Introduction

Fluorescent proteins in combination with flow cytometry have become a major asset to the biomedical field, disease research, and cell biology since the cloning of green fluorescent protein (GFP) in 1994 (Gerdes et al. 1996). Fluorescent proteins are now commonly used as markers for cellular activity, structures, transcription factors, and even apoptosis. More recently, within the past ten years, GFP has been used in combination with other fluorescent proteins, such as red fluorescing proteins (RFPs) like mCherry and mScarlet to observe similar cellular behaviors. Dual protein reporting of GFP and mCherry have been used in bacteria such as *Bacillus subtilis* to observe sporulation (Doherty et al. 2010). Reporter systems such as the ones described above have proven to be extremely beneficial in the research of *Mycobacterium tuberculosis*. Dual protein reporter systems of mCherry and GFP with constitutive or regulated promoters have been used to discover inhibitors of *M. tuberculosis* survival (Abramovitch et al. 2018).

Further research on reporter systems as tools to aid in the research of *M. tuberculosis* is necessary. Until the spread of SARS-CoV-2 (COVID-19) in 2019, *M. tuberculosis* was the leading infectious killer worldwide with more than 1 million deaths annually (World Health Organization, 2021). The tuberculosis epidemic has garnered the attention of the UN, which set out specific targets worldwide to limit the spread and severity of the infection. However, despite efforts, death and infection rates continue to rise, with an estimated 10.6 million people developing *M. tuberculosis* worldwide in 2021, and an estimated 1.6 million cases resulting in death (World Health Organization, 2021). The current methodology for the treatment of an *M. tuberculosis* infection can be effective but it is cumbersome, with multiple different antibiotics for a recommended duration of 6 months (Dartios et al. 2022).

Patient compliance with treatment is inconsistent for varying reasons, such as access to antibiotics, medical care, side effects, and financial restrictions. Another common issue with the treatment of *M. tuberculosis* continues to be drug tolerance, leading to a rise in treatment failure and a need for further research. Drug tolerance is a phenomenon in which bacteria can survive the prescribed antibiotic treatment despite lacking the mutations that allow them to grow in the presence of antibiotics.

The life cycle of *M. tuberculosis* is unique and depends on its ability to interact with the host's immune system. Granuloma formation is a hallmark of the *M. tuberculosis* infection, causing a decrease in drug susceptibility. The granuloma is an organized structure of differentiated macrophages and other immune cells. The granuloma starts in the initial stages of infections and expands by spreading to arriving macrophages. As immunity within the body of an infected individual develops, so does the granuloma, which can allow for formation of a necrotic core that promotes transmission of the infection onward to another host Cambier et al. 2014). In addition, the developing granuloma can render the bacteria drug-tolerant as a consequence of the bacterial responses to environmental stressors (Cambier et al. 2014). This life-cycle characterization makes it crucial to determine which bacteria are alive and which are dead to assess the effectiveness of an antibiotic.

Research on *M. tuberculosis* and the effectiveness of antibiotics has its challenges, including the labor-intensive method of counting colony-forming units (CFUs) to assess bacterial viability. In *M. tuberculosis* research, it is sometimes critical to calculate what percent of the bacterial population is alive or dead, especially when testing and developing antibiotic treatments. *M tuberculosis* has a unique and irregular colony morphology. This makes it difficult to count plates utilizing automated counting software. Additionally, colonies that form on solid media may either be products of a single cell or products of clumps of cells, making colony counts inaccurate representations of the number of viable cells (de Knegt et al., 2017, Sarathy et al.2013). The plating of cultures and preparing dilutions is also labor intensive and time-consuming. These factors make the manual quantification of colonies when attempting to measure the effectiveness of antibiotics an unideal method. A secondary issue to determining the proportion of alive versus dead bacteria on a solid media plate by counting is that *M. tuberculosis* can enter a dormant growth phase. Dormant cells do not maintain growth or uptake of nutrients, making them unable to form colonies on plates (Sarathy et al. 2013).

There are existing developed systems for assessing mycobacterial death. Reporter systems have been developed that can distinguish between live and dead *M. tuberculosis* (Wang et. al 2019). In this example both GFP and RFP were expressed, one from an inducible promoter and one from a constitutive promoter (Wang et al. 2019). This allowed the researchers to distinguish between bacteria that were living and bacteria that had died, as only the live bacteria can express protein from the inducible promoter.

Here we sought to develop a live-dead reporter system utilizing GFP and mScarlet. In the reporter system mScarlet would be driven by a constitutive promoter and accumulated protein would be present and remain detectable after cell death. GFP would be expressed by an inducible tet promoter (pmyc1) that can only be expressed by cells that are still alive when the inducer is added (Ehrt et al. 2005). This would allow for distinction between living and dead bacteria, as only the living bacteria would synthesize the inducible protein which can be observed using flow cytometry. This allows for measurement of the proportions of live and dead bacteria. The reporter system can then be used when introducing antibiotics to a bacterial population. This system can then be used as a tool not only to measure bacterial death in a less labor-intensive way but has further application in testing potential drug combinations.

Materials and Methods

I. Q5 (high fidelity) Polymerase Chain Reaction (PCR)

All reactions for each DNA segment were completed by following the recommended procedure from New England Biolabs (NEB). Each reaction started with a 2-minute denaturation step at 98 ℃. After this denaturation, the reaction cycle is repeated for 35 cycles, starting at 98 ℃ for 10 seconds for denaturation, and an annealing step for 20 sec, at a temperature calculated by the NEB ™ calculator, and an elongation step for 72 ℃ at 30 sec/kb. After 35 cycles the reaction undergoes a final elongation step for 5 minutes at 72 ℃, then held at 4 ℃ until use. All

PCR products were treated with 1 µl of DpnI enzyme at 37℃ for 1 hour and then 80 ℃ for 20 min. Products were then mixed with a 6X DNA loading dye and loaded into a TAE agarose gel, along with the corresponding NEB ladder depending on the expected product size. Gel electrophoresis was run between 100-120 V. The gels were observed under UV light, and DNA bands were extracted if they were the appropriate size. DNA bands were then purified using the Zymoclean Gel DNA Recovery kit according to the manufacturer's instructions.

Table I. Primer Description and Annealing Temperature

Primer Pair Description	**Primer Set Sequence**	**Annealing Temperature**
Amplification of the backbone sequence pSS470	Fwd: GGAGCGAGAAGCTTGCATGCC	72℃
	Rv: GGGCGGCCGCGTATGCTTAG	
Amplification of the mScarlet protein sequence from pSS321	Fwd: AAAGGCTAAGCATACGCGGCCGCCCTCACTTATACAGCTCGTCC	62℃
	Rv: ATGGTCTCCAAAGGTGAAG	
Amplification of the promoter sequence to be used with the mScarlet protein	Fwd: ATGACTGCTTCACCTTTGGAGACCATTGCGAAGTGATTCCTCC	62℃
	Rv: TGCAGGCATGCAAGCTTCTCGCTCCCACTCTAGAAATATTGGATCGTCG	

II. Plasmid Construction

The Hi-Fi Assembly Master Mix by New England BioLabs was used to construct the plasmid. A 5 µl volume was used with 2.5 µl of Master Mix and 2.5 µl of recovered DNA. A

2:2:1, mass ratio of DNA fragments was used to make a 3 µl master mix. This contained 1 µl of 25 ng/µl backbone, and for each insert fragment 1 µl of each insert fragment at 50 ng/µl. The 3 µl DNA fragment mix was then diluted with 3 µl of H_2O. 2.5 µl of this master mix was used in the assembly. This 5 µl reaction was incubated in the thermocycler for 1 hr at 50℃.

III. *E. coli* Transformations

The transformations of *E. coli* were done using New England Biolabs competent cells. The 5 µl volume of the plasmid construction product was added to the cells and put on ice for 30 min. The cells were then heat shocked at 42℃ for 45 seconds and then placed back on the ice for 2 minutes. 400 µl of SOC media was added and the cells were placed horizontally in the shaker for 1 hr at 37℃. The cells were then centrifuged for 1 min at 15,000 rpm, the supernatant was discarded, and the pellet was resuspended and plated on LB agar containing the corresponding antibiotic hygromycin. *E. coli* colonies from plates were grown in LB broth with hygromycin shaking at 37℃ overnight. This liquid culture was prepped using the ZR Plasmin Mini-Prep Kit and included protocol from Zymo Research. Mini-Prepped plasmid and specific primers were sent for sequencing through Quintara Biology.

IV. PCR Colony Check

When Sequence results came back inconclusive a PCR check of some of the *E. coli* colonies grown on the plate was performed looking for the mScarlet protein. PCR reactions were completed by following the New England BioLabs recommended taq protocol. A 25 µl reaction was used with a zero-template volume. The reaction started with an initial denaturing step at 95℃ for 2 minutes. This step was followed by a 30-cycle repeat of denaturing step at 95℃ for 30 seconds, an annealing step at 59℃ for 1 minute, and an extension step at 68℃ for 45 seconds. The reaction ended with a final extension at 68℃ for 5 minutes and then was held at 4℃ until the product was ready to be used.

PCR products were checked following the same TAE gel protocol as described in section I. When the correct band size was observed on the gel the corresponding colony was mini-prepped and sent for sequencing following the protocol in section III.

Results and Discussion

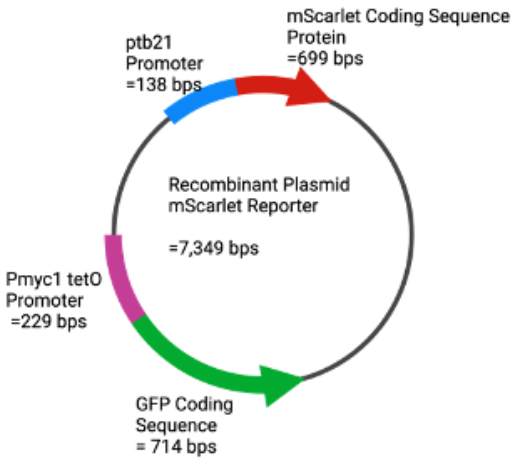
Designing of the Reporter System Plasmid

To avoid issues that we have had in the past with mCherry toxicity, we sought to construct a live-dead reporter in which the brighter RFP variant mScarlet was expressed from the mid-strength constitutive promoter ptb21 and GFP was expressed from the tet-inducible promoter pmyc1-tetO (Fig. 1, Ehrt et al. 2005). To make this recombinant plasmid, we planned to insert ptb21 with its associated 5’ UTR and the mScarlet coding sequence into the existing plasmid

pSS470 which already contains GFP driven by Pmyc1-tetO. We will refer to pSS470 as the backbone. The Pmyc1 tetO promoter is part of an inducible tet repressor system critical to the function of the reporter system. A codon-optimized mScarlet coding sequence was amplified by PCR from pSS321. The ptb21 promoter was cloned from CT56-pUVtetOR. After construction of the plasmid, it can be transformed into mycobacteria along with another plasmid that constitutively expresses a tet repressor. Adding an inducer will cause GFP to be produced by live cells but not dead cells. However, all cells will be able to fluoresce red, as mScarlet is constitutively expressed allowing for easy identification of all cells that were once living.

The location of adding the ptb21-mScarlet insert was selected primarily based off two parameters. The insert had to be placed far enough upstream from the tsynA transcriptional terminator sequence adjacent to the Pmyc1-GFP cassette in the pSS470 backbone. The insert was placed in a divergent orientation relative to the pmyc1 tetO promoter so both protein sequences (mScarlet and GFP) could be expressed independently. The resulting recombinant plasmid was 7,349 base pairs in size.

Figure 1. Plasmid Schematic



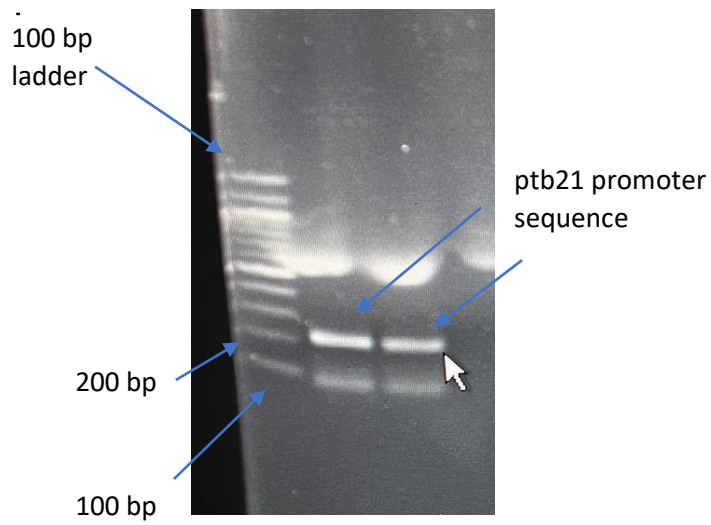
The above figure shows the proposed schematic for the mScarlet protein live-dead reporter system. The above reporter uses the pSS470 backbone containing the existing pmyc1 tetO promoter, expressing GFP coding sequence as well as the ptb21 promoter used to express the red fluorescing protein mScarlet coding sequence. The total size of the recombinant plasmid is 7,349 base pairs.

PCR and Gel Extraction of DNA Fragments for HiFi Assembly

We planned to use HiFi assembly (New England Biolabs) to construct the plasmid. PCR was used to amplify each fragment (pSS470 backbone, mScarlet coding sequence, and ptb21 promoter coding sequence) needed for assembly. The PCR reactions used NEB Q5 mix. Each

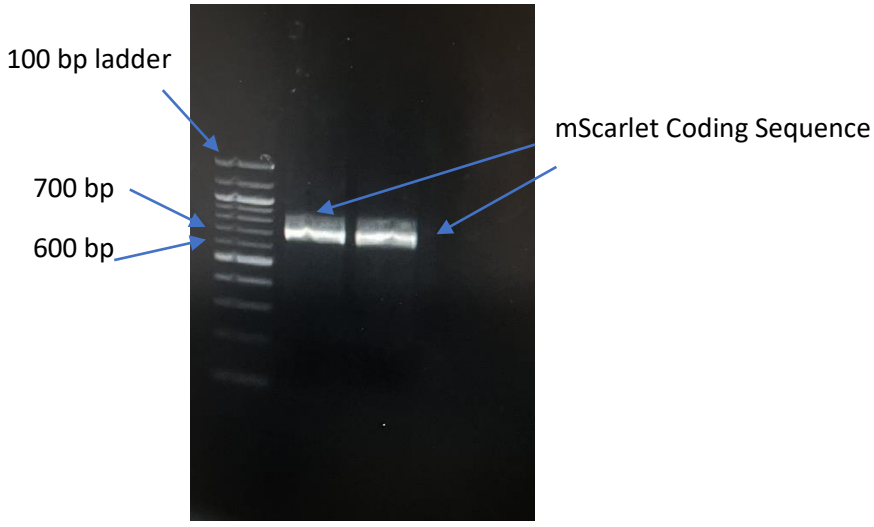
PCR product was treated with the DpnI to remove template DNA, and then ran on a 1% TAE gel with EtBr. The resulting bands were extracted and purified so that the DNA could be used in the HiFi assembly protocol. All band product sizes extracted matched with the anticipated product size identified in each figure legend below.

Figure 2. Ptb21 Promoter



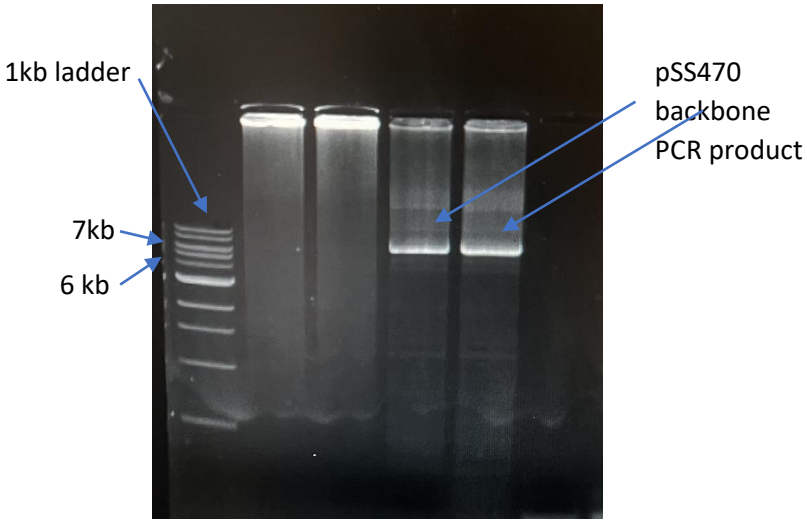
The above image shows the gel for the ptb21 promoter. The bands match the expected product size of 166 base pairs. The bands were extracted and purified to be used in the HiFi assembly of the recombinant plasmid. The resulting concentration of DNA was 37.8 ng/µl.

Figure 3. mScarlet Protein Coding Sequence



The above image shows the gel for the mScarlet Coding Sequence. The band matches the expected product size of 699 base pairs. The bands were extracted and purified to be used in the HiFi assembly of the recombinant plasmid. The resulting concentration of DNA was 133.1 ng/µl

Figure 4. pSS470 Backbone

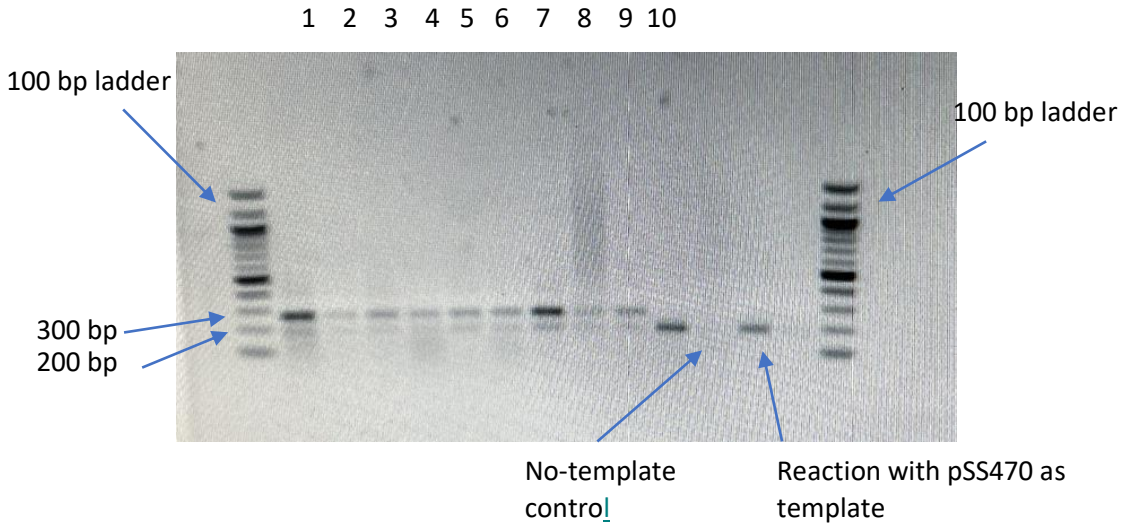


The above image shows the gel for the pSS470 backbone. Reactions that used 3 ng of template DNA (lanes 4 and 5) were proven optimal and the resulting bands were extracted. The band matches the expected band size of 6484 base pairs. The resulting concentration of the purified DNA was 37.8 ng/µl.

PCR Colony Checking *E. Coli* Colonies from Assembly

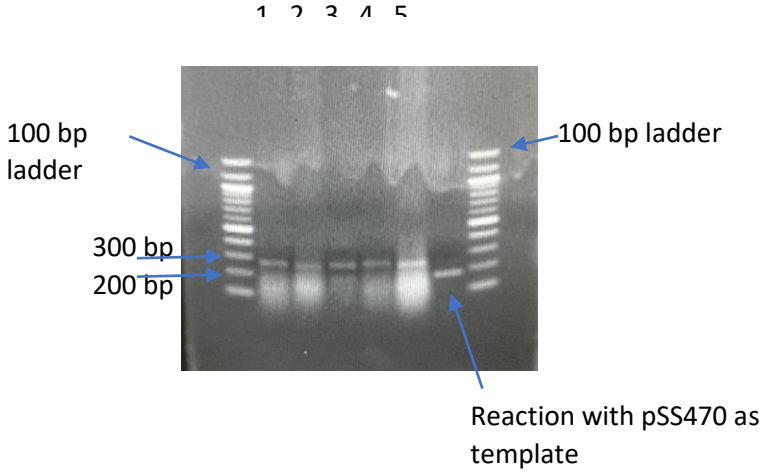
Assemblies were transformed into *E. coli*, and colonies grew. A PCR colony check was performed to confirm successful assembly. Primers were chosen that annealed upstream and downstream of the insert to check for a product of the expected size. If successful, the expected product size was approximately 865 base pairs in length. If unsuccessful (backbone only, no insert), the product size would be approximately 199 bp in length. The bands in 9 out of the 10 colonies were 100 bp larger in size than the expected product size from the backbone plasmid, but they were still much smaller than expected from a successful assembly of the inserts into the pSS470 backbone. Due to the unexpected band sizes of the colonies shown in Figure 6, they were each miniprepped using the Zymoresearch Miniprep Classic Kit and sent for Sanger Sequencing through Quintara Biosciences.

Figure 5. PCR Clone Check



The above image shows the results from the PCR colony check. 10 colonies were checked for successful insertion. A reaction with water was used as a control to check for primer interactions and a second control was done using 3 ng of pSS470 (the backbone) to confirm size of the bands.

Figure 6. PCR Clone Check Confirmation



The above image shows the PCR check of 5 colonies from the same plate used in Figure 5. (Colonies 1-3) were the same and colonies 4 and 5 were new colonies from the same plate tested. The resulting bands were the same size as in Figure 5, 100 bp larger than if there were an unsuccessful assembly resulting in recovery of the backbone plasmid pSS470, but too small to be indicative of a successful assembly.

Sanger Sequencing

In order to be able to visualize the sequence of the cloned plasmids Sanger sequencing was performed by Quintara Biosciences. Primers for sequencing were chosen based on location and proximity to the insert. Alignments of the results were performed in Benchling to both the intended recombinant plasmid and pSS470. A sequence duplicated in clones was found, accounting for the extra base pair length observed in the PCR Clone Check Gels (Figure 5 & 6). It is unclear what sort of misannealing or aberrant assembly could have produced this duplication. A future attempt to make the live-dead reporter plasmid could place the inserts at a different location in the pSS470 backbone, which may prevent the unknown aberrant events leading to the duplication and failure to produce the intended plasmid.

Figure 7. Sequence Duplicated in Clones

GCGAAAAGGCTAAGCATACGGCCGCCCTAGGTGCTAGCTTAATTAACCATGGAGC
GAGAACGTTGCATGCCTGCAG

The above is the sequence found to be duplicated in clones. Yellow indicates the divergent primer sequences that were used for the backbone PCR. Red indicates the sequence that should have been deleted during assembly.

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