Development of Luminescent Tools for Use in the Study of Mycobacterium tuberculosis

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Krista A. Moore
University of Central Florida

DEVELOPMENT OF LUMINESCENT TOOLS FOR USE IN THE STUDY OF *MYCOBACTERIUM TUBERCULOSIS*

by

KRISTA A. MOORE

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ABSTRACT

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis, is a growing problem worldwide due to the emergence of multi-drug resistant and extensively-drug resistant strains of the bacteria. A key to combatting the spread of these strains lies in the understanding of gene expression occurring in *Mtb*. This study focuses on the development and optimization of a luciferase-based bioluminescent transcriptional reporter that can be used to monitor gene expression in *Mtb*. The luminescent signal emitted from the reporter can be measured and correlated with the level of transcription of certain genes. This study focuses specifically on a gene called *whiB7* which encodes a transcription factor known to contribute to the drug resistance of *Mtb*. The drug-inducible *whiB7* promoter was cloned into various locations in the luciferase plasmid in order to determine the ideal configuration of the reporter for maximum luminescence. The optimized luciferase reporter was then compared with a fluorescent transcriptional reporter, mCherry, also under control of the *whiB7* promoter. Fluorescent reporters present some disadvantages including delayed kinetics and inability to accurately reflect gene downregulation due to long half-life of reporter proteins. It was hypothesized that the luciferase reporter would solve these problems by offering a more sensitive and dynamic tool to monitor gene expression. Quantitative real-time PCR was used to measure *whiB7* mRNA present in cultures containing either the luciferase or mCherry reporters. The luminescent and fluorescent signal given from these reporters was then compared to actual mRNA expression. It was observed that the signal from the luciferase reporter more closely matched mRNA expression at each timepoint, indicating that the luciferase reporter is a better gauge of actual gene expression levels than the mCherry reporter.

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DEDICATION

This thesis is dedicated to the memory of W. Wayne McDonald and William Donald Moore, my loving grandfathers and enthusiastic supporters.

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1. INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), the deadliest infectious disease globally. In fact, in 2016 there were 1.7 million reported deaths from TB [1]. This disease primarily affects the lungs and respiration, but may spread to other organ systems. Thanks to developments in the treatment of TB with antibiotics, the annual number of TB deaths is dropping. The standard recommended treatment for TB involves at least a six-month regimen of four antibiotics: isoniazid, rifampicin, pyrazinamide, and ethambutol [2]. However, the emergence of drug resistant strains of TB has become a growing concern among scientists and physicians. Approximately twenty percent of TB isolates globally are estimated to be resistant to at least one major antibiotic [3]. Multi-drug resistant (MDR) TB is defined by the World Health Organization as resistance to at least isoniazid and rifampicin while extensively drug-resistant (XDR) TB has the same characteristics as MDR but also has resistance to fluoroquinolones and second-line injectable drugs. XDR TB has even evolved to totally drug resistant (TDR) strains in some parts of the world that have endemic TB, including China, India, and South Africa [3].

1.1 Drug resistance

Drug resistance can arise because of mutations occurring in the TB genome that cause drug targets or certain enzymes to be altered or due to the activation of efflux pumps used to remove drug when it enters the bacterium [3]. Additionally, poor compliance of patients with the extensive drug regimen required to treat TB can be a problem that ultimately leads to the development of drug-resistant strains of TB. However, studies have shown that even when a patient follows his or her drug regimen precisely and the treatment appears curative, some active

Mtb may still be present in the patient [4]. Most patients with MDR or XDR TB present first with resistance to one antibiotic, then over time clonal selection allows these resistant bacteria to outcompete others. These more impervious bacteria will develop other single-nucleotide polymorphisms or mutations, allowing them to gain resistance to other antibiotics as well. This method of developing resistance is termed secondary resistance [3]. Primary resistance, on the other hand, occurs when already resistant strains are transmitted from an infected to non-infected host [3].

The threat to the public that MDR and XDR TB pose cannot be understated. In South Africa, for example, the MDR and XDR TB typically make up only 5% of TB cases, but these cases are estimated to utilize over one-third of the resources allocated to the management of TB [3]. With the rise of international travel and migration, TB is no longer only a threat to individual countries, but to nations around the world. Therefore, because of the global threat to public health that MDR and XDR TB pose, the need for the identification of new drugs and drug targets in TB is becoming increasingly important. In order to identify new drug targets, host-pathogen interactions involved in TB infection must be understood.

1.2 Gene Expression

Mtb has evolved extensive mechanisms to prevent eradication by the host immune system. One method that *Mtb* uses for survival is the ability to colonize and replicate within host macrophages, immune cells that typically break down pathogens to prevent them from further invading host tissue [5]. The adaptations used by *Mtb* to survive in such a hostile environment are still under investigation. However, it is known that differential gene expression is harnessed by *Mtb* over the course of infection to respond and adapt to the many challenges presented by the

host immune system [6]. These gene expression differences may also be central to the mechanisms used by *Mtb* to resist the harsh effects presented by many antibiotics.

Gene expression can be monitored *in vitro* and *in vivo* using multiple methods including quantitative real-time PCR (qRT-PCR), fluorescent reporters, and bioluminescent transcriptional reporters. This study will focus on developing and optimizing a bioluminescent transcriptional reporter for use in the study of *Mtb* and comparing the efficacy of this reporter with other commonly used reporter technologies.

1.3 Transcriptional Reporters

A transcriptional reporter is a fusion of genetic regulatory elements to reporter proteins that produce detectable outputs, allowing gene expression to be monitored [7]. There are many different types of transcriptional reporters currently in use, including those based on fluorescent proteins, colorimetric enzymes, and drug resistance genes. These reporters are often used to study how transcription is affected by different factors such as the presence of drugs or a change in environmental stimuli.

Transcriptional reporter genes have multiple uses both in the study of *Mtb* and in the study of other bacteria. For example, these transcriptional reporters have been used to identify the mechanism of action of drugs against TB [8], for measuring dynamic protein-protein interactions in bacterial systems that may be important for pathogenesis [9], and in *in vivo* in live mice to view the localization of TB throughout infection [10].

1.4 Fluorescent Transcriptional Reporters

Fluorescent transcriptional reporters are commonly used in the study of *Mtb*, including the mCherry reporter. mCherry is a red fluorescent protein derived from the *Discosoma* sp.

mushroom coral [11]. mCherry is widely used because of its monomeric structure, high brightness, and fast maturation [11]. However, mCherry does have some disadvantages when compared to other transcriptional reporter technologies. While the fluorescent signal from mCherry is bright, detectable signal does not accurately reflect transcript levels. It may take hours for this signal to build up to a detectable level as the fluorescent protein accumulates. The mCherry protein also takes time to fold and mature to an actively fluorescent form. This may hinder understanding of the kinetics of gene expression in response to certain stimuli. In addition, the mCherry reporter can only show upregulation of transcription signaling because the fluorescent protein has a long half-life and does not degrade efficiently [7]. Because of this, once the fluorescence is stimulated, its levels will not decrease again to show decreasing transcription. For this reason, alternatives to fluorescent reporters that may offer more dynamic readings of gene expression in real-time are being explored.

1.5 Bioluminescent Transcriptional Reporters

Bioluminescent transcriptional reporters may offer a solution to the problems presented by fluorescent transcriptional reporters. Bioluminescence is the production of light by chemical reactions catalyzed by an enzyme known as luciferase [10]. Luciferases are a family of enzymes that catalyze reactions in which a substrate, generally called a luciferin, is oxidized with associated production of light [10]. Light is emitted when the product, in an electronically excited state, emits a photon upon returning to ground state [12]. Multiple organisms naturally express luciferase, including fireflies, marine organisms, and certain bacteria. The luciferase system used in this study originates from the bacterium *Photorhabdus luminescens*. This

luciferase system was chosen because it does not require exogenously added substrate, making its use simpler as well as less toxic to living cells [12].

The *Photorhabdus luminescens* luciferase is encoded by an operon called *luxCDABE***.** The *luxAB* genes are responsible for encoding the luciferase enzyme, an αβ-heterodimeric luciferase [13]. The *luxCDE* gene cluster encodes the enzymatic complex that synthesizes the substrate, a long-chain aldehyde [13]. The bioluminescence reaction occurs as follows [12]:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + light$$

Luciferase binds to reduced flavin mononucleotide ($FMNH_2$), molecular oxygen, and a long-chain fatty aldehyde. A redox reaction occurs in which $FMNH_2$ is oxidized to flavin mononucleotide (FMN), and the fatty aldehyde is oxidized to a fatty acid. This process emits a blue photon with a spectral emission maximum of 490 nm [12]. The intensity of this blue light can be measured using a spectrometer. Through genetic engineering of the *luxCDABE* plasmid, the luciferase operon can be used as a tool to measure transcription.

The luciferase plasmid used in this experiment, *pMV306Lux+Hsp60+G13,* contains the luciferase operon split into two segments, *LuxAB* and *LuxCDE,* because this configuration yields the best reporter performance [13]. This plasmid includes the *LuxAB* segment under the control of a constitutive promoter *hsp60* (P_{hsp60}) which controls the formation of enzyme. The *LuxCDE* segment is under the control of the constitutive *G13* promoter (P_{G13}), controlling the formation of the substrate [13]. Previous studies performed in *E. coli* have also shown that in order to get the highest signal possible, the *LuxAB* genes should be under the control of an inducible promoter while the *LuxCDE* genes should be under the control of a constitutive promoter [13]. This result shows that the availability of aldehyde, the substrate formed by *LuxCDE,* is the rate limiting

factor in this operon, so it should be constitutively available. This study will involve replacing either of the two constitutive promoters in *pMV306Lux+Hsp60+G13* with a drug-inducible promoter. In this way, the promoter configuration that gives the highest signal and most accurate dynamic readout will be optimized for use in future studies in *Mycobacteria*.

1.6 The *whiB7* Gene

In order to evaluate different luciferase reporter configurations for optimization, a well-characterized, drug-inducible promoter, P_{whiB7}, was used. WhiB7 is a transcriptional regulator of certain genes in *Mtb* that contribute to the intrinsic multi-drug resistance seen in the bacteria [14]. P_{whiB7} responds to the presence of certain antibiotics that inhibit protein synthesis by activating its own expression. WhiB7 also acts as a transcriptional activator of many drug resistance genes [15]. Specifically, WhiB7 binds to a protein known as SigA, a primary sigma factor subunit of RNA polymerase [15]. In this way, WhiB7 is able to activate systems of intrinsic drug resistance including antibiotic export systems such as efflux pumps, antibiotic inactivation systems that modify certain drugs or drug targets, and redox balance changes within the bacteria [16]. Therefore, monitoring the gene expression of the WhiB7 transcriptional activator in response to different environmental stimuli could lead to important insights into how to block this protein from stimulating intrinsic antibiotic resistance.

A homolog for the *whiB7* gene in *Mtb* exists in *Mycobacterium smegmatis (Msm),* a relative of *Mtb* that can be safely used in Biosafety Level 2 laboratories and has been shown to be induced by similar antibiotics as *Mtb* P_{whiB7} [14]. Therefore, *Msm* P_{whiB7} can be induced by the addition of the antibiotics azithromycin (AZT) and clarithromycin (CLR). These drugs are classified as macrolides which bind to the 50S subunit of the bacterial ribosome and inhibit

protein synthesis [17]. When these antibiotics are added to cultures of *Msm* containing the luciferase operon under control of P_{whiB7}, P_{whiB7} will be induced, resulting in a higher luminescent output compared to the unmodified luciferase operon. In order to monitor *whiB7* gene expression, P_{whiB7} will be cloned into the *pMV306Lux+Hsp60+G13* plasmid in place of P_{G13} or P_{hsp60}. These modified transcriptional reporters will be used to optimize the luciferase operon for monitoring gene expression in *Mycobacteria* as well as to test the efficacy of the luciferase system in comparison to other reporters.

1.7 Measuring Real-Time Gene Expression

In order to determine which reporter—luciferase or mCherry— is most accurate, expression of *whiB7* RNA in *Msm* must be measured in real-time. Quantitative real-time PCR (qRT-PCR) will be used to determine the level of *whiB7* RNA being made by *Msm* containing the luciferase or mCherry reporters at specific time points after induction by antibiotics. This real-time expression level will be compared to the luminescent and fluorescent signals being given off by each transcriptional reporter at each time point. This will allow conclusions to be drawn about which reporter gives readings with kinetics that are most similar to real-time gene expression. This study seeks to validate that the luciferase transcriptional reporter offers measurements of transcription that are closer to real-time than those seen using the fluorescent mCherry reporter.

2. OBJECTIVES

Objective 1: Development of a Luciferase-Based Transcriptional Reporter

A luciferase-based transcriptional reporter will be developed by modifying the *lucCDABE* operon in the plasmid *pMV306Lux+Hsp60+G13*. The constitutive promoters controlling the *LuxAB* genes and the *LuxCDE* genes will be replaced with the inducible, P_{whiB7}, causing the reporter to respond to the presence of antibiotics. The luminescence of bacteria containing one of these new reporter plasmids will be measured after exposure to certain antibiotics.

Objective 2: Comparative Analysis of the Luciferase Transcriptional Reporter

The signaling of the luciferase reporter will be compared to that of another commonly used fluorescent transcriptional reporter, mCherry, also under the control of the drug-inducible P_{whiB7}. Quantitative real-time PCR will be performed on the bacteria containing either the luciferase or mCherry reporters to compare luminescent and fluorescent readouts with actual mRNA levels.

Objective 3: Enhancing Luminescent Signaling

To make the luminescent signal brighter, the *pMV306Lux+Hsp60+G13* plasmid will be modified further by cloning an additional FMN reductase into the operon. This new FMN reductase is expected to add more reducing power to the operon, creating a brighter luminescent signal that is easier to detect.

3. METHODS

3.1 FastCloning

pMV306Lux+hsp60+WhiB7, pMV306Lux+WhiB7+G13, and *pVVRG+WhiB7* were created through modification of the *pMV306Lux+Hsp60+G13* plasmid. This plasmid, derived from *Photorhabdus luminescens*, contains the *luxAB* genes under control of constitutive P_{hsp60} and the *luxCDE* genes under control of constitutive P_{G13} [10]. The promoters in this plasmid were replaced by P_{whiB7} using a method called FastCloning [18]. FastCloning is a ligation-free cloning technique that can be used to insert a segment of DNA, referred to as an insert, into a larger vector. Primers with overhangs corresponding to the reverse complement of the desired insertion site in the vector were designed and used to amplify the insert piece through polymerase chain reaction (PCR). To amplify the vector piece, a pair of primers were created flanking the site of insertion. The following components were used in the FastCloning PCR reaction: 5 μL 1x GC Buffer, 0.2 mM dNTPs, 0.25 μL Phusion, 16.25 μL deionized water, 1 μL of 10 mM forward primer, 1 μL of 10 mM reverse primer, and 20 ng/μL template DNA. The following PCR conditions were used to amplify both the insert and the vector pieces:

Table 1: PCR Reaction Conditions

PCR Stage	Time (s)	Temperature (°C)	Repeats
Initial Melting	30	98	1x
Melting	10	98	20x
Annealing	30	Varies according to annealing temperature of primers	20x
Extension	15/ kilobase of DNA replicated	72	20x
Final Extension	180	72	1x

After PCR amplification, the sizes of the vector and insert pieces were verified by gel electrophoresis. Once the desired pieces were obtained, they were combined in varying ratios: 4:1 vector to insert, 1:4 vector to insert, and 1:1 vector to insert. Each mixture was then incubated with an enzyme called Dpn1 for at least one hour. Dpn1 acted to digest any remaining unamplified plasmid DNA by targeting methylated DNA. The length of the Dpn1 incubation may be extended overnight in order to ensure that all unamplified plasmid DNA was removed. Next, the recombinant plasmids were transformed into *E. coli* cells. 2 μL of each reaction mix was introduced to NEB 10-Beta Competent *E. coli* and heat shocked at 42°C for 30 seconds. The cells were allowed to incubate on ice for 5 minutes before 250 μL of SOC outgrowth media was added. The cells were then shaken at 250 rpm and 37°C for one hour before plating. The plasmids used in this study contained kanamycin resistance genes to allow for the selection of colonies containing the recombinant plasmid. Therefore, LB agar plates containing 50 μg/μL

kanamycin (K50) were used to select for positive colonies containing the plasmid of interest. After growth of the *E. coli* at 37°C for 24 hours, colony PCR was used to further identify positive clones containing the plasmid with the correct insertion. A small portion of each colony was removed from the plate with a sterilized toothpick, placed in 20 μL deionized water, and boiled for 5 minutes. The lysate that resulted was used as template DNA for PCR reactions using primers flanking the region of insertion. Gel electrophoresis was performed to identify the size of the insertion, with one specific size correlating to proper gene insertion. The bacteria containing the verified recombinant plasmids were grown up in culture and the plasmid DNA was purified using a Qiagen Miniprep kit. This purified plasmid DNA was then sent for sequencing by Eurofins.

3.2 Blunt Cloning

The *pMV306Lux+WhiB7+LuxG13* plasmid was created through modification of the *pMV306Lux+Hsp60+G13* using a different cloning technique called Blunt Cloning. Primers were designed to match each end of the piece of DNA that was inserted into the vector. These primers were then phosphorylated using the following reaction mixture:

Table 2: Primer Phosphorylation Components

Component	Volume (μL)
Deionized Water	37
10X Kinase Buffer	5
$MgSO_4$ (50 mM)	1
Primer (10 μM)	5
ATP (100 mM)	1
T4 Polynucleotide Kinase	1

These components were then incubated for 60 minutes at 37°C, then the T4 polynucleotide kinase was inactivated by incubation for 5 minutes at 65°C. The insert and vector pieces were then amplified with the same PCR components and conditions as used in FastCloning, except phosphorylated primers were used. The vector and insert fragments were visualized through gel electrophoresis to verify that they were the correct size. The vector and insert samples were then mixed in equal proportions and digested with Dpn1 for at least one hour at 37°C. The vector and insert were ligated using the following reaction mix:

Table 3: Blunt Cloning Ligation Components

Component	Volume (μL)
Deionized Water	8.75
Vector/Insert Mix	3
ATP (10 mM)	0.75
10X Fast-Link Buffer	1.5
Fast-Link DNA Ligase	1

The reaction mixture was incubated for 30 minutes at room temperature, then heat inactivated at 70°C for 15 minutes. The recombinant plasmid was then transformed into *E. coli* as described previously and screened appropriately to verify proper insertion.

3.3 Luminescence Assay

The luminescence of the bacteria containing the newly created luciferase plasmids was tested using the luminescence assay and compared to the luminescence levels of bacteria containing the unmodified luciferase plasmid. The luciferase reporters were first transformed by electroporation into *M. smegmatis (Msm)* competent cells. The *Msm* competent cells were prepared by growing *Msm* in 15 mL of 7H9 media until an optical density (OD) of 0.6. The cells were then pelleted and the supernatant was removed. The cells were washed with 7.5 mL of cold glycerol, pelleted, and washed again with 7.5 mL of cold glycerol. The cells were then pelleted and washed with 1.5 mL of 7H9. The cells were pelleted once more and resuspended in 0.6 mL of 7H9. 100 mL aliquots of these cells were made and stored at -80°C.

To transform the luciferase reporter plasmids into these electrocompetent *Msm*, 200-500 ng of DNA was added to 100 μL of *Msm* competent cells. These cells were electroporated using a BioRad Gene Pulse X at 2500 V, a capacitance of 25 μF, and a resistance of 1000Ω. The electroporated cells were added to 250 μL of LB Tween 80 media, and shaken for 4 hours at 37°C. Positive clones were selected for by plating on LB agar containing K50. The *Msm* containing the luciferase reporter were able to grow on these plates, and these colonies were picked and grown up in 5 mL of LB Tween containing 5 μL of K50. These cultures were grown until an OD of 0.6 was reached. The *Msm* culture was then added to a white 96-well plate along with two drugs known to induce the macrolide-sensitive P_{whiB7}: azithromycin (AZT) and clarithromycin (CLR) [14]. 90 μL of culture was added to each well along with 10 μL of each drug at the appropriate concentration (determined in the MIC experiment in section 3.5). The plate was incubated at 37°C and luminescence and optical density readings of each well was taken by the Synergy H4 plate reader. These measurements were recorded at 0, 2, 4, 24, 48, and 72 hours post-induction.

3.4 Fluorescence Assay

A fluorescent transcriptional reporter called mCherry was used to compare luminescent and fluorescent reporters. Using FastCloning, the P_{whiB7} was placed 17 base pairs upstream of the mCherry gene in place of the *smyc* promoter in the *pVVRG* plasmid, creating the *pVVRG+WhiB7* reporter [19]. This recombinant plasmid was then transformed into *E. coli* competent cells and positive clones were identified by colony PCR and verified by sequencing. The reporter plasmid was purified using a Quiagen Miniprep Kit. The purified reporter plasmid was then electroporated into *Msm* as previously described. The *Msm* were grown up in culture to an OD

of 0.6 and placed in a black 96-well plate along with AZT and CLR. Fluorescence (excitation 580 nm and emission at 615 nm) and optical density measurements were taken on a Synergy H1 plate reader to parallel the luciferase assay at 0, 2, 4, 24, 48, and 72 hours post-induction.

3.5 Alamar Blue MIC Assay

The induction of P_{WhiB7} depends on the concentration of antibiotic to which the bacteria are exposed. For optimal performance of the transcriptional reporter, the concentration of antibiotic used in the luciferase assay must be enough to induce the activation of P_{WhiB7} without killing the bacteria themselves. This concentration was found by creating a minimum inhibitory concentration (MIC) curve using an Alamar Blue assay. The bacteria containing the luciferase plasmid were grown up to an optical density of 0.6. They were then filter sterilized and diluted to an optical density of 0.0005. 24 μL of culture was placed in a black, clear bottom 384 well plate along with 6 μL of AZT and CLR in descending concentrations. The plate was incubated at 37°C for two days. After this time, 6 μL of reduction indicator Alamar Blue (0.02 w/v resazurin) was added to each well and allowed to incubate for 4 hours. Alamar Blue was used to monitor the health and metabolic functioning of the cells. The dye changes from an oxidized blue state to a reduced pink state in the presence of living cells. Living cells maintain reducing power and turn the solution pink, increasing its fluorescence, while dead cells will not change the color of the dye [20]. The fluorescence of the bacteria in the presence of multiple concentrations of antibiotic was used to determine the MIC for each drug. At 4 hours, the fluorescence (excitation: 560nm, emission: 590 nm) was read using a Synergy 4 plate reader. The level of fluorescence indicated whether the bacteria were dead or alive. The data, expressed as percent growth, were fitted by a modified Gompertz model [21], and the MIC curve for each drug was generated using GraphPad

Prism (version 7.0). The MIC was defined as the lowest drug concentration at which 99% of the bacteria were killed.

3.6 Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was used to directly measure *whiB7* mRNA transcript levels for comparison with luminescent and fluorescent reporter readouts. First, RNA was isolated from broth culture containing either the luciferase or mCherry reporter and 0.01 μg/mL AZT. At timepoints of 0, 4, 24, 48, and 72 hours after addition of AZT, 15 mL aliquots of broth culture containing each reporter were collected and centrifuged at 3,500 xg for 20 minutes. The supernatant was removed and discarded, and the pellet was then washed and resuspended in 1 mL of GTC buffer (4 M guanidine thiocynate, 0.5% Na N-lauryl sarcosine, 25 mM sodium citrate, and 0.1 M β-mercaptoethanol). Each sample was then spun down again and washed with 1 mL of PBS + 0.1% Tween 80. Finally, the pellet was resuspended in 200 μL of 5mg/mL lysozyme and incubated for 15 minutes at room temperature. 0.75 mL of prewarmed Trizol was then added to each sample. The samples were then transferred to screw cap tubes containing 0.5 mL of 0.1 mm silica beads and beaten in the Mini-BeadBeater 16 by Biospec for 2 minutes at maximum speed. Then, 200 μL of chloroform was added to each tube, and tubes were spun for 15 minutes at 12,000 rpm. The upper aqueous phase was removed from each sample, collected, and placed in an RNase-free tube. 500 μL of 100% RNase free EtOH was added to each sample. The Qiagen RNeasy kit was then used to isolate the RNA. To ensure that no DNA contamination was present in the RNA, Invirogen's TurboDNAfree kit was used twice to DNase the samples. The RNA concentration was then measured with a Nanodrop.

The RNA samples were then converted to cDNA using BioRad's iScript cDNA Synthesis Kit. All RNA samples were first diluted to 50 ng/μL. Then, cDNA was synthesized and a no reverse transcriptase control was made for each sample according to the following reaction mixture:

Table 4: cDNA Reaction Components

+ Reverse Transcriptase Volume (μL)	- Reverse Transcriptase Volume (μL)	Component
5	5	5x iScript Reaction Mix
1.25	0	iScript Reverse Transcriptase
1.00	1.00	RNA (50 ng/μL)
12.75	14	RNase – Free Water
20	20	Total Volume

The following PCR conditions were used to amplify the cDNA:

Table 5: cDNA PCR Conditions

Temperature (°C)	Time (min)
25	5
42	30
85	5
4	∞

To detect the amount of *whiB7* RNA present in each sample, primers were created flanking a segment of the *sigA* gene (normalization control gene) and the *whiB7* gene in *Msm* chromosomal DNA. Then, BioRad's iTaq Universal SYBR Green Supermix Kit was used to create the following reaction: 1 μL of each cDNA was combined with 5 μL of iTaq SYBR green, 3 μL of RNase-free water, and 0.5 μL of forward primer and 0.5 μL of reverse primer. This mixture was placed in a Microamp Fast Optical 96-well plate. qRT-PCR of the samples was then run on the QuantStudio 7 Flex System using the following PCR cycle:

Table 6: qRT-PCR Conditions

Time (s)	Temperature (°C)	Ramp (°C/s)	Repeats
20	95	2.05	1x
10	95	2.05	40x
20	56	1.71	40x

3.7 Addition of FMN Reductase

Msm gene *2921*, an FMN reductase, was cloned using upstream of the luciferase operon using FastCloning in order to improve brightness of the luminescent signal emitted. Previous research by Gregor *et. al.* has shown that the addition of an FMN reductase to a luciferase reporter in *E. coli* improves reporter brightness by regenerating additional $FMNH_2$ for oxidation in the luciferase reaction [12]. Therefore, the addition of *Msm2921*, an FMN reductase, was hypothesized to increase the brightness of the luminescent signal from the luciferase reporter in *Msm.* First, *Msm2921* was placed under the control of constitutive P_{G13} using FastCloning as

described previously. Then, *G13+Msm2921* was cloned into *pMV306Lux+Hsp60+G13* upstream of *LuxAB,* creating a new reporter: *pMV306Lux+Hsp60+G13+Msm2921*. The recombinant plasmid was then transformed into *E. coli*, Miniprepped, sequenced, and transformed into *Msm.* Positive clones were selected for using growth on LB Tween K50 plates. Positive clones were then grown to an optical density of 0.6 in 5 mL of LB Tween 80 containing 5 μL K50. 100 μL of *Msm* containing *pMV306Lux+Hsp60+G13+Msm2921* were placed in a white 96-well plate and luminescence of the samples was read by a Synergy H1 Plate Reader.

4. RESULTS

4.1 Optimization of the Luciferase Transcriptional Reporter

pMV306Lux+Hsp60+G13 was edited so that P_{whiB7} would replace one of the two constitutive promoters located in the plasmid: P_{Hsp60} or P_{G13}. First, P_{G13} was replaced by the drug-inducible P_{whiB7}, creating a new reporter called *pMV306Lux+Hsp60+WhiB7*. The goal of this reporter was to emit a luminescent signal that correlates to the induction of P_{whiB7} due to the stress caused by AZT or CLR. Figure 1 below shows a single replicate of the experiment showing fold induction of the luminescent signal after addition of AZT (0.1 μg/mL) or CLR (0.01 μg/mL).

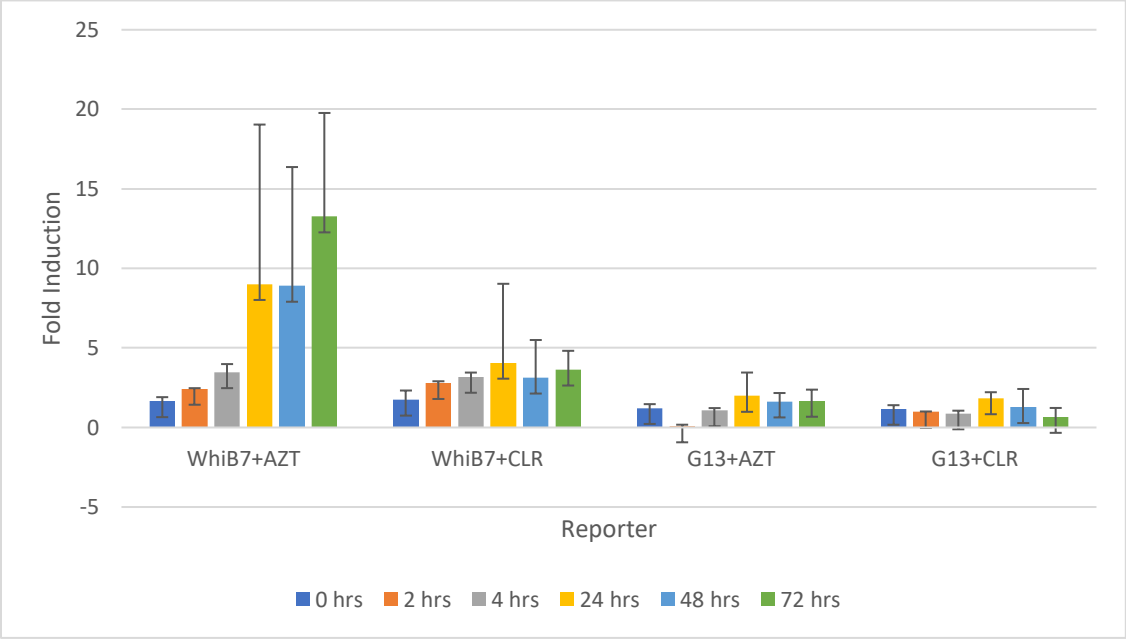

Figure 1: Induction of Luminescence of Unmodified Reporter Versus *pMV306Lux+Hsp60+WhiB7*. Fold induction refers to luminescence normalized to optical density and compared to the signal from the same reporter without induction by drugs. Error bars signify standard deviation in all figures, unless otherwise stated. A single replicate of the luminescence assay was performed in triplicate wells. The unmodified reporter was exposed to 0.1 μg/mL AZT (G13+AZT) and 0.01 μg/mL CLR (G13+CLR). The inducible substrate reporter was exposed to the same concentrations (WhiB7+AZT and WhiB7+CLR).

The fold induction values were normalized to the optical density of the culture at each timepoint. The cultures containing the unmodified *pMV306Lux+Hsp60+G13* reporter (labeled G13+AZT and G13+CLR) showed little induction of luminescent signal due to the lack of $P_{whiB7.}$ The cultures containing the *pMV306Lux+Hsp60+WhiB7* reporter (labeled WhiB7+AZT and WhiB7+CLR) showed a large induction in response to AZT and a moderate induction in response to CLR. This decreased level of induction was thought to be due to the use of a concentration of CLR that was too large, killing the bacteria.

4.2 Optimization of Drug Concentration

Because of the unexpected inconsistencies in luminescent signaling seen in reporters stimulated by AZT versus CLR, it was hypothesized that the concentration of CLR may be too high, causing the bacteria to die. Therefore, a Minimum Inhibitory Concentration (MIC) curve was created to determine the proper CLR concentration needed to induce P_{whiB7} without killing the bacteria. An MIC curve for AZT was also created to verify that the proper concentration of AZT was being used in the assay. The MIC experiment utilized the unmodified *pMV306Lux+Hsp60+G13* reporter (referred to as G13) and *pMV306Lux+Hsp60+WhiB7* reporter (referred to as WhiB7). The resulting MIC curves are shown in Figures 2 and 3. Table 7 shows the calculated MIC values for AZT and CLR.

Table 7: AZT and CLR MIC Values

	AZT	CLR
WhiB7 MIC (μg/mL)	0.4452	0.05669
G13 MIC (μg/mL)	0.4993	0.03085

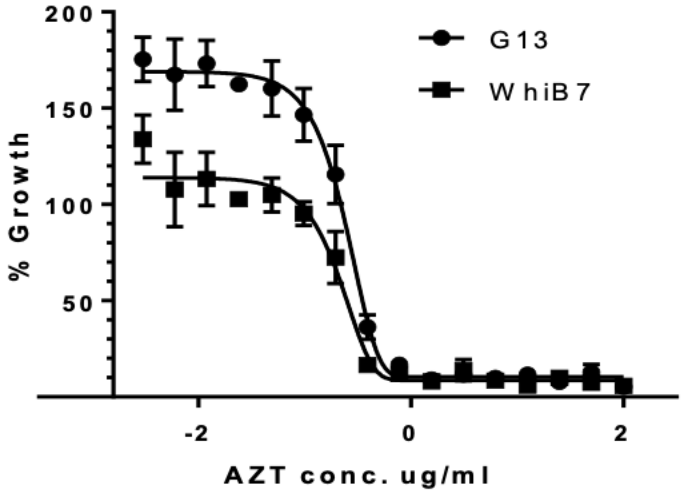

Figure 2: MIC Curve for AZT. Percent growth is shown for *Msm* containing either the unmodified reporter (G13) or the inducible substrate reporter (WhiB7) when exposed to decreasing concentrations of AZT.

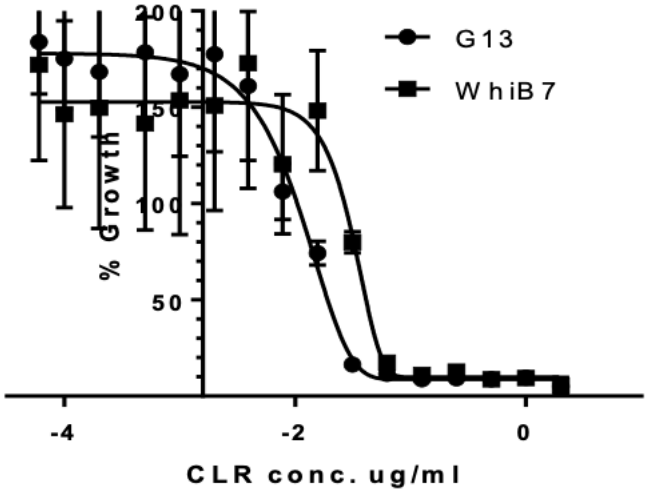

Figure 3: MIC Curve for CLR. Percent growth is shown for *Msm* containing either the unmodified reporter (G13) or the inducible substrate reporter (WhiB7) when exposed to decreasing concentrations of CLR.

Because the MIC value shows the lowest concentration of drug that prevents bacterial growth, it was confirmed that the appropriate concentration of AZT was used in the luminescence assay of less than 0.4 µg/mL. The CLR MIC was reported as 0.04 µg/mL, but 0.01µg/mL CLR as used in the experiment in Figure 1 was still not inducing the reporter. To further optimize the CLR concentration needed for maximum induction, a luminescence assay was run on the unmodified *pMV306Lux+Hsp60+G13* reporter (referred as G13+CLR) and the inducible *pMV306Lux+Hsp60+WhiB7* reporter (referred to as WhiB7+CLR). This assay was used to measure induction of these reporters in response to various concentrations of CLR. Results of this experiment can be seen in Figure 4.

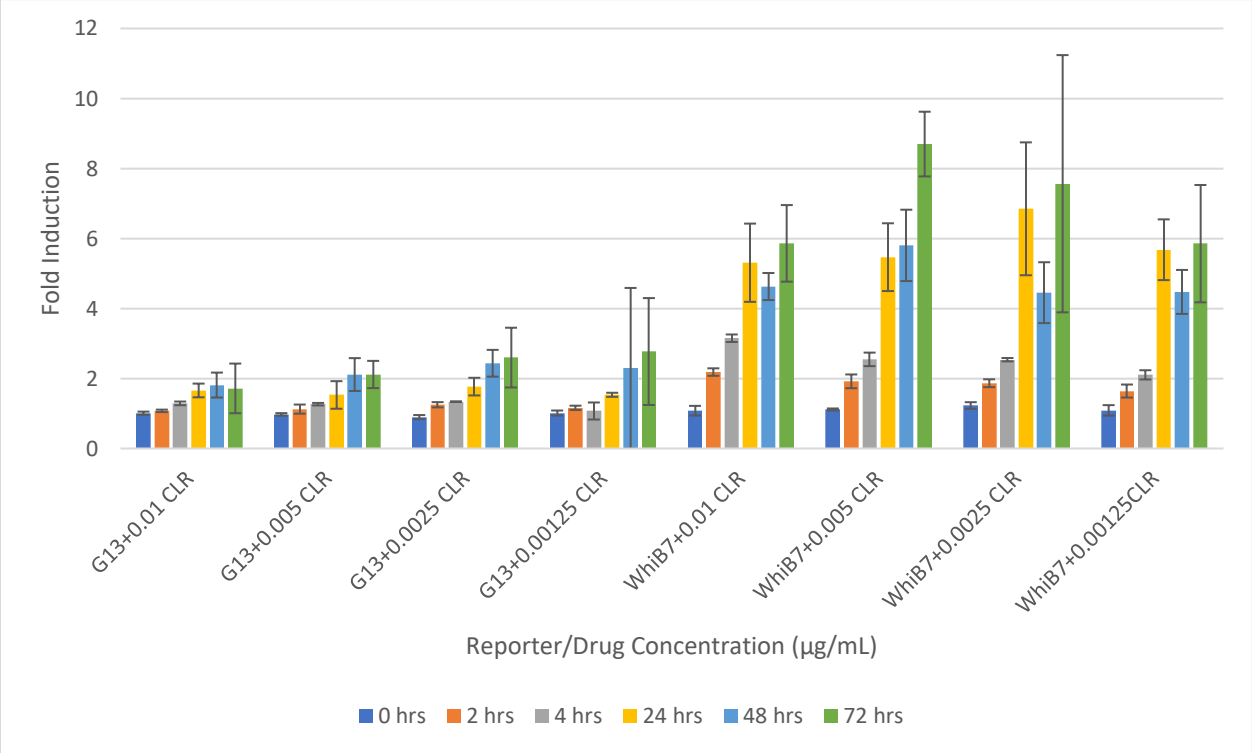

Figure 4: Optimization of CLR Concentration for Luciferase Induction. Fold induction refers to luminescence normalized to optical density and compared to the signal from the same reporter without induction by drugs. A single replicate of the luminescence assay was performed in triplicate wells. The unmodified reporter was exposed various concentrations of CLR (G13+AZT). The inducible substrate reporter was exposed to the same concentrations (WhiB7+CLR).

This experiment showed that 0.005 μg/mL CLR gave the largest induction of luminescent signal in the *pMV306Lux+Hsp60+WhiB7* reporter, so this concentration of CLR was used in future experiments.

4.3 Comparison of Promoter Localization in the Luciferase Plasmid

P_{Hsp60} in *pMV306Lux+Hsp60+G13* was then replaced by P_{whiB7} in order to compare which promoter configuration gives the best signaling. A new reporter was created called *pMV306Lux+WhiB7+G13.* The performance of this reporter was tested against the unmodified reporter and *pMV306Lux+Hsp60+WhiB7.* AZT was used at 0.1 μg/mL and CLR was used at 0.005 μg/mL as determined in the MIC experiment in section 4.2. Figure 5 represents a single replicate of the experiment showing induction of luminescent signal for each reporter normalized to the optical density of each sample.

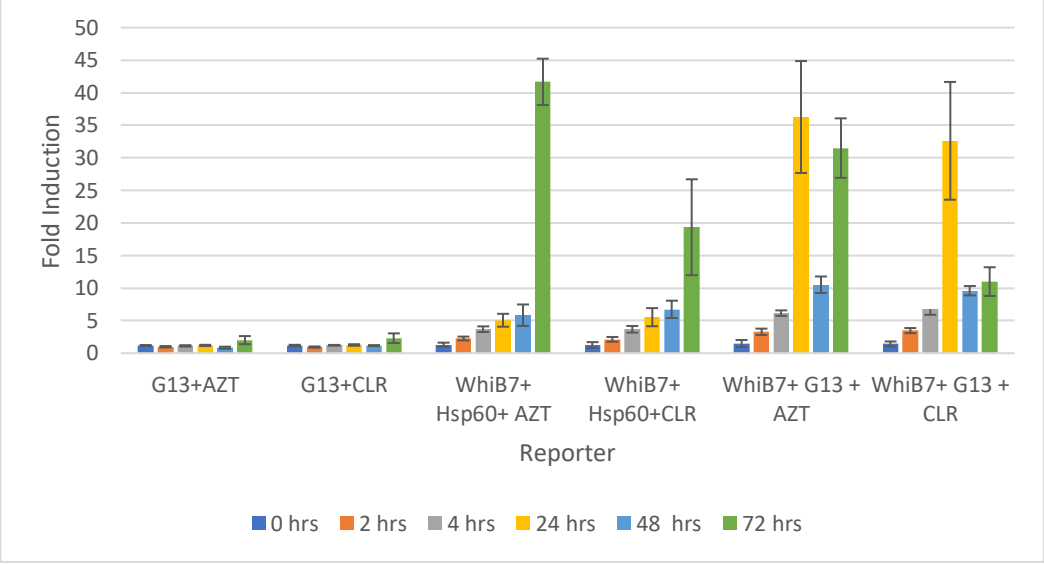

Figure 5: Induction of Luminescence of *pMV306Lux+Hsp60+WhiB7* Versus *pMV306Lux+WhiB7+G13.* Fold induction refers to luminescence normalized to optical density and compared to the signal from the same reporter without induction by drugs. A single replicate of the luminescence assay was performed in triplicate wells. The unmodified reporter was exposed to 0.1 μg/mL AZT (G13+AZT) and 0.005 μg/mL CLR (G13+AZT). The inducible substrate reporter was exposed to the same concentrations (WhiB7+Hsp60+AZT/CLR). The inducible enzyme reporter was exposed to the same concentrations (WhiB7+G13+AZT/CLR)

The unmodified reporter (labeled G13+AZT and G13+CLR) showed little induction in response to drugs due to the lack of $P_{whiB7.}$ Samples containing *pMV306Lux+Hsp60+WhiB7* (labeled WhiB7+Hsp60+AZT and WhiB7+Hsp60+CLR) showed small increasing induction over time until the 72 hour timepoint when the signal increased dramatically. Samples containing *pMV306Lux+WhiB7+G13* (labeled WhiB7+G13+AZT and WhiB7+G13+CLR) showed increasing induction that peaked at 24 hours. In the case of each reporter, the induction caused by CLR was not as strong as the induction caused by AZT. However, the lower concentration of CLR did improve induction in comparison to that seen with 0.01 μg/mL CLR (Figure 1). The *pMV306Lux+WhiB7+G13* reporter showed a large increase in induction at 24 hours while the *pMV306Lux+Hsp60+WhiB7* reporter was delayed in indicating this increase. Therefore, the *pMV306Lux+WhiB7+G13* reporter was used in the remaining luminescence assays because of its superior kinetics.

4.4 Comparison of Luciferase and mCherry Transcriptional Reporters

In order to compare the efficacy of luminescent and fluorescent transcriptional reporters, the mCherry plasmid was modified so that P_{whiB7} controlled the expression of fluorescent protein mCherry in response to stimulation by AZT and CLR. The fluorescence assay utilized the *pVVRG+WhiB7* reporter stimulated by AZT (0.1 μg/mL) and CLR (0.005 μg/mL). Fluorescence values were normalized to the optical density of each sample as shown in the single experiment in Figure 6. The fluorescence stimulated by AZT and CLR increases slowly over time, reaching a peak at 48 hours followed by a decrease in signal at 72 hours. Once again, AZT stimulated a greater fluorescent signal than CLR.

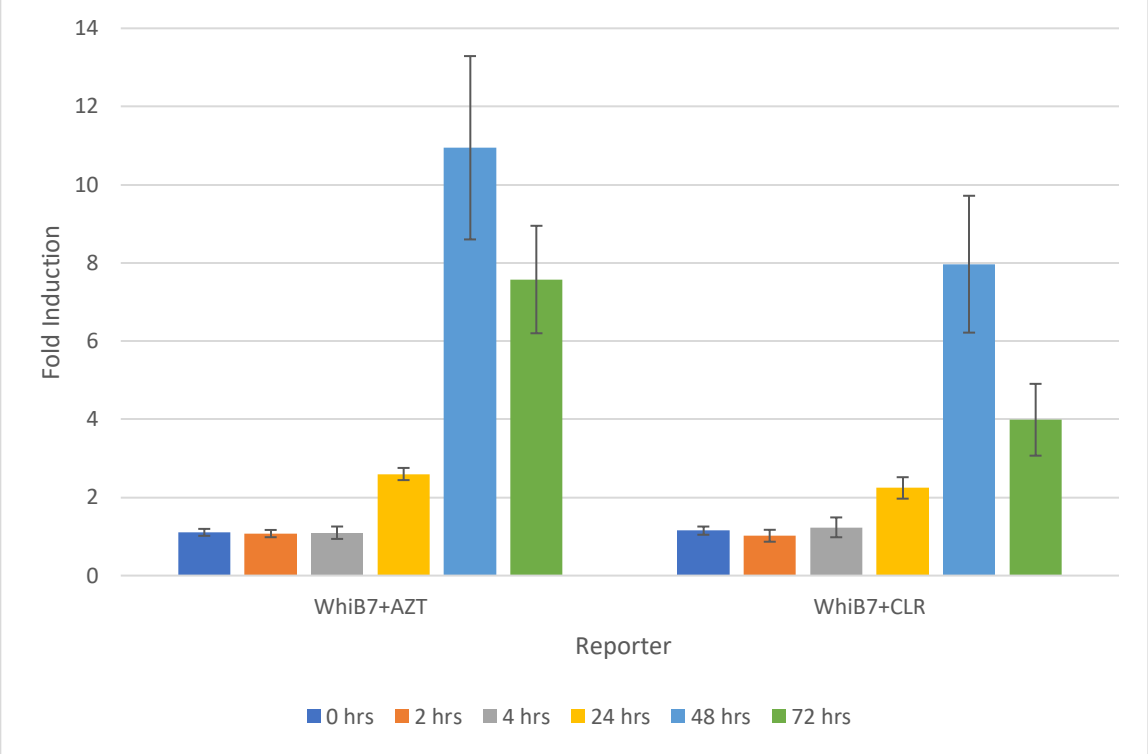

Figure 6: Fluorescence of *pVVRG+WhiB7*. Fold induction refers to fluorescence normalized to optical density and compared to the signal from the same reporter without induction by drugs. A single replicate of the fluorescence assay was performed in triplicate wells. The *whiB7* reporter was exposed to 0.1 µg/mL AZT (WhiB7+AZT) and 0.005 µg/mL CLR (WhiB7+CLR).

4.5 Comparison of Transcriptional Reporters and Real-Time Transcription

In order to determine which reporter most accurately reflected actual gene expression in terms of magnitude and kinetics, qRT-PCR was used. A portion of each culture was first used to measure luminescence and fluorescence in a single experiment as shown in Figure 7. The kinetics of the luciferase and mCherry reporters look quite different. As can be seen in Figure 7, the *pMV306Lux+Hsp60+G13* reporter was not induced by AZT because it lacked $P_{whiB7.}$ The *pMV306Lux+WhiB7+G13* reporter was chosen for comparison with the unmodified reporter because it showed improved induction over the *pMV306Lux+Hsp60+WhiB7* reporter (Figure 5). The induction of this reporter peaked at 24 hours and decreased thereafter at both 48 and 72 hours. The fluorescence of the *pVVRG+WhiB7* reporter was compared with the luciferase reporter. Figure 7 also shows that the fluorescent reporter's fold induction increased throughout the experiment, but only reached a maximum of less than 4 fold induction. This is about 3 times smaller than the maximum induction seen in the *pMV306Lux+Hsp60+WhiB7* reporter. Additionally, the signal emitted by the mCherry reporter could not be detected at the four hour timepoint, while the luciferase reporter already showed 4 fold induction at this time. In order to examine the kinetics of these reporters with actual mRNA expression, the remaining culture was used to extract RNA for qRT-PCR. The fold regulation for each culture stimulated by AZT (0.1 μg/mL) is represented by the average values of two experiments shown in Figure 8.

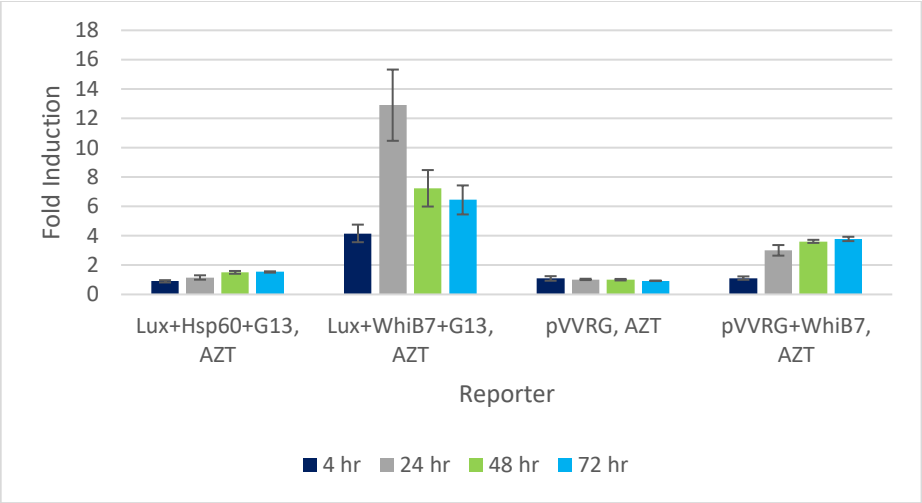

Figure 7: Luminescent vs. Fluorescent Reporter. Fold induction refers to luminescence normalized to optical density and compared to the signal from the same reporter without induction by drugs. A single replicate of each assay was performed in triplicate wells. The unmodified luciferase reporter was exposed to 0.1 μg/mL AZT (Lux+Hsp60+G13, AZT). The inducible enzyme luciferase reporter was exposed to the same concentrations (Lux+WhiB7+G13, AZT). The signal unmodified fluorescent reporter (pVVRG, AZT) was also measured compared to the signal from the inducible fluorescent reporter (pVVRG+WhiB7, AZT) when exposed to 0.1 μg/mL AZT.

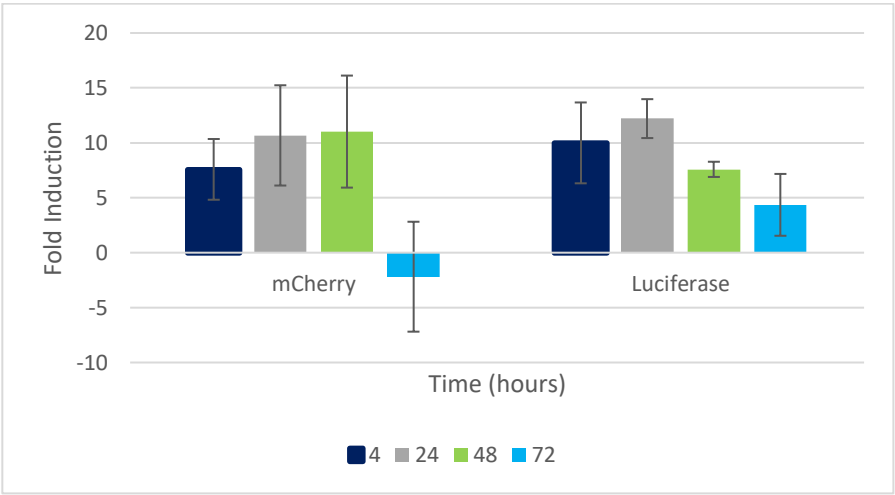

Figure 8: qRT-PCR Fold Induction of *Msm* Containing mCherry vs. Luciferase Reporter. Fold induction refers to measurements of *whiB7* mRNA levels in comparison to mRNA levels of the housekeeping gene *sigA*. Two replicates of the experiment were performed in triplicate wells and averaged. Levels of *whiB7* mRNA were measured from Msm containing the fluorescent reporter (mCherry) and the luminescent reporter (Luciferase).

The *pMV306Lux+WhiB7+G13* reporter stimulated by AZT shows maximum luminescence at 24 hours, and the fold induction seen in the qRT-PCR experiment also peaks at 24 hours for these bacteria. After this point, fold induction values decrease in the qRT-PCR experiment, and luminescence decreases as well. The *pVVRG+WhiB7* reporter shows increasing fluorescence throughout the fluorescence assay with the highest induction reported at 72 hours. However, the fold induction values seen in the qRT-PCR for the bacteria containing the mCherry reporter seem to increase until 24 and 48 hours and decrease drastically at 72 hours. The mCherry reporter used did not indicate this decrease in mRNA expression, instead showing increased fluorescence over the course of 72 hours.

4.6 Enhancing Luminescent Signaling

One drawback that has been noted with luciferase-based transcriptional reporters is their relatively low brightness. It was hypothesized, based on the research of Gregor *et al.,* that the addition of an extra FMN reductase would add more reducing power to the *LuxCDABE* operon by regenerating additional $FMNH_2$ for oxidation as part of the luciferase reaction [12]. This addition was shown to increase the brightness of the luminescent signal produced by the reaction. Therefore, *Msm2921*, an FMN reductase, was placed under the control of constitutive P_{G13} and cloned into *pMV306Lux+Hsp60+G13*, creating the *pMV306Lux+Hsp60+G13+Msm2921* reporter. *Msm* containing this reporter were cultured and their luminescence level was read and compared to the unmodified *pMV306Lux+Hsp60+G13* reporter. Figure 10 shows the luminescence values normalized to the optical density of each sample performed in a single experiment. *pMV306Lux+Hsp60+G13+Msm2921*(referred to as

Lux+Msm2921 in Figure 10) showed a drastically decreased luminescent signal when compared to unmodified *pMV306Lux+Hsp60+G13*.

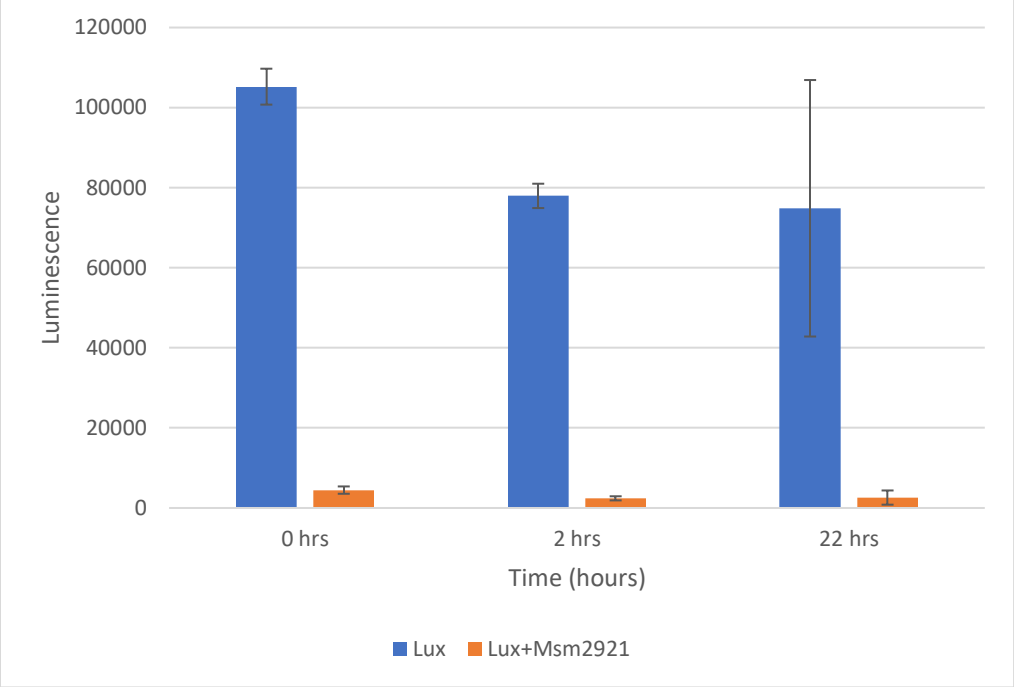

Figure 9: Luminescence of *pMV306Lux+Hsp60+G13+Msm2921.* Luminescence refers to luminescent signal normalized to optical density. Luminescence was measured for the unmodified luciferase reporter (Lux) as well as the luciferase reporter containing the additional FMN reductase (Lux+Msm2921)

5. DISCUSSION

The goal of this study was to determine whether luminescent transcriptional reporters offer more sensitive and dynamic readings than fluorescent reporters. This project also hoped to optimize a luciferase-based transcriptional reporter for use in *Mtb.* The understanding of gene expression is vital to the study of the pathogenesis of *Mtb,* so an effective transcriptional reporter is an important tool for scientists to have available to them. Furthermore, transcriptional reporters are versatile in that they can be used in drug discovery, in studying protein-protein interactions, and in imaging bacteria in live animal models. This study hypothesized that a luciferase-based transcriptional reporter emits signal that is closer to real-time gene expression than fluorescent reporters and could be a better, more accurate tool to monitor gene expression in *Mycobacteria.*

Two new luciferase-based transcriptional reporters were created as a part of this study: *pMV306Lux+Hsp60+WhiB7* and *pMV306Lux+WhiB7+G13.* The difference in these reporters lies in which promoters control the creation of the enzyme and substrate used in the luciferase reaction. The first reporter, *pMV306Lux+Hsp60+WhiB7,* contained constitutive P_{Hsp60} controlling the creation of the luciferase enzyme, and drug-inducible P_{whiB7} controlling the formation of the reaction substrate, a long-chain aldehyde. The results of the luciferase assay comparing these two reporters (Figure 5) showed that *pMV306Lux+Hsp60+WhiB7* did not give as high of a signal as *pMV306Lux+WhiB7+G13.* The *pMV306Lux+WhiB7+G13* contained drug-inducible P_{whiB7} in control of the formation of the luciferase enzyme, and the constitutive P_{G13} promoter in control of the formation of the substrate. This reporter showed a much faster increase in signal, reaching a peak at 24 hours as opposed to the continuous increase in signal

seen in *pMV306Lux+Hsp60+WhiB7*. Additionally, the fold induction of *pMV306Lux+WhiB7+G13* was about 7 times higher than that of *pMV306Lux+Hsp60+WhiB7* at 24 hours. Figure 5 also shows a decrease in the luminescent signal of *pMV306Lux+Hsp60+WhiB7* at 48 hours that recovers at 72 hours. This decrease at the 48 hour timepoint was not repeated in future luminescence experiments, so it was determined that this result was most likely an outlier due to technical error. This data indicates that in order to give the brightest luminescent signal, the production of the enzyme should be under the control of an inducible promoter, while the substrate concentration should be saturating as it is controlled by a constitutive promoter. This result is in agreement with the conclusions seen in a similar experiment performed by Yagur-Kroll *et. al.* in *E. coli* [13]. In future studies, both the P_{Hsp60} and P_{G13} will be replaced with drug-inducible $P_{whiB7,}$ and the luminescent output will be compared with the other reporters.

The luciferase transcriptional reporters created in this study were also compared to a fluorescent mCherry reporter. The production of the mCherry protein was placed under the control of P_{whiB7} in the *pVVRG+WhiB7* reporter. The fold induction seen in this reporter in response to drugs was compared its luminescent counterparts. Fluorescence levels in *pVVRG+WhiB7* increased more slowly than luminescence, with fluorescence first detectable at 24 hours induction and peaking at 48 hours (Figure 6). This is in contrast with the *pMV306Lux+WhiB7+G13* reporter, which emitted signal that was detectable at as early as 2 hours and peaked at 24 hours (Figure 5). Additionally, *pVVRG+WhiB7* had a maximum fold induction of 10 (Figure 6) compared to the maximum fold induction of *pMV306Lux+WhiB7+G13* at 35 (Figure 5). This 3.5 times increase in induction at a timepoint

24 hours earlier shows that the luciferase reporter can be induced more quickly and to a greater extent than the mCherry reporter under the control of the same promoter.

Both mCherry and luciferase reporter signals were compared to *whiB7* mRNA expression using qRT-PCR. This experiment was used to determine which reporter's signaling most accurately represented actual mRNA expression occurring in the bacteria. The signal emitted by the luciferase reporter correlated well with RNA expression, with the highest level of both RNA expression and luminescence reached at 24 hours and continuously decreasing at 48 and 72 hours (Figures 7 and 8) . The mCherry reporter was not as accurate. Fluorescence values consistently increased over 72 hours (Figure 7) for this reporter, but real-time fold induction values seemed to peak at 24 and 48 hours, then decrease drastically at 72 hours (Figure 8). As expected, *whiB7* mRNA expression followed the same basic trend in the bacteria containing mCherry and luciferase reporters. However, the expression seen 72 hour timepoint differed between the two reporters (Figure 8). The negative fold induction value for this timepoint in mCherry is most likely an outlier due to technical error, but further testing would be required to determine if this difference is consistent over multiple trials. Despite this uncertainty, it is clear that the luciferase reporter more accurately models the kinetics of real-time gene expression than the mCherry reporter. In the future, it is important that more replicates of qRT-PCR along with the luminescence and fluorescence assays are performed to verify that this result is repeatable.

This study also attempted to enhance the brightness of the luminescent signal from *pMV306Lux+Hsp60+G13.* The addition of *Msm2921*, an FMN reductase, was expected to increase the luminescent signal by adding more reducing power to the luciferase reaction. However, the addition of *Msm2921* actually drastically decreased the luminescent signal as seen

in Figure 9. Further testing needs to be performed to determine why this decrease in signal occurs. It is possible that when *Msm2921* was inserted into the plasmid, it may have disrupted the expression of certain vital components in the *LuxCDABE* operon. In the future, *Msm2921* should be cloned into different locations within *pMV306Lux+Hsp60+G13* to see if it could induce a higher luminescent signal when placed elsewhere.

This experiment yielded a transcriptional reporter for use in *Mtb* that is more sensitive and more dynamic than previously used tools such as mCherry. This luciferase reporter more accurately models real-time gene expression than mCherry. The luciferase reporter is also advantageous because it allows researchers to visualize both upregulation and downregulation of transcriptional activity. This advantage will be further demonstrated in future experiments examining the kinetics of the luciferase versus mCherry reporter after antibiotic stimulation is removed. Additionally, since the operon *luxCDABE* allows both enzyme and substrate transcription to be controlled, this system is more easily manipulated and optimized. This reporter also does not require the addition of exogenous substrate as other luciferase reporters do. This is an advantage because these substrates are often toxic and may kill the cells that are being studied. Keeping the cells alive throughout the visualization process allows the reporter to study transcriptional activity in real time. In these ways, the luciferase reporter offers distinct advantages over other methods.

The luciferase transcriptional reporter is also an adaptable tool that can be used for many purposes in the study of *Mtb.* In future studies, the luciferase reporter can be used in macrophages or in live animal models to monitor gene expression as infection progresses. Additionally, luciferase can be used in methods such as bioluminescence resonance energy

transfer (BRET). BRET can be used to monitor protein-protein interactions in real-time by converting the blue signal given off in the luciferase reaction to a red signal if two protein binding partners come into close proximity [9]. This method could be used as a screening tool for inhibitors of protein-protein interactions which are important drug targets in *Mtb.* In the future, the *pMV306Lux+WhiB7+G13* reporter could be used to perform mechanism of action screenings of potential new drugs. Using the reporter with the macrolide-inducible $P_{whiB7,}$ new drugs that could target the ribosome in *Mtb* could be identified.

In the study of *Mtb,* information about gene expression is vitally important. This study yielded an optimized luciferase-based transcriptional reporter for use in *Mtb.* This study also showed that this luminescent reporter gives signal that is more indicative of actual mRNA level than its fluorescent counterpart. Using this tool, researchers can study how transcription is induced by a variety of factors, including the presence of antibiotics, and the function of a gene can be deduced. Additionally, this method may help to identify transcription factors needed for a gene to be expressed, making the identification of new drug targets possible. In the future, this tool could be used *in vivo* to monitor gene expression during infection of an animal or in a macrophage. This deeper understanding of how genes are transcribed in *Mtb* could lead to the identification of new drug targets and antibiotics, an important step in combating the rise of drug-resistant TB.

APPENDIX A: PLASMID MAPS

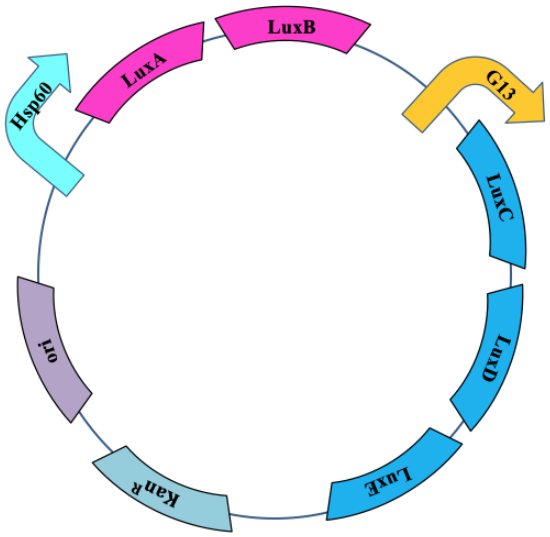

Figure A1: ***pMV306Lux+Hsp60+G13*** **Transcriptional Reporter**

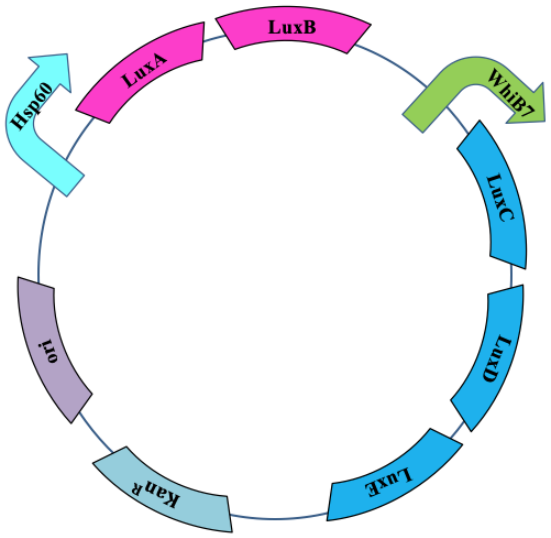

Figure A2: ***pMV306Lux+Hsp60+WhiB7*** **Transcriptional Reporter**

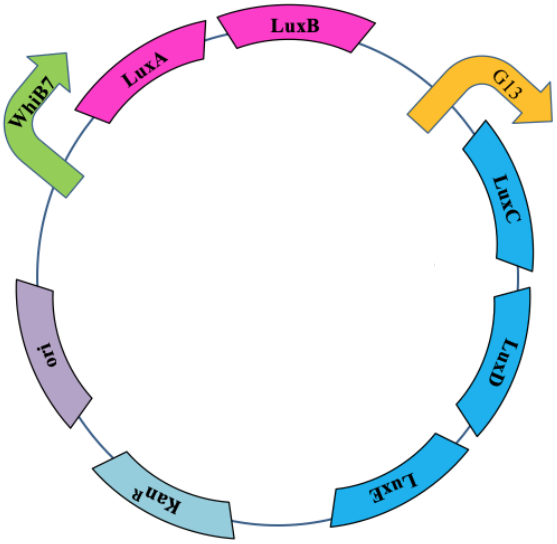

Figure A3: ***pMV306Lux+WhiB7+G13*** **Transcriptional Reporter**

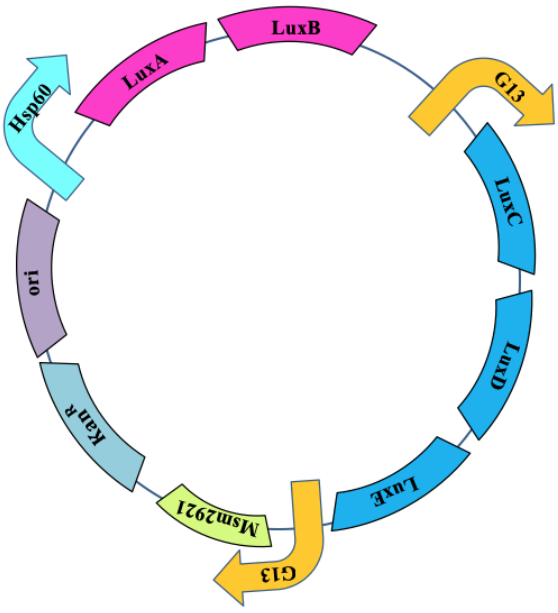

Figure A4: ***pMV306Lux+Hsp60+G13+Msm2921***

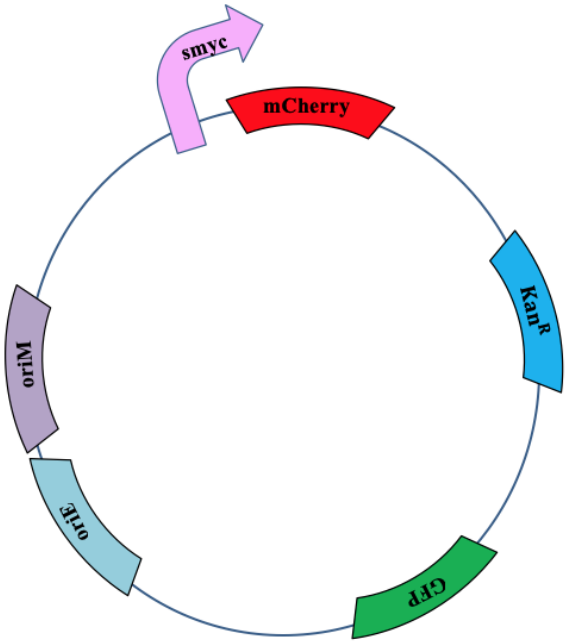

Figure A5: *pVVRG*

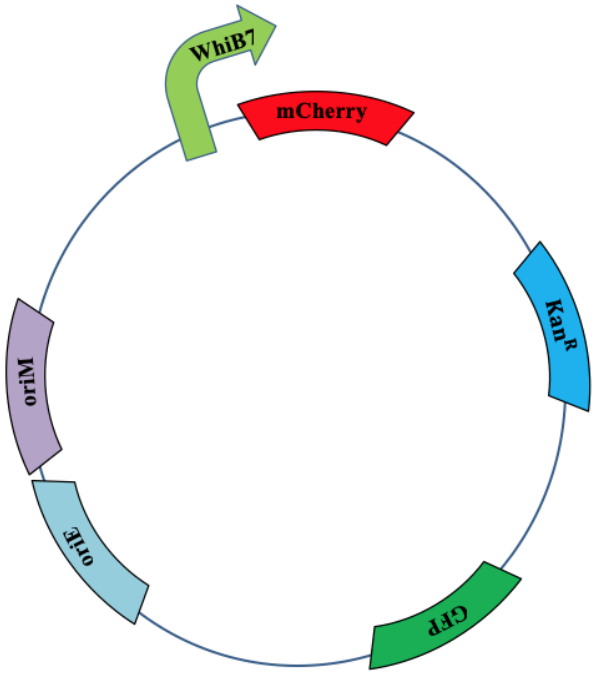

Figure A6: *pVVRG+WhiB7*

APPENDIX B: PRIMER TABLES

Table B1: Cloning Primers

Primer Name	Primer Sequence	Function
Lux_hsp60_FC_F	ggccaagacaattgcgg	Forward primer to amplify luciferase vector without h*sp60*
Lux_hsp60_FC_R	gcgtcgttgtggtcacct	Reverse primer to amplify luciferase vector without *hsp60*
WhiB7_hsp60_FC_F	Aggtgaccacaacgacgcgtagaaaataggttgtgcgattcag	Forward primer to amplify the w*hiB7* promoter and insert in place of *hsp60*
WhiB7_hsp60_FC_R	ccgcaattgtcttggccgtgtttttccccctgcatct	Reverse primer to amplify the *whiB7* promoter and insert in place of *hsp60*
WhiB7_G13_FC_FLong	aaatatacctaataggtaccgttcacggacaccacgct	Extended forward primer to amplify the *whiB7* promoter and insert in place of *G13*
Lux_whiB7_FC_F	Atgactaaaaaaatttcattcattattaacgg	Forward primer to amplify luciferase vector and remove *G13*
Lux_whiB7_FC_R.2	cggtacctattaggtatatttcatgtgg	Reverse primer to amplify luciferase vector and remove *G13*

Primer Name	**Primer Sequence**	**Function**
WhiB7_Lux_FC_F	aaatatacctaataggtaccggtagaaaataggttgtgcgattcag	Forward primer to amplify the *whiB7* promoter and insert in place of *G13*
WhiB7_Lux_FC_R	atgaaatttttttagtcatgtgtttttccccctgcatct	Reverse primer to amplify the *whiB7* promoter and insert in place of *G13*
Msm2921_LuxCDE_FC_F.2	gtaccaggaggatggcaaatatgacggtcatcgcgc	Forward primer to amplify *Msm2921* and attach to *G13*
Msm2921_LuxCDE_FC_R.2	atcgataagcttgattgcaggatatcagcggattcccagg	Reverse primer to amplify *Msm2921* and attach to *G13*
Lux_Msm2921_FC_F	ctcagcgcagcggga	Forward primer to amplify luciferase vector and insert *G13+Msm2921*
Lux_Msm2921_FC_R	gacgtgccaactaggtctcct	Reverse primer to amplify luciferase vector insert *G13+Msm2921*
WhiB7_Blunt_FC_F	gtagaaaataggttgtgcgattcag	Blunt cloning forward primer to amplify w*hiB7* promoter

Primer Name	**Primer Sequence**	**Function**
WhiB7_Blunt_FC_R	gtgtttttcccccctgcatct	Blunt cloning reverse primer to amplify w*hiB7* promoter
pVVmCh_FC-F	atggtcagcaagggcga	Forward primer to amplify *pVVRGcor* and remove *smyc*
pVVmCh_FC-Rpro	ggtgccgacgatcctcta	Reverse primer to amplify *pVVRGcor* and remove *smyc*
WhiB7_pVV16_FC_F	tagaggatcgtcggcaccttcacggacaccacgct	Forward primer to amplify *whiB7* promoter and insert into *pVVRGcor*
WhiB7_pVV16_FC_R	tcgcccttgctgaccatgtgtttttcccccctgcatct	Reverse primer to amplify *whiB7* promoter and insert into *pVVRGcor*

Table B2: Sequencing Primers

Primer Name	Primer Sequence	Function
Lux_Msm2921_G13_Seq_F	ctctaagtggaaagaaattgcagg	Forward primer to sequence *G13+Msm2921* in luciferase vector
Lux_Msm2921_G13_Seq_R	gcgtccatcttgttgtcgtag	Reverse primer to sequence *G13+Msm2921* in luciferase vector
Lux_hsp60_seq_F	cgttatcccctgattctgtgg	Forward primer to sequence around *hsp60* in luciferase vector
Lux_hsp60_seq_R	ctgtcgacgatcattgcaatt	Reverse primer to sequence around *hsp60* in luciferase vector
Lux_G13_Seq_F	gtgtggtgcgaaaagtgtattg	Forward primer to sequence around *G13* in luciferase vector
Lux_G13_Seq_R	gagagtcattcaatattggcagg	Reverse primer to sequence around *G13* in luciferase vector
Lux_hsp60_seq_F.2	ggataaccgtattaccgcctt	Forward primer to sequence around *hsp60* in luciferase vector
Lux_hsp60_seq_R.2	ggtcatagagtcctcctgtcaa	Reverse primer to sequence around *hsp60* in luciferase vector

Primer Name	Primer Sequence	Function
Lux Hsp60 seq F.3	ttggagcgaacgacctacac	Forward primer to sequence around *hsp60* in luciferase vector
Lux Hsp60 seq R.3	aaactccgtgaaatgatgctcc	Reverse primer to sequence around *hsp60* in luciferase vector
pVV16seqF2	gaactggcgcagttcctct	Forward primer to sequence around *smyc* in *pVVRGcor*
pVVmChseqR	ggttcaaggtgcacatggag	Reverse primer to sequence around *smyc* in *pVVRGcor*

Table B3: qRT-PCR Primers

Primer Name	Primer Sequence	Function
Msm_SigA_qRT_F.3	gaggaagaagaagctgatgc	Forward primer to amplify *sigA*
Msm_SigA_qRT_R.3	ctcttcctcgtcccacac	Reverse primer to amplify *sigA*
Msm_WhiB7_qRT_F.2	atgacatgcgagacgcg	Forward primer to amplify *whiB7*
Msm_WhiB7_qRT_R.2	cacggttcctgccgttcg	Reverse primer to amplify *whiB7*

REFERENCES

1. Floyd, K., et al., *The global tuberculosis epidemic and progress in care, prevention, and research: an overview in year 3 of the End TB era.* Lancet Respir Med, 2018. **6**(4): p. 299-314.

2. Nahid, P., et al., *Official American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America Clinical Practice Guidelines: Treatment of Drug-Susceptible Tuberculosis.* Clin Infect Dis, 2016. **63**(7): p. e147-e195.

3. Dheda, K., et al., *The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis.* Lancet Respir Med, 2017.

4. Malherbe, S.T., et al., *Corrigendum: Persisting positron emission tomography lesion activity and Mycobacterium tuberculosis mRNA after tuberculosis cure.* Nat Med, 2017. **23**(4): p. 526.

5. Stanley, S.A. and J.S. Cox, *Host-pathogen interactions during Mycobacterium tuberculosis infections.* Curr Top Microbiol Immunol, 2013. **374**: p. 211-41.

6. Rohde, K.H., et al., *Linking the transcriptional profiles and the physiological states of Mycobacterium tuberculosis during an extended intracellular infection.* PLoS Pathog, 2012. **8**(6): p. e1002769.

7. Allen, M.S., et al., *A destabilized bacterial luciferase for dynamic gene expression studies.* Syst Synth Biol, 2007. **1**(1): p. 3-9.

8. Naran, K., et al., *Bioluminescent Reporters for Rapid Mechanism of Action Assessment in Tuberculosis Drug Discovery.* Antimicrob Agents Chemother, 2016. **60**(11): p. 6748-6757.

9. Cui, B., et al., *Bioluminescence resonance energy transfer system for measuring dynamic protein-protein interactions in bacteria.* MBio, 2014. **5**(3): p. e01050-14.

10. Andreu, N., et al., *Optimisation of bioluminescent reporters for use with mycobacteria.* PLoS One, 2010. **5**(5): p. e10777.

11. Shen, Y., et al., *Engineering of mCherry variants with long Stokes shift, red-shifted fluorescence, and low cytotoxicity.* PLoS One, 2017. **12**(2): p. e0171257.

12. Gregor, C., et al., *Strongly enhanced bacterial bioluminescence with the ilux operon for single-cell imaging.* Proc Natl Acad Sci U S A, 2018. **115**(5): p. 962-967.

13. Yagur-Kroll, S. and S. Belkin, *Upgrading bioluminescent bacterial bioreporter performance by splitting the lux operon.* Anal Bioanal Chem, 2011. **400**(4): p. 1071-82.

14. Burian, J., et al., *The mycobacterial transcriptional regulator whiB7 gene links redox homeostasis and intrinsic antibiotic resistance.* J Biol Chem, 2012. **287**(1): p. 299-310.

15. Burian, J., et al., *The mycobacterial antibiotic resistance determinant WhiB7 acts as a transcriptional activator by binding the primary sigma factor SigA (RpoV).* Nucleic Acids Res, 2013. **41**(22): p. 10062-76.

16. Burian, J., et al., *WhiB7, a transcriptional activator that coordinates physiology with intrinsic drug resistance in Mycobacterium tuberculosis.* Expert Rev Anti Infect Ther, 2012. **10**(9): p. 1037-47.

17. Whitman, M.S. and A.R. Tunkel, *Azithromycin and clarithromycin: overview and comparison with erythromycin.* Infect Control Hosp Epidemiol, 1992. **13**(6): p. 357-68.

18. Li, C., et al., *FastCloning: a highly simplified, purification-free, sequence- and ligation-independent PCR cloning method.* BMC Biotechnol, 2011. **11**: p. 92.

19. Rodrigues Felix, C., et al., *Selective Killing of Dormant Mycobacterium tuberculosis by Marine Natural Products.* Antimicrob Agents Chemother, 2017. **61**(8).

20. Rampersad, S.N., *Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays.* Sensors (Basel), 2012. **12**(9): p. 12347-60.

21. Lambert, R.J. and J. Pearson, *Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values.* J Appl Microbiol, 2000. **88**(5): p. 784-90.