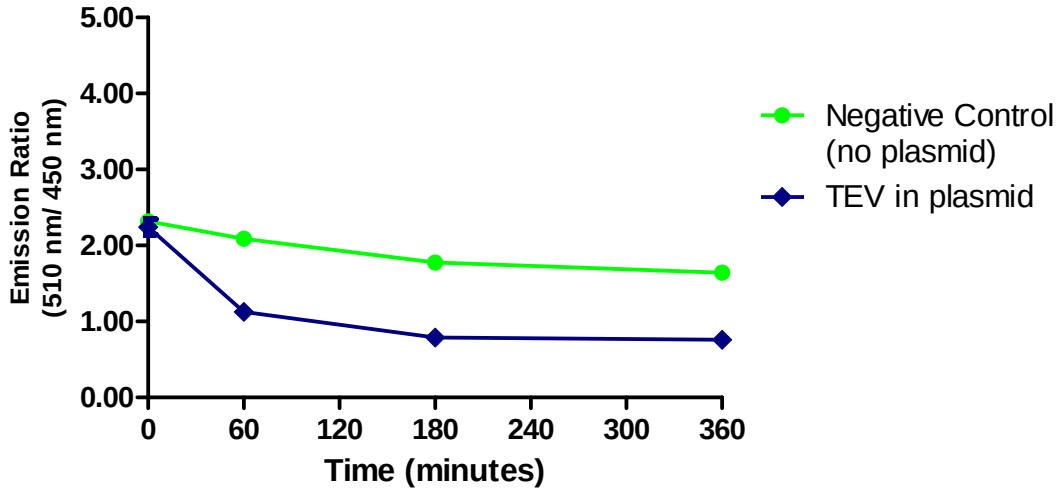


Figure : TEV Plasmid Cleavage of 1.38 µM FRET Protein in IVTT system
Excitation at 365 nm. n=2, error bars represent s.e.m. Data analyzed using column statistics of tabulated data on GraphPad Prism version 5.01 for Windows. Data provided by Mr. John Lynch (AFRL/RHPB).

Comparison of emission ratios between the negative control (0 mM TEV protease) and the TEV plasmid product was evaluated using the student's one-tailed t-tests with Welch's correction assuming unequal variances (refer to Table 4). Statistical significance, indicated by an asterisk (*) based on $p < 0.05$, was observed for all time

points except 0 minutes. The emission ratio at 0 minutes was expected to be similar since the TEV protease must be expressed from the TEV plasmid using the components in the IVTT reaction buffer. The statistical significance at the later time points indicated that TEV protease was successfully expressed within the IVTT system.

Table : Statistical Analysis for TEV Plasmid in IVTT System

Time (minutes)			
0	60	180	360
0.3179	0.0148*	0.0173*	0.0117*

Probability values from student's one-tailed t-test using Welch's correction between Negative Control (no plasmid) and TEV plasmid in IVTT system. Data analyzed on GraphPad Prism version 5.01 for Windows. Legend: * $p < 0.05$.

Comparison of the negative controls for all experimental designs demonstrated significant differences between the Buffer and IVTT systems (Figure 17).

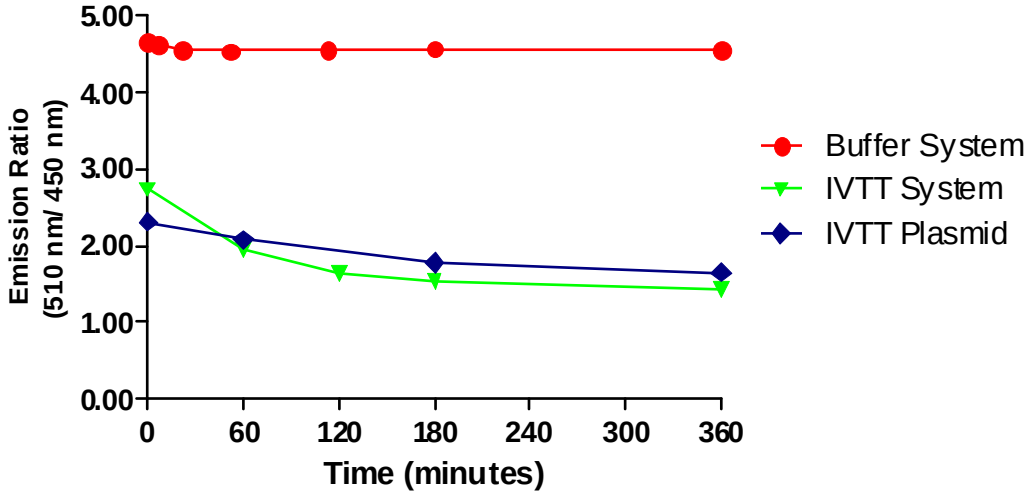


Figure : Comparison for Negative Controls between Different *In Vitro* Systems
Excitation at 365 nm. n=2, error bars represent s.e.m. Data analyzed using column statistics of tabulated data on GraphPad Prism version 5.01 for Windows

Evaluation of the emission ratio difference between the negative controls of the Buffer system and both the IVTT system experiments verified that the FRET signal dropped significantly in the IVTT system (see Figure 18).

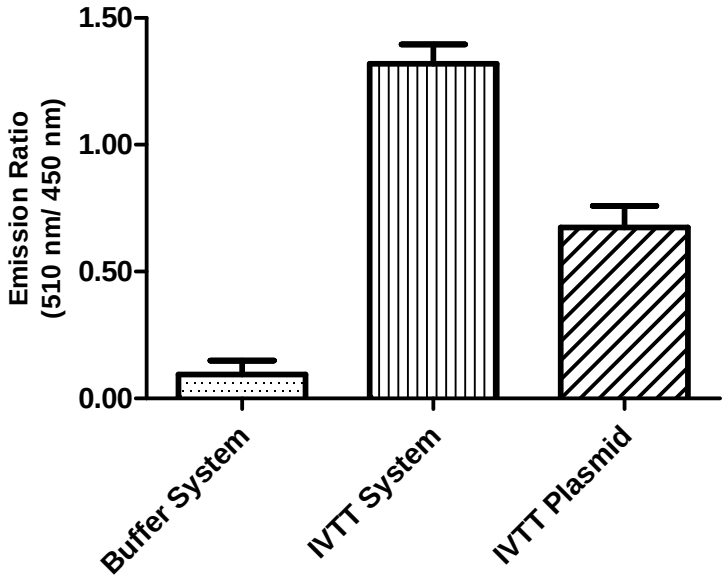


Figure : Emission Ratio Differences (t_0-t_{360}) for *In Vitro* Systems
Excitation at 365 nm; Buffer system, x = 0.09, n=2; IVTT System, x = 1.32, n=3; IVTT Plasmid, x = 0.67, n = 3; error bars represent s.e.m. Data analyzed using column statistics of tabulated data on GraphPad Prism version 5.01 for Windows.

The emission ratio of the negative control in all experiments should have remained constant throughout the 360 minute time frame because no enzyme-substrate reaction should exist. Using the two way ANOVA, there was statistical significance between the buffer system and IVTT System/IVTT Plasmid experiments (p= 0.026 and

0.038 respectively) but no statistical significance between within the IVTT system (p = 0.242). Possible explanations for the large emission change include degradation of the FRET protein due to side reactions in the IVTT reaction mixture or accumulation of by-products from the IVTT reaction that mask the FRET protein emission. It is also possible that the complex matrices of the IVTT system induce a conformational change in the FRET protein and shift the fluorophores outside the Förster resonance distance of 100 Å.

In addition to the large emission range displayed by the negative control in IVTT system experiments, the emission ratio of all concentrations was lower than that of the Buffer system by a factor of two as illustrated in Figure 19.

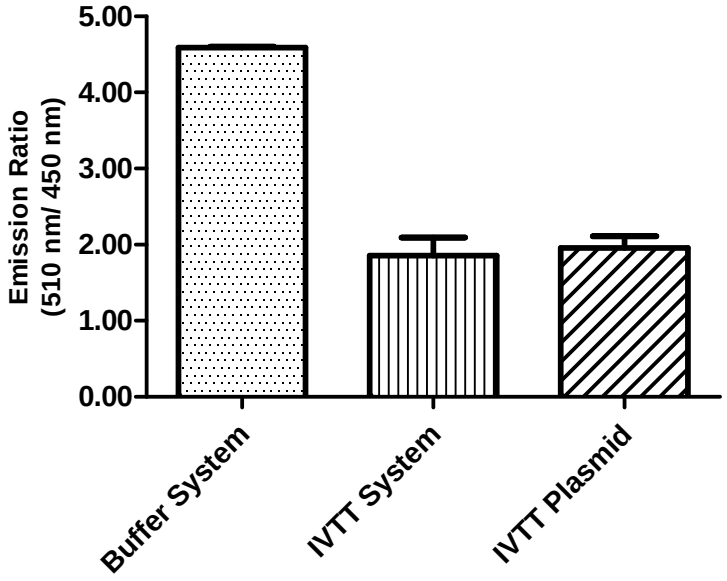


Figure : Mean Emission Ratios of 0 μM TEV Protease for *In Vitro* Systems
Buffer system, x = 4.59, n=2, IVTT System, x = 1.86, n=3, IVTT Plasmid x = 1.96, n = 2; error bars represent s.e.m. Data analyzed using GraphPad Prism version 5.01 for Windows.

The mean emission ratios of both IVTT system experiments was not statistically significant ($p = 0.3566$), where as the mean between the Buffer and IVTT systems was statistically significant ($p = 0.002$). Natural molecules often contain chromophoric moieties that have characteristic spectra (Boyer, 1993). In addition, proteins absorb light as well as diffract light when bombarded with light. Because the IVTT system utilized components from cell extracts, the background fluorescence of the IVTT reaction buffer components interfered with the FRET signal in a non-uniform manner over time.

To determine if the negative control emission change in the IVTT System (using purified TEV protease) was due to FRET protein cleavage, degradation, or conformational change, non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed (Figure 20). Purified FRET protein (30 μg) was used as a standard and each well contained 20 μl reaction mixtures (16 μg FRET protein). No significant degradation of the negative control occurred from zero minutes to 360 minutes since the band intensity remained visually constant.

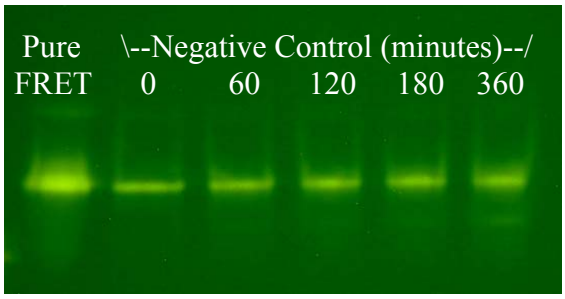


Figure : Non-denaturing PAGE of IVTT System with FRET protein
12% Tris-HCl non-denaturing gel; Pure FRET: 30 μg FRET protein; Negative Control: 16 μg FRET protein. Photo taken on Dark Reader (ex: 400-500 nm) with Fuji Camera.

These results reinforce the supposition that the background fluorescence of the IVTT components were interfering with the FRET protein emission and that the lowered emission ratio was not a result of FRET protein cleavage.

The same gel was stained with Coomassie Blue staining solution (Figure 21) indicated numerous other proteins of different molecular weights were present in the negative control reaction mixture when using the IVTT system. These other proteins contributed to the background fluorescence that masked the FRET protein emission when taken on a spectrophotometer.

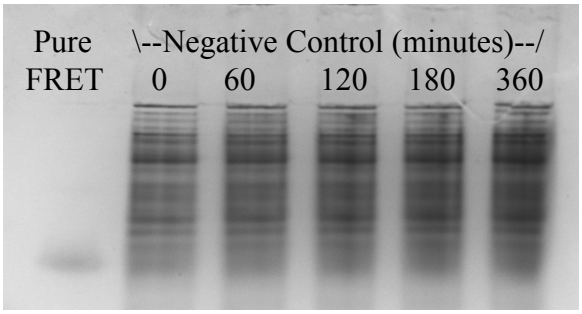


Figure : Non-denaturing PAGE of IVTT System with Coomassie Blue Stain
12% Tris-HCl non-denaturing gel; Pure FRET: 30 μg FRET protein; Negative control: 16 μg FRET protein. Photo taken on Bio Imaging Systems G-Box.

Though the negative control displayed anomalous behavior and emission ratio was lower than the buffer system due to background fluorescence, it was assumed that the IVTT reaction behavior would be consistent within all reactions mixtures and comparisons could be made between the different TEV protease concentration and the negative control (0 μM TEV protease). Comparisons were made using the student's one-tailed t-tests with Welch's correction (assuming unequal variances). Statistical significance is indicated by an asterisk (*) based on $p < 0.05$ in Table 5 below. Statistical

significance was observed between 0 μM and both 1.148 μM and 2.962 μM TEV protease concentrations for all time points. These results were consistent with results obtained when using the buffer system.

Table : Statistical Analysis for IVTT System

TEV Protease Concentration Comparisons (uM)			Time (minutes) 0	60	120	180	360
0	&	0.296	0.318	0.072	0.017*	0.016*	0.013*
0	&	0.781	0.108	0.018*	0.005*	0.006*	0.008*
0	&	1.481	0.000*	0.010*	0.003*	0.005*	0.007*
0	&	2.962	0.000*	0.010*	0.003*	0.005*	0.008*
1.481	&	2.962	0.017*	0.015*	0.388	0.059	0.156

Probability values from student's one-tailed t-test using Welch's correction between negative control (0 uM TEV protease) and varied concentrations of TEV protease in IVTT system. Data analyzed on GraphPad Prism version 5.01 for Windows. Legend: * $p < 0.05$.

An additional analysis was performed between 1.148 μM and 2.962 μM TEV protease concentrations because of their graphical results. Statistical significance was observed only at 0 and 60 minute time points, indicating the higher TEV protease concentration produced a more discriminating signal than the lower concentration. Limited inferences were made for TEV activity in the IVTT system given that the negative control could not be validated and inability to remove background fluorescence. Additional insight into TEV activity was obtained by comparing results between the buffer and IVTT system, and the purified TEV protease and the TEV plasmid in the IVTT system (Figure 22). All experiments used the same concentration of purified FRET protein (1.38 μM). To maintain consistency with comparisons, parameters were matched

where possible. Results could only be inferred for the IVTT-pET28a TEV assay because the exact concentration of TEV protease was not determined.

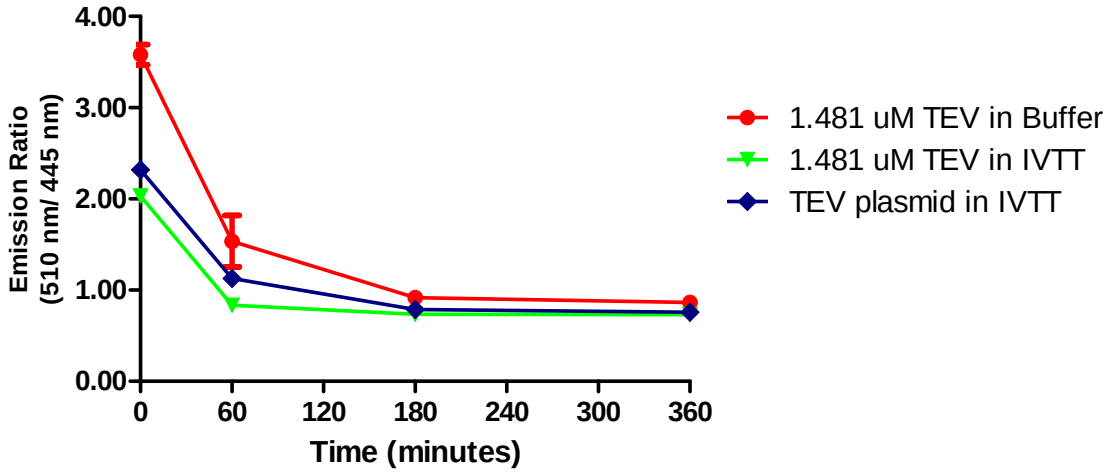


Figure : TEV Protease Cleavage of 1.38 μM FRET Protein in Different Systems
Excitation at 365 nm. n=2, error bars represent s.e.m. Data analyzed using column statistics of tabulated data on GraphPad Prism version 5.01 for Windows

Two-way analysis of variance (ANOVA) with a Bonferroni post test was used to evaluate the different experiments. Two-way ANOVA indicated statistical significance between all interaction ($p < 0.0001$) of time and experiment type. As expected, time demonstrated statistical significance ($p < 0.0001$). Variances between the experiments types resulted in $p = 0.0023$, indicating each experiment produced statistically different results; however, results of the Bonferroni post test (Table 6) indicated no statistical significance between experiments performed within the IVTT system. For all experiment types, no statistical significance was noted after 180 minutes, indicating that the all FRET protein in the reaction had been cleaved.

Table : Statistical Analysis for All *In Vitro* Systems

Bonferroni Comparisons	Time (minutes) 0	60	180	360
1.48 uM TEV in Buffer & 1.48 uM TEV in IVTT	***	***	ns	ns
1.48 uM TEV in Buffer & TEV Plasmid in IVTT	***	*	ns	ns
1.48 uM TEV in IVTT & TEV Plasmid in IVTT	ns	ns	ns	ns

Data analyzed using two-way ANOVA with Bonferroni post-test on GraphPad Prism version 5.01 for Windows. Legend: ns = not significant, * $p < 0.05$; *** $p < 0.001$

In summary, the *in vitro* experiments verified that the TEV protease cleaved the FRET protein as designed, and the reaction output signal was strongest at a TEV protease: FRET protein ratio of 0.5 or higher. Though reactions in the Buffer System demonstrated larger emission ratios, the both IVTT system experiments successfully confirmed a discernable output signal, indicating the reporter pathway might be successful when utilized within *in vivo* (cell-based) studies. Given that the IVTT system displayed a non-uniform pattern of fluorescence interference as well as a lack of TEV protease production when placed under the control of the riboswitch (data not shown), determination of the interfering IVTT components was not pursued further.

In Vivo Analyses of TEV Protease

In vivo assays offer more flexibility for development of modular sensor-reporter systems because machinery of the host cell can be used to translate and transcribe the reporter pathway components; thereby reducing reliance upon expensive IVTT systems. However, cell-based assays introduce greater complexity to the analysis of enzyme reactions because the output signal is affected by side-reactions and background fluorescence of the entire system.

TEV protease activity was examined through the use of two different *in vivo* assays. The TEV Plasmid Assay evaluated the kinetic activity of the enzyme whereas the Two Plasmid Assay evaluated the behavior of the TEV protease when co-expressed with the FRET protein. Table 7 summarizes the assays and their end products.

Table : Summary of *In Vivo* Assays

Assay Title	Plasmid	End Products
Two Plasmid Assay	pET28a pHWG640	TEV & RS-TEV proteins FRET protein
TEV Plasmid Assay	pET28a	TEV & RS-TEV proteins

The goals of these experiments were to evaluate TEV protease activity in an *in vivo* setting and introduce the riboswitch as a genetic regulator to determine its kinetics via the output of the reporter pathway.

Two-Plasmid Assay

In the Two Plasmid Assay, TEV protease and FRET protein were co-expressed from plasmids in the same cell (Figure 23). Protein was expressed for 30 minutes prior to introducing experimental treatments and analysis. TEV protease and TEV protease containing the riboswitch (RS-TEV) were prepared from two separate cell cultures. Treatments to the RS-TEV design included riboswitch activation by theophylline and its caffeine relative, and no riboswitch activation. It was expected that the RS-TEV On treatment with theophylline would closely match results from the TEV positive control (no riboswitch) because both would express the TEV protease. The RS-TEV On treatments with theophylline and caffeine were expected to be different since the riboswitch design was based on research presented by Lynch *et al.* (2007) that reported

reactions specific to theophylline but not caffeine. Caffeine differs from theophylline by a methyl group (refer to Figure 5, Chapter II) and was used to gauge riboswitch specificity for the ligand. The RS-TEV treatment with caffeine was expected to overlap the RS-TEV Off treatment because the conformation of the riboswitch for both treatments should inhibit translation initiation.

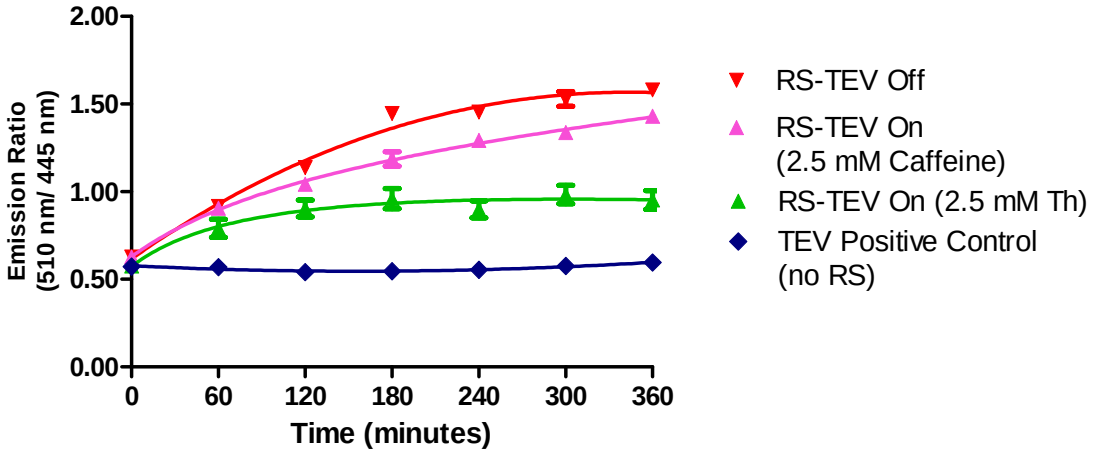


Figure : Emission Ratio from Two Plasmid Assay
Excitation at 365 nm. n = 3 for RS Off, RS On (Caffeine); n=6 for RS On (Th) and TEV Positive Control; error bars represent s.e.m. Analysis performed using Non-linear regression on GraphPad Prism version 5.01 for Windows.

The higher emission ratios of the RS-TEV On with theophylline treatment when compared to the TEV positive control indicated less FRET cleavage. One possible explanation could be that insertion of the riboswitch upstream of the TEV protease gene hindered translation of the TEV protease, resulting in a lower protein yield.

The RS-TEV On treatment with theophylline demonstrated higher TEV protease activity than that with its caffeine competitor. Statistical analysis performed using two-way ANOVA indicated significance between time points ($p < 0.0001$) and treatments (p

< 0.0001); however, the Bonferroni post test (Table 8) implied some treatments produced similar results for 180 minutes, and then became statistically different. The inference from these results indicated the riboswitch does not provide absolute specificity for theophylline; there is a slight conformational change of the riboswitch in the presence of caffeine which allowed ribosome binding and translation initiation. At least 240 minutes were required to discriminate between riboswitch on, off, and a non-specific ligand (caffeine). In essence, this time distinguishes between a positive and false positive reading. In addition, the RS-TEV On with caffeine treatment appeared to express some quantity of TEV protease because its emission ratio was lower than the RS-TEV Off treatment, though the Bonferroni post test indicated no statistical significance.

Table : Statistical Analysis for *In Vivo* Two Plasmid Assay

	Time (minutes)						
Bonferroni Comparisons	0	60	120	180	240	300	360
TEV Ctrl & RS-On	ns	***	***	***	***	***	***
TEV Ctrl & RS Off	ns	***	***	***	***	***	***
TEV Ctrl & RS-On (Caff)	ns	***	***	***	***	***	***
RS On (Th) & RS-OFF	ns	ns	***	***	***	***	***
RS On (Th) & RS On (Caff)	ns	ns	ns	***	***	***	***
RS On (Caff) & RS-OFF	ns	ns	ns	***	ns	*	ns

Data analyzed using two-way ANOVA with Bonferroni post-test on GraphPad Prism version 5.01 for Windows. Legend: ns = not significant, * $p < 0.05$; *** $p < 0.001$.

A difference between RE-TEV On with theophylline and RS-TEV Off treatments was expected after 60 minutes of protein expression, but was not statistically significant until 120 minutes, indicating the RS-Off treatment also expressed low levels of TEV protease. This observation was supported by results reported by Lynch *et al.* (2007) that

the riboswitch did not completely repress protein translation of the TEV protease, resulting in background noise.

To validate measurements from the spectrophotometer, 20 µl aliquots RS-TEV on with theophylline were extracted at specified time points for non-denaturing gel electrophoresis (Figure 24). The TEV assay consisted of purified FRET protein, BFP, and eGFP proteins. The gel illustrates the production of FRET protein and TEV protease over time as well as the TEV protease activity cleaving the FRET protein into its BFP and eGFP constituents. The emission signal became discernable to the naked eye after 240 minutes, suggesting this is the minimal reaction time required to verify TEV protease activity in the presence of theophylline.

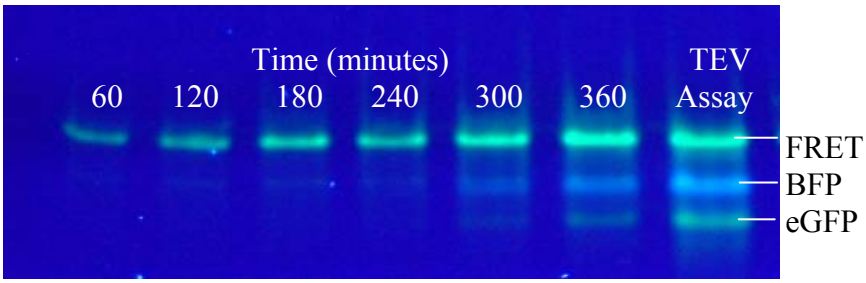


Figure : Non-denaturing Gel of TEV Protease Activity with Riboswitch On
Riboswitch On (2.5 mM theophylline). 12% Tris-HCl Native gel. Photo taken on UVP TW-26 white light transilluminator (ex: 365 nm) with Fuji Camera.

Analysis of purified total RNA on a 1% agarose gel indicated the presence of the two ribosomal subunits, 50S (top) and 30S (bottom), in *E. coli* cellular mRNA (Figure 24). Visualizing the mRNA in the Agarose gel assists in assessing presence and purity of the mRNA. Bands that were not well defined or appeared to have smeared in the gel indicated degradation of the mRNA (Figure 25, top). Well-defined bands indicated

mRNA with higher level of purity (Figure 25, bottom). The level of intensity was an indication of concentration. Determination of concentration and purity were quantified on the NanoDrop® spectrophotometer.

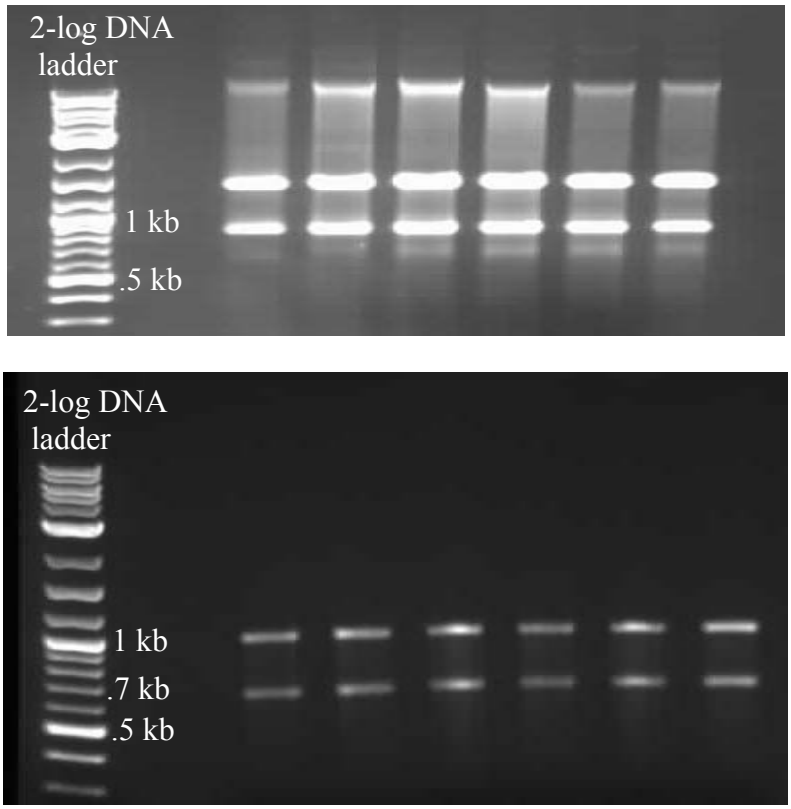


Figure : Purified Total RNA for RS-TEV On
Top band of both photos represent 50S ribosomal subunit while lower band is 30S subunit. Top photo indicates mRNA degradation while lower photo indicates higher purity, but lower concentration. mRNA evaluated on 1% agarose gel. Photos taken on Bio Imaging Systems G-Box.

Purified mRNA was used to produce complementary DNA (cDNA) for analysis by real time polymerase chain reaction (qPCR), which evaluates lifespan of the mRNA through multiple polymerase cycles as well as differentiating mRNA from different genes. Unfortunately, there were challenges developing a standard curve for the TEV

protease and FRET protein, so further evaluation was discontinued for the duration of this study.

TEV Plasmid Assays

In the TEV Plasmid Assay, TEV protease was expressed for 360 minutes to maximize concentration for kinetic experiments (Figure 26). Kinetic reactions were analyzed after addition of 0.25 μM FRET protein. All data was normalized to account for total protein concentration (see appendix B for process). In these experiments, the RS-TEV On treatment was expected to closely align with the TEV positive control (no riboswitch) because TEV protease should be produced in both designs. The RS-TEV off treatment should produce the highest emission signal ratio because TEV protease should not be expressed and the FRET protein should be prevalent.

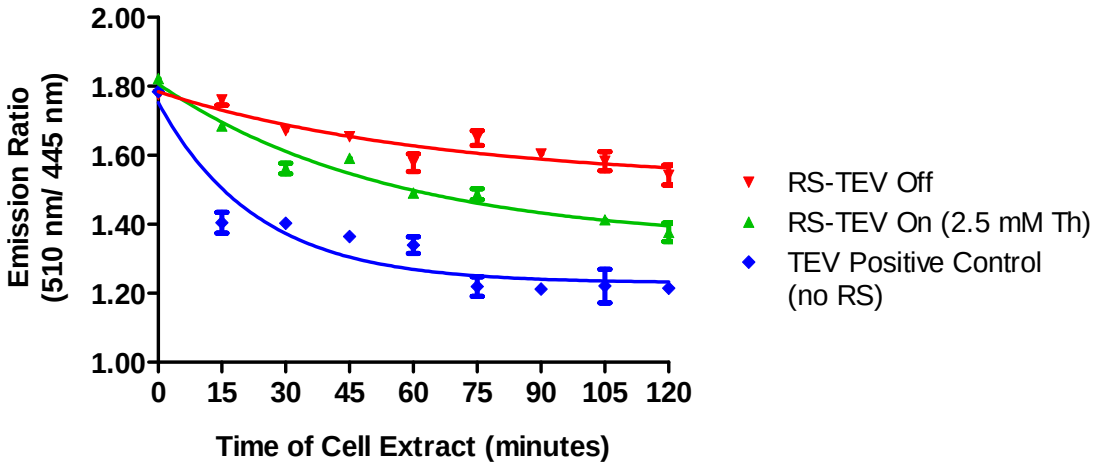


Figure : Emission Ratio from TEV Plasmid Assay
Excitation at 365 nm. n=2, error bars represent s.e.m. Data analyzed using non-linear one phase exponential decay on GraphPad Prism version 5.01 for Windows.

Results from the TEV Plasmid assay and subsequent statistical analysis further validated the effectiveness of the riboswitch to regulate gene expression in the presence of theophylline. Results also demonstrated that the presence of the riboswitch on the TEV plasmid reduced protein expression, resulting in a high signal ratio than the TEV positive control which does not harbor the riboswitch.

Two-way ANOVA indicated significance between time points ($p < 0.0001$), treatments ($p = 0.0009$), and all interactions ($p < 0.0001$). The Bonferroni post tests indicated statistical difference between the TEV positive control and both RS-TEV On and RS-TEV Off treatments (see Table 9). The mixed significance levels between the RS-TEV On and RS-TEV Off are likely due to the small number of replicates and the early time points. The results of the TEV Plasmid Assay should parallel the *in vitro* analyses; however, comparisons could not be generated due to the differing experimental treatments.

Table : Statistical Analysis for *In Vivo* TEV Plasmid Assay

	Time (minutes)								
Bonferroni Comparisons	0	15	30	45	60	75	90	105	120
Positive Control TEV & RS-TEV On	ns	***	***	***	***	***	md	***	***
Positive Control TEV & RS-TEV Off	ns	***	***	***	***	***	md	***	***
RS-TEV On & RS-TEV Off	ns	ns	**	ns	*	***	md	***	***

Data analyzed using two-way ANOVA with Bonferroni post-test on GraphPad Prism version 5.01 for Windows. Legend: ns = not significant, * $p < 0.05$; ** $p < 0.01$;*** $p < 0.001$, md = missing data

The data indicated that the riboswitch in the RS-TEV off treatment did not completely repress expression of the TEV protease; otherwise there should be no change in emission signal over the 120 minute time course.

Summary

In summary, both *in vivo* assays demonstrated the ability of the sensing element and reporter pathway to signal the presence of an analyte of interest. At least 60 minutes were required to distinguish the reporter signal from a control, whereas 240 minutes were required to distinguish between the on, off, and non-specific analyst reporter signals. These studied also provided evidence that introduction of the riboswitch impacts the rate of TEV protease production, but no protease-substrate activity. Background noise lowered the signal output but did not significantly affect interpretation of the results.

V. Conclusions

Overview

The research outlined in this thesis supports the larger research project by AFRL/HE to develop a modular synthetic biology-based system that detects and reports the presence of a threat agent. The focus of this thesis was to characterize the enzyme-substrate interactions in the synthetic system to accurately report the presence of a molecule of interest. Specifically, I investigated the signal output resulting from tobacco etch virus (TEV) protease cleavage of a fluorescence resonance energy transfer (FRET) protein. The investigation included evaluation of the theophylline-dependent riboswitch sensing element to produce a reportable signal. This chapter provides a summary of the study by revisiting the research questions initially posed in addition to a description of continuing studies and recommendations for future research.

Research Questions Revisited

1. Does the TEV protease and fluorescent fusion protein interaction result in a robust output signal?

Both *in vitro* and *in vivo* analyses demonstrated detectable and differentiated signals when compared to a control. For *in vitro* evaluation, the fluorescent signal strength was dependent on enzyme concentration. A minimal enzyme: substrate ratio of 1:2 was required for a valid signal. The *in vivo* reactions also emitted a differentiated signal but produced background noise which interfered with the signal output.

2. How will enzyme-substrate interaction behave in different systems?

a. Buffer System, in vitro transcription translation system (IVTT), and in vivo system

The TEV protease and FRET protein interactions effectively demonstrated a defined and differentiated output signal to be of value in a sensing and the reporter system. *In vitro* assays were more effective for screening modified enzymes or substrates in the reaction mixture. Should any components of the reporter pathway be changed, *in vitro* analysis using the Buffer system would validate the reaction, while the IVTT system would provide insight into expected signal output using *in vivo* techniques. Both analyses can be performed in one day without the time consuming culture growth and protein extraction steps. *In vivo* studies validated production of TEV protease and FRET protein using the cell's machinery to translate and transcribe their genes. The cellular environment may be more adaptable to platform design for field use or pathway encapsulation.

b. What is the time factor for the earliest distinguishable output signal?

Differentiated output signals from the reporter pathway reaction were detectable as early as 60 minutes using *in vitro* techniques. With the introduction of a genetic sensing element for the *in vivo* studies, a detectable signal was observed after 60 minutes when compared to a negative control, but 240 minutes was required to distinguish activity between the target analyte and a competitor.

3. *Will introduction of a sensing element (riboswitch) negatively affect the downstream reporter system and output signal?*

Riboswitch insertion only applied to the *in vivo* assessment. The sensing element was able to regulate transcription activity in a ligand-dependent fashion, but required a minimum reaction time of 240 minutes reaction to ensure the signal could be distinguished from a false positive result. The presence of the riboswitch reduced TEV protease expression and impacted signal output as compared to the TEV protease gene without the upstream riboswitch.

The TEV Protease Assay demonstrated the impact of signal interference from background fluorescence. It reinforced results for the Two Plasmid Assay by demonstrating the riboswitch gene regulation in the presence or absence of theophylline. Because the replications for this experiment were low (n=2), the experiment should be repeated to gain more insight into the kinetic behavior of the TEV protease generated from different plasmid designs.

The background fluorescence from cellular molecules found in the IVTT and *in vivo* experiments was a major factor that interfered with the reporter system design.

Continuing and Future Research

Experimentation performed for this thesis supported a larger research design evaluated by the Air Force Research Laboratory (AFRL). Progress on the sensing and reporter system proof-of-concept was reported to the Air Force Office of Scientific Research (AFOSR) in January 2008. AFOSR authorized additional studies on encapsulation systems and riboswitch design modifications to recognize different ligands. AFRL/RH is evaluating an alternate FRET protein construct, based on research

performed by Ganesan *et al.* (2006), which squelches output signal in its fusion protein form but emits a signal after catalytic cleavage.

This research validated genetic control by riboswitch insertion upstream of an enzyme gene. One recommendation for future research is to evaluation of alternative reporter designs, such as substituting the enzyme-substrate pathway with a direct riboswitch control of the fluorescent protein gene. Yen *et al.* (2004) reported insertion of a self-cleaving ribozyme upstream of the reporter gene. In the absence of a regulator ("off" mode), the reporter protein was not expressed because the mRNA coded for self-cleavage and destruction. In the presence of a regulator, the self-cleaving RNA motif was inactivated and protein translation proceeded. Simplifying the sensing and reporter system potentially reduces interference from other biological activities and may increase modularity. Such evaluations would need to be considered when investigating encapsulation systems.

Appendix A: Acronyms and Abbreviations

Acronym		Definition
AFOSR		Air Force Office of Scientific Research
AFRL		Air Force Research Laboratory
DNA		deoxyribonucleic acid
	cDNA	complementary DNA
DTRA		Defense Threat Reduction Agency
E. coli		*Escherichia coli*
FRET		fluorescence resonance energy transfer
GFP mutants		green fluorescent protein
	BFP	blue fluorescent protein
	CFP	cyan fluorescent protein
	eGFP	enhanced green fluorescent protein
	YFP	yellow fluorescent protein
IED		improvised explosive device
IVTT		*in vitro* transcription/translation
N		nitrogen
NIa		Nuclear Inclusion a
p		probability
PAGE		polyacrylamide gel electrophoresis
PCR		polymerase chain reaction
	qPCR	real-time polymerase chain reaction
	RT-PCR	real time polymerase chain reaction
PURE		Protein synthesis Using Recombinant Elements
QDR		Quadrennial Defense Review
RBS		ribosome binding site
RH		Human Effectiveness Directorate
RNA		ribonucleic acid
	mRNA	messenger RNA
rpm		revolutions per minute
RS-TEV		riboswitch-tobacco etch virus
s.e.m.		standard error of the mean
SPAWAR		Space and Naval Warfare Center
TEV		tobacco etch virus

Appendix A: Acronyms and Abbreviations (continued)

	Acronym	Definition
Amino Acids:		
	Asn (N)	asparagine
	Asp (D)	aspartic acid
	Gln (Q)	glutamine
	Glu (E)	glutamic acid
	Gly (G)	glycine
	His (H)	histidine
	Ile (I)	isoleucine
	Leu (L)	leucine
	Phe (F)	phenylalanine
	Pro (P)	proline
	Ser (S)	serine
	Thr (T)	threonine
	Tyr (Y)	tyrosine
Chemicals:		
	BCA	bicinchoninic acid
	β-ME	2-mercaptoethanol
	DEPC	diethylpyrocarbonate
	DMSO	dimethyl sulfoxide
	DTT	dithiothreitol
	EDTA	ethylene diaminete traacetic acid
	EtBr	ethidium bromide
	EtOH	ethanol
	HCl	hydrogen chloride
	IPTG	isopropyl-beta-D-thiogalactopyranoside
	KCl	potassium chloride
	LB media/agar	Luria-Bertani media/agar
	$MgCl_2$	magnesium chloride hexahydride
	$MgSO_4$	magnesium sulfate
	NaCl	sodium cloride
	Ni^{2+}	Nickel (elemental)
	SDS	sodium dodecyl sulfate
	SOC	Super Optimal Catabolite
	TAE	Tris-Acetate-EDTA
	TBE	Tris-Borate-EDTA
	TE	Tris-EDTA
	Tris	trishydroxymethylaminomethane

Appendix A: Acronyms and Abbreviations (continued)

	Acronym	Definition
measurements		
	Å	angstrom
	^{o}C	degrees Celsius
	kb	kilo base
	kDa	kilo Dalton (measure of atomic mass)
	OD_{600}	optical density at 600 nm
	pH	measure of acidity or alkalinity
	mg/mg	milligram/milliliter (10^{-3})
	μg/μg	microgram/microliter (10^{-6})
	ng/nm	nanogram/nanometer (10^{-9})
	μM/mM	micromolar/millimolar

Appendix B: Normalizing Kinetic Data

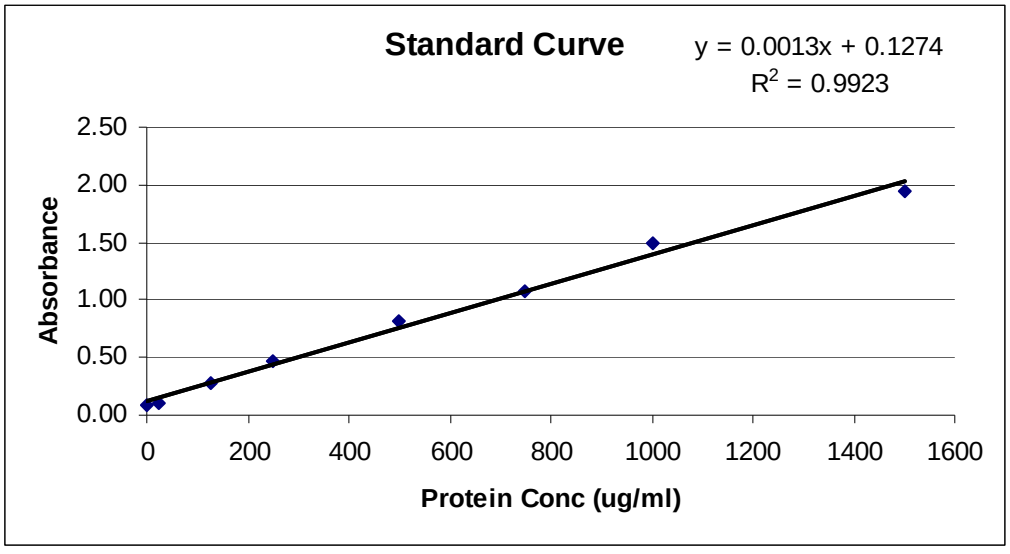
Materials: Bicinchoninic acid (BCA®) Protein Assay was purchased from Pierce.

Step 1: Measure absorbance readings of standard protein concentration over a range of concentrations.

Results:

Standard concentration (ug/ml)	Abs	Abs	Avg Abs
0	0.0860	0.0854	0.0857
25	0.1118	0.1105	0.1112
125	0.2833	0.2799	0.2816
250	0.4682	0.4710	0.4696
500	0.8246	0.8142	0.8194
750	1.0889	1.0812	1.0851
1000	1.4888	1.4963	1.4926
1500	1.8201	2.0673	1.9437

Step 2: Develop standard curve by plotting average absorbance by standard concentration. Plot a trend line and define formula



Appendix B: Normalizing Kinetic Data (continued)

Step 3: Use the slope of the line and y-intercept to determine total protein concentration in sample.

Results:

Sample	Abs	Abs	Avg Abs	Calculated Concentration (ug/ml)	**Conc (mg/ml)**
TEV Positive Control	0.7045	0.6649	0.6847	4286.9	**4.29**
RS-TEV Off	0.7831	0.8277	0.8054	5215.4	**5.22**
RS-TEV On	0.7985	0.7230	0.7608	4871.9	**4.87**

Step 4: Calculate total protein concentration per sample. To equalize protein concentrations between treatments, use a ratio of protein concentration of sample ÷ protein concentration of control, then multiply ratio by emission ratio of sample. For example:

$$\left(\frac{\text{RS-TEV Off}}{\text{TEV Positive Control}}\right) * \text{RS-TEV Off Emission Ratio}$$

$$= \text{Normalized RS-TEV Off Emission Ratio}$$

Sample emission ratio data:

	RS-TEV Off	
raw data	1.3685	1.3817

$$\left(\frac{5.22}{4.29}\right) * (1.368) = 1.665$$

Sample Normalized emission ratio data:

	RS-TEV Off	
raw data	1.3685	1.3817
normalized	1.6652	1.6812

Step 5: Plot and analyze normalized data.

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Air Force Institute of Technology
Graduate School of Engineering and Management (AFIT/ENV)
2950 Hobson Way
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Kelley-Loughnane, Nancy. AFRL/RHPB
Air Force Research Laboratory
WPAFB, Bldg 837, Area B, 2729 R St.
WPAFB OH 45433-7765
Tel: 937-904-9498

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14. ABSTRACT

Chemical and biological threats are ever present and attacks have occurred throughout the world in both war and peace-time. Multiple government agencies, academia, and private industry are developing detection capabilities to address such threats. The research presented in this paper supports development of a modular synthetic biology-based system that detects and reports the presence of a threat agent. Synthetic biology builds upon past research in genetic engineering and seeks to combine broad applications within biotechnology in novel ways. This basic research project will help to demonstrate a proof-of-concept design which will guide future studies on the development of a modular sensor platform. In this study, *in vitro* and *in vivo* techniques were used to evaluate a reporter system composed of the Tobacco Etch Virus (TEV) protease and an engineered fluorescent fusion protein for their combined effectiveness as a reporter system. The TEV protease demonstrated the ability to cleave the fusion protein construct to produce a measureable output signal. The coupling of this reporter system with a riboswitch recognition element showed that this system can be applied to detecting chemicals. Further studies to modify this recognition element suggest modularity for future biosensors to detect chemicals of military interest.

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U	**U**	**U**	**UU**	97	(937) 255-3636, ext 4721 email: Charles.bleckmann@afit.edu