Detection of Drug-Resistance Conferring SNPs in Mycobacterium Tuberculosis using Binary DNAzymes

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DETECTION OF DRUG-RESISTANCE CONFERRING SNPS IN MYCOBACTERIUM TUBERCULOSIS USING BINARY DNAZYMES

by

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ABSTRACT

*Mycobacterium tuberculosis* (*Mtb*) is the pathogen that causes Tuberculosis (TB) and is responsible for an average of 1.5 million deaths annually. Although a treatment regimen does exist, Multi-Drug Resistant (MDR-TB) and eXtremely Drug Resistant (XDR-TB) TB strains are becoming a more prevalent concern partly due to failure of patient compliance with the current six to nine month drug treatment regimen. The current diagnostic methods are not able to identify these MDR and XDR-TB strains efficiently therefore more effective point-of-care (POC) diagnostics and drug susceptibility testing (DST) are urgently needed to detect drug resistance and facilitate prompt, appropriate treatment plans. In order to detect TB and efficiently identify drug resistance, this project seeks to develop a novel diagnostic technology based on deoxyribozyme (DNAzyme) sensors.

The overall goal of this project is to create an assay which combines Polymerase Chain Reaction (PCR) and DNAzymes to identify drug resistance conferring Single Nucleotide Polymorphisms (SNPs). To safely test the ability of DNAzyme sensors to detect SNPs indicative of multi-drug resistant TB, we have constructed a panel of drug resistant (drug*R*) nonpathogenic *M. bovis* BCG. We have designed a multiplex PCR that amplifies 6 chromosomal regions of the genome necessary for the species specific detection of TB and determination of a drug susceptibility profile based on the presence of SNPs. To improve the sensitivity and selectivity of the detection and DST of *Mtb*, we have designed and optimized DNAzyme sensor assays combined with multiplex PCR analytes that will enable the rapid, POC detection of drug resistance. This work aims to develop novel tools for the prompt and specific diagnosis of TB allowing for the implementation of an
effective treatment regimen that will ultimately lessen transmission and control the emerging global threat of MDR and XDR-TB.
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INTRODUCTION

*Mycobacterium tuberculosis* (*Mtb*) is the pathogen that causes Tuberculosis (TB). One-third of the world’s population is currently infected with TB and it causes an average of 1.5 million deaths annually [1]. TB is spread through aerosolized droplets expelled from the lungs of an infected individual through talking, coughing, or sneezing wherein it may travel to the lungs of a new host and reside in pulmonary tissue [2]. Upon infection with *Mtb*, a patient can develop either the active or latent form of the disease. Active TB occurs when the body fails to control *Mtb* within alveolar macrophages and can result in pulmonary damage and dissemination to other areas of the body, both of which may ultimately result in death [3]. Individuals with active TB are infectious, will present with symptoms, and may test positive in a sputum smear microscopy test. Symptoms include a chronic cough, chest pain, fatigue, and weight loss [4]. Approximately 2 million people (or 90% of patients infected with *Mtb*) are latently infected with *Mtb* [5]. Latent TB infections result when the body effectively contains, but does not clear the infection. The patient will not present with symptoms because the bacilli become dormant within granulomas and the disease cannot be spread to others [6, 7]. The development of the granuloma, an immune structure formed to contain the infection, results in difficulties treating the infection [2]. Many of the currently used drugs are unable to effectively penetrate this barrier and bacilli in the granuloma are typically characterized by a phenotypically nonreplicative, dormant state [6, 7].

Treatment of drug-susceptible TB consists of a four drug cocktail taken over a six to nine month period, however, due to the lengthy treatment regimen many patients fail to comply [8]. The current first-line drugs are isoniazid and rifampicin and when those become ineffective second-line drugs such as fluoroquinolones are used [5]. While a treatment regimen does exist, Multi-Drug
Resistant (MDR-TB) and eXtremely Drug Resistant (XDR-TB) TB strains are becoming increasingly prevalent due to failure to complete the drug regimen as well as the development of antibiotic tolerance. In 2012, approximately 450,000 people were diagnosed with MDR-TB and there were approximately 170,000 deaths due to MDR-TB that year [1]. Of those people with MDR-TB, 9% of the cases were XDR-TB [4]. Treatment for drug resistant tuberculosis is even more expensive, difficult to administer, accompanied by dangerous side effects, and requires a prolonged treatment of 18 to 24 months [5]. Due to these factors, noncompliance with treatment and mortality rates are even higher among these patients than those with drug-susceptible TB [1]. Single nucleotide polymorphisms (SNPs) in the drug targets of the TB genome are the major cause underlying the development of drug resistance [9]. Other mechanisms that may confer resistance occur through mutations that disrupt pro-drug activation or lead to up-regulation of the target gene [5].

Currently we rely on suboptimal diagnostic technologies for the detection of *Mtb* which include sputum smear microscopy, culture based testing, and molecular diagnostics such as line-probe assays and RT-PCR techniques like the GeneXpert. Sputum smear microscopy is the most common diagnostic test for TB [10]. It is ineffective due to the fact that it detects less than fifty percent of TB cases and even less in children and patients with HIV [10]. Also sputum smear microscopy is unable to differentiate between different mycobacterium species, such as *Mycobacterium abscessus* (*M. abs*), for example, which requires its own multi-drug treatment regimen [11]. Culture-based testing is extremely slow and presents a risk of infection to those conducting the protocol [10]. Molecular diagnostics, including line-probe assays and RT-PCR techniques like the GeneXpert, are the optimum methods for the detection of drug resistance, however they are not feasible due to
the high cost, technical expertise, and requirement of expensive instruments and cold storage of reagents which are typically not available in high burden areas [12].

Due to the limitations listed above, more efficient POC diagnostics and drug susceptibility testing (DST) are urgently needed to detect drug resistance and facilitate prompt, appropriate treatment plans. This project seeks to develop a novel diagnostic technology based on deoxyribozyme (DNAzyme) sensors for detection of TB and the identification of drug resistance. The overall goal is to create a POC assay which combines Polymerase Chain Reaction (PCR) and DNAzymes for the identification of tuberculosis and drug resistance.
BACKGROUND

To understand the need to create a new diagnostic assay for the detection of TB and identification of drug resistance, a deeper knowledge of the pathology of the disease, the rising global threat of drug resistance, and current diagnostic technology available will be necessary. The discussion of methods that will be utilized for the development a novel diagnostic tool that can identify TB and its drug susceptibility, will follow.

The Bacterium

The evolution of *Mycobacterium tuberculosis* dates back thousands of years ago, beginning from tubercle bacilli in East Africa. This suggests that the pathogenic bacterium has affected the human race from the beginning of time, coevolving with its host to survive [13]. Members of the *Mtb* complex include *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canetti*, *Mycobacterium microti*, and *Mycobacterium bovis* [13]. While they differ widely in their preferred environment, phenotype, and pathogenicity, all cause pulmonary TB-like disease in mammals [14]. The bacteria of the *Mtb* complex are 99.9% similar at the DNA level and share identical hypervariable regions on their 16S and 23S rRNA sequences [15, 16]. Before the entire genome was sequenced, it was proposed that *M. tuberculosis* went through a steep reduction in the size of its genetic variation, otherwise known as a bottleneck, due to environmental events that date back to about 15-20,000 years ago and was thought to have evolved to the human host from *Mycobacterium bovis* (*M. bovis*), the agent of bovine tuberculosis due to the high conservation within the species, however this is still up for debate [17-19]. The similarity between *M. tuberculosis* and *M. bovis* has enabled the development of
an *M. bovis* bacilli Calmette-Guerin (BCG) vaccine for the disease that is administered in countries with a high burden of TB [20, 21].

*M. tuberculosis* is a member of the Actinomycete family and can be characterized by its unusually thick mycolic acid-rich cell wall that allows it be acid-fast stained [22]. *Mt* is a slow-growing, intracellular pathogen, whose generation time of about 15-20 hours is thought to contribute to its virulence [22]. *Mt* are exclusively mammalian pathogens, needing no environmental reservoir [22]. The bacterial adaptations of *Mt* which allow for its survival in host immune cells have enabled it to become one of the most successful human pathogens and the mycobacteria continues to remain a major cause of death worldwide [23].

**Pathology of Tuberculosis**

*M. tuberculosis* was the cause of White Plague of Europe in the 17th century and is still the leading cause of death due to a bacterial infectious disease worldwide [22]. The bacterium is spread through aerosolized, hydrophobic droplets expelled from a patient with the active disease that may be inhaled by another host [24]. Upon reaching the alveoli, the pathogen is taken up by alveolar macrophages and resides within the lung [24]. The macrophages, while unable to kill the mycobacteria, are able to produce an immune response that summons more macrophages and other immune cells to aggregate and form a granuloma, the hallmark lesion of TB [2]. The immune cells of the granuloma form a barrier around the bacteria in an effort to prevent the spread of the pathogen [25]. Studies have shown that the virulent mycobacteria are able to replicate within the macrophage and eventually cause macrophage cell death by inducing TNF suppression [26]. The pathogen is able to survive within dead and dying macrophage at the necrotic center of the granuloma [2].
Mtb’s ability to survive and replicate in macrophages is key to its survival with the host [2]. Once internalized by the macrophage, the pathogen resides within a phagosome [2]. The phagosomes role is to eradicate the pathogen through mechanisms such as acidification within a lysosome, reactive oxygen and nitrogen intermediates (ROS, RNI), proteolytic activity, and antimicrobial peptides [27]. Mtb is able to inhibit these phagosomal processes to avoid its death and create a favorable environment for replication. Their ability to avoid acidification is due to glycolipids known as sulfatides that prevent phagosome-lysosome fusion [28]. The cell wall of the pathogen consists mostly of lipids, specifically mycolic acids, which form a highly impermeable shell that prevents the deadly attack of cationic proteins, lysozyme, and oxygen radicals in the phagosome [22]. The pathogen also manipulates the phagosome to gain access to nutrients and creates an advantageous atmosphere for replication [27]. Mtb replicates very slowly within macrophages, which contributes to its virulence because the immune system may not recognize the bacteria right away [22]. The mycobacterium has also been known to infect other cell types, such as dendritic cells, neutrophils, and adipocytes as well [29]. The high burden of the increasing number of unrestricted, replicating mycobacteria cause the macrophage to undergo apoptosis, spreading the bacteria to new macrophages [2]. Once the initial macrophages burst, Mtb may be taken up by other migrating cells, such as lymphocytes and dendritic cells, which have been shown to facilitate its dissemination to distal tissues, like the spleen, liver, and central nervous system [22]. At this stage, the lymphocytes that come in contact with the pathogen are able to activate the immune system in an effort to destroy the life-threatening bug [22].

If the host’s immune system is not able to control the pathogen, the TB infection progresses to an active disease. Active TB occurs in 10% of infected patients due to immune suppressing
circumstances like HIV-coinfection, the use of immunosuppressive drugs, re-infection, or old age, which allows the bacilli to grow and multiply inside the host [30, 31]. Individuals with active TB are infectious, will present with symptoms, and may test positive in a sputum smear microscopy test [6]. Symptoms include a chronic cough, chest pain, production of sputum, weight loss, and fever, and may be fatal if not treated properly [32].

However, not everyone who breathes in the aerosolized pathogen develops the active form of the disease. About 90% of infected individuals are able to contain the bacteria within the granuloma in a noninfectious form known as latent tuberculosis, which is asymptomatic, sputum-smear negative, and does not pose a health threat until the immune system becomes compromised and the TB can be reactivated [6]. The chance of reactivation creates an enormous reservoir of infection and makes the goal of eradicating this disease seem almost unattainable.

**The Challenge of Drug Resistance**

Each year, approximately 8 million people become infected with TB and over 95% of deaths due to the pathogen occur in low and middle income countries [33, 34]. Treatment regimens for active tuberculosis consists of combination of four drugs taken daily for 6-9 months [35]. The failure of patients to adhere to this lengthy treatment, as well as irregular drug supply and improper prescriptions, make the global eradication of TB difficult and contribute to its drug resistance [36].

*M. tuberculosis* has evolved many intrinsic and molecular mechanisms that also make it difficult to treat. It is difficult for antibiotics to penetrate the bacterium due to the impermeability of its mostly lipid cell envelop, therefore extended treatment is needed to be effective [37]. In its latent form, the non-replicating state of tuberculosis within the granuloma allows it to be tolerant to
antibiotics whose main mechanism of action is to attack the replication process of the mycobacteria [28]. The induction of efflux pumps within the macrophage can facilitate the survival of active TB within the granuloma, allowing antibiotic resistance to develop. [38, 39]. The localization of \textit{Mtb} within host cells in various tissue sites contributes to the poor efficacy of therapeutics. The wall of the granuloma and poor vascularization also make it difficult for adequate levels of antibiotics penetrate to reach the tuberculosis, enabling the pathogen to slowly develop a resistance to the sub-therapeutic treatment [27].

On the molecular level, positive selection of spontaneous mutants harboring single nucleotide polymorphisms (SNPs) are the major contributor of drug resistance [9]. In recent years, the prevalence of drug resistant strains has been increasing, especially in China, India, and South Africa [23]. Multidrug-resistant tuberculosis (MDR-TB) is resistant to the first-line drugs, isoniazid (INH) and rifampin (RIF) and is caused by the inappropriate or incorrect use of antibiotics as described above. MDR-TB can be treated with second-line drugs such as fluoroquinolones, kanamycin, and capreomycin [35, 40]. However, second-line treatment, when available, is more costly and time extensive leading to even greater lack of compliance. The selective pressure created by the failure to complete the second-line treatment leads to eXtremely drug-resistant TB (XDR-TB) which is resistant to both first-line drugs, as well as at least one fluoroquinolone (FQ) and one second-line, injectable drug [41]. In 2013 alone, 480,000 people developed MDR-TB and 9% of these cases were estimated to be XDR-TB [36].

\textbf{Mechanism of Drug Resistance}

The severity of the rising drug resistance epidemic is due, in part, to the many mechanisms that \textit{Mtb} can use to gain resistance. As mentioned above, mechanisms of drug resistance include
intrinsic factors such as phenotypic drug tolerance mediated by efflux pumps, characteristic of its cell wall, and SNPs.

SNPs within the drug targets of the TB genome can confer resistance to specific first- and second-line drugs. This project specifically focuses on the *katG*, *inhA*, *rpoB*, and *gyrA* genes as they contain the most commonly occurring drug resistance mutations [5]. Mutations in the sequence of *katG*, a catalase-peroxidase enzyme, cause decreased activation of the prodrug INH, the most widely used first-line drug against tuberculosis, and contribute to 75-90% of the strains resistance to INH [5, 42]. Mutations in the promoter region of the drug target gene *inhA*, which functions as a NADH-dependent enoyl-acyl carrier protein, account for an additional 10% of strains resistance to INH [5, 42]. Mutations in the beta subunit of RNA polymerase, encoded by the *rpoB* gene, are present in 90% of strains that are resistant to RIF, another first-line drug used to treat TB [5, 42].

Resistance to fluoroquinolones, a second-line anti-tuberculosis drug, is conferred by mutations in DNA gyrase, an enzyme involved in regulating the supercoiled topology of double-stranded DNA (dsDNA) [5]. DNA gyrase consists of two alpha and two beta subunits encoded by *gyrA* and *gyrB* respectively [5]. Over 90% of fluoroquinolone resistant *M. tuberculosis* strains have mutation in these two genes, contributing to XDR TB [5]. Currently other members of the Rohde lab are developing MDR sensors for INH and RIF. This project specifically focuses on the development of an XDR assay that will identify resistance conferring SNPs on the *gyrA* gene [5]. Mutations within a highly conserved area of *gyrA* known as the quinolone-resistance-determining-region (QRDR) have been shown to confer fluoroquinolone resistance on TB [5]. Codons 90 and 94 of *gyrA* contain the most prevalent resistance-conferring mutations, particularly A90V (GCG→GTG) and D94G (GAC→GGC) [5]. The goal of one of the aims of this project is to
design sensors that will identify the D94G mutation in gyrA. The development of a molecular assay with the ability to detect these SNPs indicative of a drug\textsuperscript{R} profile could enable the rapid and efficient treatment of TB.

**Current Diagnostic Methods**

Rapid and affordable POC detection of tuberculosis and drug resistance is imperative to improve treatment and control the spread of TB. Current methods of detection include microscopy, culture and drug susceptibility testing, as well as molecular testing [43]. Sputum smear microscopy, is the most widely used method to detect infectious TB, especially in low and middle income countries because it takes advantage of inexpensive equipment and materials. This diagnostic method is accomplished through the collection and analysis of sputum specimens from persons suspected of having TB [10]. Due to the acid-fast nature of its cell wall, tuberculosis bacilli are able to be stained using the Ziehl-Neelson staining method and examined microscopically [22]. However, acid-fast organisms other than *M. tuberculosis* may appear [10]. Less than 50% of cases are usually detected because an excess of 10,000 microbes/mL of sputum are needed to visualize the bacilli and many positive TB patients have been shown to have negative smears (i.e. children and co-infected-HIV patients) because of their pauci-bacillary sputum samples (low bacilli numbers in sputum below detection ability of assay) [10, 22]. The pathogen may also be undetectable with this method depending on how advanced the infection is, because some *Mtb* lose their acid-fastness. Other limitations of smear microscopy include its time consuming protocol and inability to detect drug resistance [10, 44, 45]. Due to all of these factors, sputum smear microscopy can only be used as a preliminary measure.
The culture methods may be used in diagnosis of *Mtb* but further testing is needed to differentiate *Mtb* from Non-tuberculosis Mycobacteria (NTM) and it requires highly technical expertise and expensive machinery. It is important to specifically identify the *M. tuberculosis* species because other NTM may require their own specific treatment regimens. This method is also at a disadvantage because it can take up to 6 weeks to produce results, allowing for the possibility of inappropriate treatment if patients are started on a typical treatment regimen before results are obtained. If the patient is indeed infected with MDR-TB, the treatment will be ineffective allowing the spread and amplification of drug resistant TB in the meantime. Drug susceptibility testing, through the culture-based observation of growth or inhibition in a medium containing antituberculosis drug, can provide a definite diagnosis of drug-resistant TB but its methods are also costly and not readily available in high burden areas.

Molecular testing involves examining the DNA of specific genes that are known to cause drug resistance if mutated. Methods such as conventional DNA sequencing, pyrosequencing, real-time PCR, and line-probe assays are the optimum diagnostic methods available due to their rapid, accurate, and safe detection strategies however these techniques are still accompanied by limitations. All of the current molecular diagnostics are extremely labor intensive and expensive. DNA sequencing and pyrosequencing require very complex machinery and costly reagents that may not be readily available in POC environments. Real-time PCR techniques such as the fully automated GeneXpert MTB/RIF have been proven to rapidly detect the presence of *M. tuberculosis* as well as identify rifampin resistance. It does so by employing multiple fluorescent molecular beacons to detect the presence of *rpoB* mutations, which confer 95% of rifampin resistance. Line Probe Assays, such as the Hain Line Probe Assay MRBDRplus, employ immobilized membrane-
bound probes that result in a color change upon binding to specific PCR products that are able to identify mutations in the \textit{rpoB}, \textit{katG}, and \textit{inhA} genes that confer resistance to RIF and INH [5, 12]. However, both of these molecular methods can yield inaccurate results due to silent mutations, require multiple beacons/probes, and, besides being very expensive, are not amenable for POC use [12, 43]. The pitfalls of these current techniques reinforce the urgent need for more efficient diagnostics and drug susceptibility testing (DST) that can be used to detect drug resistance and facilitate prompt, appropriate treatment plans. This project seeks to address the lack of quick, specific, and affordable diagnostics through the development of a novel diagnostic technology based on deoxyribozyme (DNAzyme) sensors for detection of TB and identification of drug resistance.

**DNAzyme Sensor Technology for specific detection and differentiation of nucleic acids**

Deoxyribozymes (DNAzymes) are catalytically active DNA enzymes that were identified by \textit{in vitro} selection [48]. Originally, DNAzymes were designed as a single DNA strand consisting of a catalytic domain flanked by two substrate recognition arms [49]. In the presence of a specific analyte, the DNAzyme folds into a catalytically active secondary structure that hybridizes to the analyte DNA and cleaves a ribonucleotide within the middle of the sequence. However, the length of the original DNAzyme contributes to its stability, enabling it to hybridize to mismatched sequences and therefore preventing its specificity [50]. A binary approach, developed by our collaborator, Dr. Dmitry Kolpashchikov, divides the DNAzyme into two parts, splitting the catalytic core in half, and allowing for extremely selective nucleic acid recognition and improved sensitivity of SNP detection down to a single base substitution [50].

We take advantage of this binary DNAzyme design to create DNAzymes, specific for \textit{Mtb} analytes, which will have the ability to distinguish drug\textsuperscript{a}-conferring SNPs. As shown in Figure 1, the
binary DNAzyme assay consists of a substrate, analyte, and two sensor strands each containing an analyte binding arm, half of the catalytic core, and a substrate binding arm. The substrate is a dual-labeled synthetic oligonucleotide with a fluorophore on the 5’ end and a quencher at the 3’ end. When a specific analyte is present, the sensor strands will bind adjacently on the analyte. After binding the analyte, the sensors will then bind the substrate, reforming the catalytic core, and subsequently cleave the substrate. This cleavage separates the quencher and the fluorophore resulting in the production of a fluorescent signal/output [51].

**Figure 1: Binary DNAzyme Sensor Assay**

Deoxyribozyme sensors can be used for the detection and differentiation of DNA/RNA analytes. This binary DNAzyme approach splits the catalytic core and allows for the attachment of two short sequences complementary to the analyte binding arms. In the presence of a DNA/RNA analyte the substrate will be cleaved releasing a fluorescent/visual output. A) Binary DNAzyme sensor bound to a matched analyte. The analyte binding arms hybridize to the matched analyte allowing the formation of the catalytic core. This results in cleavage of the substrate and the production of a fluorescent output. B) Binary DNAzyme sensor in the presence of mismatched analyte. Due to the presence of a SNP, one of the analyte binding arms will be unable to bind and the catalytic core cannot form. Therefore, there will be no cleavage of the substrate.

DNAzymes provide several advantages for the specific and sensitive detection of DNA/RNA analytes. Their catalytic activity allows for activation of approximately eighty substrate molecules/minute, resulting in signal amplification, unlike the Gene Xpert and molecular beacon based approaches that are only able to bind a single fluorescent probe/analyte molecule [52]. The simple design requires only two short DNA strands for each new analyte designed because the double-labeled reporter substrate is universal for all analytes, reducing the cost of this technology.
significantly [50, 53]. The combination of a short analyte binding arm and a long analyte binding arm allows for high sensitivity and selectivity [50]. The presence of a single SNP prevents the binding of the short analyte binding arm, inhibiting the activation of the DNAzyme and substrate cleavage [50]. Longer oligonucleotides are more likely to bind mismatched DNA. The overall goal is to create an assay which combines Polymerase Chain Reaction (PCR) and DNAzymes to identify drug resistance conferring SNPs.

The goals for the project are as follows:

**Objective 1: To construct a panel of drug resistant (drugR) nonpathogenic M. bovis BCG.**

MDR-TB is dangerous, therefore creating a library of attenuated drugR *Mycobacterium bovis* BCG allows us to safely test different DNAzyme sensors on real bacteria. This will be accomplished by cloning regions surrounding the SNP of interest, making the mutation by QuickChange site directed mutagenesis, and transforming it into wild-type *M. bovis* BCG, and selecting for resistance. This project will focus on drugR conferring mutations in the *gyrA* gene.

**Objective 2: To design a multiplex PCR that will amplify chromosomal regions necessary for TB detection and drug susceptibility testing (DST)**

The overall goal is to use six sets of primers to simultaneously amplify regions of the genome that will be used later in the multiplex assay for the species-specific detection of TB and determination of a drug susceptibility profile based on the presence of SNPs. Nucleic acid amplification (NAA) through PCR is imperative to increase the sensitivity of the assay, because the current limit of detection is not sensitive enough to detect a single copy on the chromosome. The PCR conditions will be optimized to achieve optimal specificity, yield, and speed.
Objective 3: To design and optimize DNAzyme sensor assays of PCR analytes for detection and DST of Mtb.

The binary design of the DNAzyme assay allows for improved sensitivity of SNP detection. The sensors will be optimized to balance selectivity and sensitivity. This will be accomplished by constructing sensors that will discriminate between single SNPs and still produce a good signal. The limit of detection (LOD) and specificity will be determined. The sensors will be tested on synthetic analytes, DNA from MDR- and XDR-TB clinical isolates. Combining the DNAzyme sensors with the multiplex PCR analytes as an optimized assay will enable the rapid, POC detection of drug resistance.
METHODOLOGY

Preliminary Studies

Originally it was thought that single stranded DNA (ssDNA) was the preferred DNAzyme analyte. To generate the ssDNA analyte, we explored the use of Linear After The Exponential PCR (LATE-PCR), an asymmetric PCR method which employs unequal concentrations of primers that run logarithmically until one of the primers run out [54].

Our preliminary data comparing LATE-PCR and conventional symmetric PCR revealed that the generation of ssDNA is not necessary for detection by DNAzymes (data collected by B. Rosenkrantz not shown). This is advantageous because asymmetric PCR requires many cycles while the symmetric approach can be completed in less cycles, meaning less time. As shown in Figure 2, using the symmetric PCR we were able to detect as few as 50 pg of DNA or 5000 colonies forming units (CFU).

![Figure 2: Limit of Detection for DNAzyme detection of Symmetric PCR Analytes.](image)

Serial dilutions of template DNA (x-axis) were amplified by 30-cycles of symmetric PCR and detected by binary DNAzyme assay. Wild-type specific rpoB binary sensors were used for the detection of PCR products. The Signal to Background (S/B) ratio (y-axis) shows that Symmetric PCR (30 cycles) and DNAzyme sensors can detect as little as ~50 pg of DNA or ~5000 CFU. (Data provided by B. Rosenkrantz)
General Overview of Bacterial Cell Culture

NEB 10-Beta Competent *Escherichia coli* (*E. coli*) High Efficiency and electrocompetent *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) were used to conduct the experiments for this project. *E. coli* was used to amplify plasmids containing genes and DNA fragments PCR amplified from CDC1551 chromosomal *Mtb* DNA (20 ng/μL) during cloning [55]. *E. coli* was also used as a DNA source for transformation into *M. bovis* BCG for the development of a panel of nonpathogenic drug resistant strains.

*E. coli* was grown up in Luria broth (LB) within a 5 mL culture tube overnight at 37°C, shaking at 250 revolutions per minute (rpm). When constructing plasmids, 50 μg/mL of the antibiotic Kanamycin (Kan) was used to select clones containing the vectors of interest.

Creating a panel of drug resistant (Drug\(^R\)) nonpathogenic *M. bovis* BCG.

The conservation of DNA between *Mtb* and the vaccine strain *M. bovis* BCG has enabled BCG to be used as a surrogate model for *Mtb*. Designing a panel of nonpathogenic BCG strains with specific SNPs that confer distinct drug\(^R\) will provide a safe template that has the same genetic background as the real drug\(^R\) *Mtb*. The variety of strains containing drug\(^R\) mutants will be used to develop an assay that can differentiate individual mutations in a single sample. This project specifically focuses on the development of a strain containing a drug\(^R\) conferring *gyrA* D94G (GAC\(\rightarrow\)GGC) mutation.
Construction of *gyrA* 94 WT Fragment

First, primers, shown in Table 2, were designed to specifically amplify a 2 kilobase (kb) *gyrA* gene fragment using “ApE – A plasmid Editor”, a molecular biology program used for sequence editing and design, and information from Mycobacterial DNA databases including the “Myco DB - xBASE” and TbDb databases [56-59]. Then, amplification of the *gyrA* gene insert was achieved by Polymerase Chain Reaction (PCR). The DNA is PCR amplified through a few general steps. First, the double stranded DNA must be denatured to separate the complementary strands of DNA by heating it to 95°C [60]. After this primary denaturation, a secondary denaturation is necessary to ensure complete separation [60]. The third step is an annealing phase in which the temperature lowers to allow the DNA primers to hybridize to their complementary sites on the DNA template [61]. The annealing temperature is dependent on the melting temperature (Tm) of the specific primers used [61]. The primers are then extended using DNA polymerase, an enzyme that catalyzes the synthesis of new DNA by adding nucleotides onto the annealed primer that are complimentary to the template DNA [60]. This step is conducted at a temperature optimal for the Phusion® DNA polymerase, allowing the enzyme to replicate the strand of template DNA. These three steps are repeated for many rounds to enable the exponential amplification of the DNA of interest. Lastly, there is a final extension step that allows for the most recent DNA copies to finish replicating [60].

<table>
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<tr>
<th>Cycle Number</th>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
<th>PCR Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98</td>
<td>30</td>
<td>Initial Denaturation</td>
</tr>
<tr>
<td>2 (20X)</td>
<td>98</td>
<td>10</td>
<td>Secondary Denaturation</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>30</td>
<td>Annealing Phase – based on the primers’ melting temperature</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>25</td>
<td>Initial Extension</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>60</td>
<td>Final Extension</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>∞</td>
<td>Hold Time</td>
</tr>
</tbody>
</table>

Table 1: Phusion PCR Reaction Settings
The protocol followed was “PCR Protocol for Phusion® High-Fidelity DNA Polymerase” [62]. The reaction was ran in a temperature-controlled BioRad thermocycler, allowing for the proper denaturation and annealing of DNA fragments and enabling the Phusion® enzyme to activate at specific temperatures. The typical Phusion PCR Cycle used is shown in Table 1. The \( \text{gyrA} \) specific primers were used to clone 1.0 kilobase (kb) on both sides of the well-characterized \( \text{gyrA} \) SNP location.

The reaction consists of a mixture containing buffers, nucleotides, primers, enzyme, and the DNA of interest. A typical PCR reaction contains 1.0 \( \mu \text{L} \) of template DNA (~20 ng/\( \mu \text{L} \)), 1.0 \( \mu \text{L} \) of forward primer (100 ng/\( \mu \text{L} \)), 1.0 \( \mu \text{L} \) of reverse primer (100 ng/\( \mu \text{L} \)), 5.0 \( \mu \text{L} \) of 5X GC Buffer, 0.25 \( \mu \text{L} \) of Phusion® Enzyme, 0.5 \( \mu \text{L} \) of 10mM deoxynucluotides (dNTPs), and 16.25 \( \mu \text{L} \) of sterile water, for a total volume of 25 \( \mu \text{L} \) per reaction tube. After amplification, the presence and correct size of the PCR products were verified using agarose gel electrophoresis. For detection, 5.0 \( \mu \text{L} \) of the PCR product was resolved on 1% agarose gel using TAE buffer and stained with Promega Gel-red.

**TOPO Cloning**

Once the insert containing the amplified wild type (WT) \( \text{gyrA} \) fragment was obtained, a plasmid containing the WT sequence encompassing SNPs in the target gene was constructed. This was accomplished using Zero Blunt® TOPO® cloning, an extremely efficient, 5-minute, one-step cloning process for the direct insertion of blunt-ended PCR products into a plasmid vector. The enzyme, Topoisomerase, is linked to the end of the linearized TOPO vector and catalyzes the ligation of an insert into the vector [63, 64]. This process is efficient because no ligase, post-PCR procedures, or PCR primers containing specific sequences are required. The TOPO process is also
advantageous because it allows direct selection of recombinants. The pCR4Blunt-TOPO® plasmid contains a Kan® gene that is used to select for colonies that have incorporated the vector and cells containing the non-recombinant vector, lacking the resistance, will be killed upon plating [65].

The TOPO Cloning reaction mixture consists of 1.0 μL of the TOPO Cloning vector, 1.0 μL of a salt solution, 2.0 μL of the 2 kb gyrA PCR product, and enough sterile water was added to bring the final volume up to 6.0 μL. The reaction was incubated for 5 minutes at room temperature 25°C then placed on ice. 2.0 μL of this TOPO Cloning reaction mix was pipetted into a vial of competent E. coli, mixed gently, and incubated on ice for 30 minutes. After incubating, the cells were heat shocked at 42 °C for 30 seconds and immediately transferred to ice for recovery. 250 μL of room temperature SOC medium was added and the tube was capped and shook horizontally at 37°C for 1 hour. 10-50 μL of the transformation was spread on a Kan antibiotic selective plate and incubated at 37°C overnight.

Colonies present on the pTOPO-gyrA transformation were PCR screened using M13 forward and reverse primers (100 ng/μL), shown in Table 2, to verify that the insert was present in the vector. Colony PCR screening involves bacterial lysis and DNA extraction for use in a PCR reaction. To lyse the bacteria, an isolated colony was labeled, picked from the plate, and added to a tube containing 20 μL of deionized water (diH2O). The tube was boiled at 100°C for 10 minutes and centrifuged at 13,000 rpm for 10 minutes to pellet the bacterial debris. 2.0 μL of the resulting supernatant was used as the DNA template for PCR amplification. Positive colonies were identified based on the amplification of the band at an anticipated size of 2 kb on a 1% agarose gel.

The positive colonies were picked and grown overnight in 7 mL LB broth containing 7.0 μg/mL Kan to amplify the DNA. The plasmid was then isolated using the Plasmid Mini-Prep
Protocol. [66] First, the pellet was harvested from the *E. coli* culture using centrifugation. The supernatant was discarded and the pellet was suspended in 250 μL of an RNase A Buffer (100 μg/mL) [66]. After adding 250 μL of MX2 Buffer, the tube was gently inverted 4-6 times, and allowed to incubate at room temperature for 5 minutes until the solution became clear [66]. 350 μL of MX3 Buffer was then added, the tube was inverted 4-6 times afterwards, and centrifuged at 12,000 rpm for 10 minutes [66]. The supernatant was then transferred to a spin column and centrifuged at 4,000 rpm for 1 minute and the flow through was discarded. 500 μL of WS Buffer was added to the spin column and centrifuged for 1 minute, discarding flow through [66]. This was done twice to wash the mixture. The resulting mixture was centrifuged again to remove residual ethanol and the column was placed in a new microcentrifuge tube. 50 μL of EB Buffer was added to the membrane and, after sitting for 5 minutes, the tube was centrifuged at 12,000 rpm for 1 minute to elute the DNA [66]. The concentration of the resulting mini-prep DNA was measured using a Nanodrop spectrophotometer and the plasmid was submitted to Genewiz for sequencing to verify the presence of the 2 kb *gyrA* fragment. Then glycerol stocks were made to store aliquots of the DNA by inoculating 500 μL of 50% glycerol with 1.0 mL of the culture. The purified plasmid was then used as a template for QuikChange mutagenesis to create the *gyrA* mutant (MT).
Table 2: Cloning Primers

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Size (bp)</th>
<th>Tm</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{gyrA} 2kb</td>
<td>\textit{gyrA}_2kb\textsubscript{F}</td>
<td>ATTGCGCTCCACGGGATC</td>
<td>18</td>
<td>66</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>\textit{gyrA}_2kb\textsubscript{R}</td>
<td>GGGCGATATCGACGGTCTT</td>
<td>18</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>\textit{gyrA} 94 QuikChange</td>
<td>\textit{gyrA}_{QC}\textsubscript{F}</td>
<td>CGATCTACGGGCCACCTGG</td>
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<td>67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{gyrA}_{QC}\textsubscript{R}</td>
<td>CCAGGCTGCGTAGATCG</td>
<td>18</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>M13Long</td>
<td>M13L\textsubscript{F}</td>
<td>TGTAAAACGACGGCCAGTGAATTT</td>
<td>23</td>
<td>68</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>M13L\textsubscript{R}</td>
<td>CAGGAAACAGCTATGACCATGAT</td>
<td>23</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3: Creation of a drug\textsuperscript{R} BCG strain using TOPO Cloning and Site-Directed Mutagenesis

A) \textit{gyrA} specific primers were used to clone 2 kb fragment containing a \textit{gyrA} gene and Phusion PCR was used to amplify the WT \textit{gyrA} 94 gene of interest within CDC1551 \textit{Mtb} DNA (20 ng/\muL). B) TOPO cloning was used to insert the 2 kb WT \textit{gyrA} 94 fragment into the pCR4Blunt-TOPO plasmid containing Kan\textsuperscript{R} antibiotic resistance marker. C) QuikChange PCR primers designed to contain the D94G SNP in the center of their sequences were used for the rapid insertion of the mutation. D) Once the mutation was inserted, the 2 kb PCR Primers were used to amplify the \textit{gyrA} mutation. E) Butanol Precipitation was used to prepare the MT product for transformation into BCG. F) After making competent cells, electroporation was used to transform the 2 kb linear PCR product containing the mutation into WT BCG. G) The drug\textsuperscript{R} \textit{gyrA} D94G BCG strain can then be selected for on Ciprofloxacin plates.
QuikChange Site-Directed Mutagenesis

After successfully TOPO cloning, methodology similar to QuikChange (QC) Site-Directed Mutagenesis was performed to insert the mutation, as shown in Figure 3. This was accomplished through a series of steps. First, individual mutagenic primers needed to be designed. Both the forward and reverse primers were designed to contain the gyrA D94G SNP of interest and to anneal to the same sequence on opposite strands of the plasmid, as shown in Table 2. The desired mutation was designed to be in the middle of the primers with ~10-15 nucleotides of the correct sequence on each side [67]. This reaction followed the protocol by Stratagene, in which 2.0 μL of lysate from colonies containing the gyrA insert was combined with 1.0 μL of each gyrA Mutation QuikChange forward and reverse primer (100 ng/μL), 0.25 μL of Phusion® polymerase, 0.5 μL of dNTP’s, 5.0 μL of 5X G.C. Buffer, and brought to a final volume of 25 μL using 16.25 μL of diH2O. The thermocycler PCR settings used for the QuikChange are shown in Table 3.

Table 3: QuikChange PCR Reaction Settings

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
<th>PCR Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98</td>
<td>30</td>
<td>Initial Denaturation</td>
</tr>
<tr>
<td>2 (30X)</td>
<td>98</td>
<td>10</td>
<td>Secondary Denaturation</td>
</tr>
<tr>
<td>2 (30X)</td>
<td>65</td>
<td>30</td>
<td>Annealing Phase – based in the primers’ melting temperature.</td>
</tr>
<tr>
<td>2 (30X)</td>
<td>72</td>
<td>90</td>
<td>Initial Extension</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>60</td>
<td>Final Extension</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>∞</td>
<td>Hold Time</td>
</tr>
</tbody>
</table>

After performing the QuikChange PCR and verifying the successful amplification and size of the plasmid on a 1% gel, 1.0 μL of Dpn 1 restriction enzyme was added to the positive QuikChanged PCR sample, mixed thoroughly, and incubated for 1 hour at 37°C to digest the
nonmutated (parental) supercoiled dsDNA. Failure to do so results in a high false-positive rate of pTOPO-\textit{gyrA} Kan\textsuperscript{8} colonies.

After thawing an aliquot of 10-beta Competent \textit{E. coli} cells, 4.0 \(\mu\text{L}\) of digested \textit{gyrA} Mutant QuikChanged fragment was added. The cells incubated on ice for 30 minutes and were then heat shocked for 45 seconds at 42\(^\circ\)C. After recovering on ice for 5 minutes, 250 \(\mu\text{L}\) of SOC medium was added and the cells were shaken for 1.5 hours to recover. Then, the entire volume of the transformation reaction was plated on LB-Kan (50 \(\text{ug}/\mu\text{L}\)) agar to select for antibiotic resistance and incubated overnight at 37\(^\circ\)C.

The colonies that grew were PCR screened for the insert of interest using M13 forward and reverse primers and verified on a 1\% agarose gel. The PCR products of the \textit{E. coli} colonies determined to contain the insert of interest were then prepared for direct sequencing using a PCR clean-up method that removes excess dNTPs and unincorporated primers from the PCR mixture. The PCR mixture was incubated at 37\(^\circ\)C for 20 minutes in an Exo-SAP mixture consisting of Antarctic alkaline phosphatase, buffer, exonuclease (20 units/\(\mu\text{L}\)), and \text{dH}_{2}\text{O}, to purify the DNA. The pure DNA was then sent for sequencing to determine if the correct mutation was present. The sequence was analyzed using ApE to align the mutated \textit{gyrA} D94G sequence with the original WT \textit{gyrA} sequence to verify that the correct mutation was present.

**Transformation into Wild Type BCG**

Once the mutated \textit{gyrA} PCR product was amplified and the sequence was verified, butanol precipitation was performed to prepare the product for transformation into wild type BCG. This method removes salt from the ligation buffer to improve transformation efficiency. 10 \(\mu\text{L}\) of
unsaturated n-Butanol was used to precipitate every 1.0 μL of PCR reaction. The mixture was spun down in at 13,000 rpm for 5 minutes to remove supernatant. The tube was then washed with 100 μL of 70% ethanol and centrifuged for 5 more minutes to further remove any leftover supernatant. A speed vacuum was used to dry the pellet and then the pellet was suspended in 10 μL of water. The concentration of the MT gyrA 2 kb PCR product was then quantified using the Nanodrop spectrophotometer.

30 mL of M. bovis BCG was pelleted with a 20 mL 10% glycerol wash to rid of excess salt. This was repeated in smaller and smaller increments until 1.0 mL of BCG was left. Then, 2.0 μg of gyrA MT DNA was transformed into 100 μL of electrocompetent BCG cells. The electroporation was accomplished in a 2.0 mL cuvette using the Biorad GenePulser Xcel Microbial System at 250 Volts, 25 μF, and 1,000 Ω. After shocking the cells, they were placed in 5 mL 7H9 and allowed to recover for 3 days.

After the recovery, 2 mL of electrocompetent drug\textsuperscript{R} BCG was plated on 2 μg/mL Ciprofloxacin (Cipro) LB agar to select for resistant colonies that have incorporated the SNP-containing DNA. Colonies were picked at 3 weeks and grown in 5 mL of 7H9 media. Then after growing for another 3 weeks, a well-established method of nucleic acid isolation from mycobacteria known as a hexadecyltrimethylammonium bromide (CTAB) treatment, was used to purify the DNA [68]. Once purified, the DNA product was sent for sequencing to confirm the transformation. The resulting engineered drug\textsuperscript{R} M. bovis BCG DNA was used as a template in the multiplex PCR which will later be tested in the DNAzyme assay.
Design of the Multiplex PCR

The goal of this aim is to develop a multiplex PCR with the ability to simultaneously amplify six targets, including MTC-specific loci and drug\(^R\) loci for MDR and XDR-TB, in a single reaction. The multiplex PCR will be combined with DNAzyme sensors in an assay to specifically detect TB and identify first and second line drug\(^R\) in a single sample. PCR amplification is important because the current limit of detection is not sensitive enough to detect a single copy on the chromosome.

Primer Design

First, primers were designed to specifically amplify the regions of interest. For species specific identification of TB, two of the primer pairs were designed to target the 16S and 23S rRNA genes, which are completely identically in BCG and TB. The targeted regions of the 16S and 23S rRNA genes are hypervariable regions unique to \(Mtb\) and therefore are capable of distinguishing TB and BCG from other members of the \(Mycobacterium\) \(tuberculosis\) complex (MTC).

Then 4 other primers pairs were designed to target genes associated with drug\(^R\). As stated in the background section, the target genes, \(gyrA\), \(katG\), \(rpoB\), and \(inhA\), were picked to cover the most common SNPs conferring resistance to first and second line drugs. Mutations in \(inhA\) and \(katG\) both confer isoniazid resistance (Inh\(^R\)), a frontline drug for TB. Mutations in \(rpoB\) confer rifampin\(^R\) (Rif\(^R\)), another first line drug, and \(gyrA\) mutations confer fluoroquinolone\(^R\), a second-line drug used in TB treatment [69]. The 12 multiplex primers that were designed are shown in Table 4 with their corresponding locus, sequence, nucleotide length, and Tm. The 6 multiplex PCR products are shown in Table 5 with their description, amplicon size, and mutations. The amplicons were designed to be approximately 50 base pairs apart to allow for gel resolution.
### Table 4: Multiplex PCR Primers

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Size (bp)</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>inhA</td>
<td>inhA_F1</td>
<td>CCGGAATCGCAGGCA</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>inhA_R1</td>
<td>CGGTTTCCTCCGGTAACCA</td>
<td>19</td>
<td>65</td>
</tr>
<tr>
<td>rpoB</td>
<td>rpoB_F</td>
<td>GTCGCCGCGGCTACAAGGAGTT</td>
<td>20</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>rpoB_R</td>
<td>CCCTCAGGGGTTTTCGATCGGG</td>
<td>21</td>
<td>74</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16s_rRNA_F1</td>
<td>GCGATGCCGCGGAGGTTA</td>
<td>17</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>16s_rRNA_R1</td>
<td>ACTTCGTCCCAATCGCCG</td>
<td>18</td>
<td>66</td>
</tr>
<tr>
<td>katG</td>
<td>katG_F1</td>
<td>ATGGCCATGAACGACGTC</td>
<td>18</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>katG_R1</td>
<td>GTGTATTGCGCAAGGCGCA</td>
<td>18</td>
<td>66</td>
</tr>
<tr>
<td>gyrA</td>
<td>gyrA_F1</td>
<td>GACTCGCTCGACCGGATC</td>
<td>18</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>gyrA_R1</td>
<td>GGGCTTCCGGGTACCTCATC</td>
<td>20</td>
<td>66</td>
</tr>
<tr>
<td>23S rRNA</td>
<td>23s_rRNA_F1</td>
<td>CTCTGCTGCAAGAAAAGGC</td>
<td>20</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>23S_rRNA_R1</td>
<td>CAGGTCGGAACATTACCCGAC</td>
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<td>66</td>
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### Table 5: Multiplex PCR products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Amplicon Size (bp)</th>
<th>Mutation</th>
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</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>Species specific TB detection</td>
<td>239</td>
<td>Multiple</td>
</tr>
<tr>
<td>23S rRNA</td>
<td>Species specific TB detection</td>
<td>410</td>
<td>Multiple</td>
</tr>
<tr>
<td>inhA</td>
<td>isoniazid R</td>
<td>149</td>
<td>Promoter -15 nt C→T</td>
</tr>
<tr>
<td>katG</td>
<td>isoniazid R</td>
<td>299</td>
<td>Codon 315</td>
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<tr>
<td>rpoB</td>
<td>rifampin R</td>
<td>200</td>
<td>AGC→ACC</td>
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<tr>
<td>gyrA</td>
<td>fluoroquinolone R</td>
<td>356</td>
<td>Codon 94</td>
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**Philisa® Thermal Cycler PCR Optimization**

Conventional PCR is very time consuming due to slow ramp rates. In an effort to develop a fast and convenient assay, the Philisa® Thermal Cycler PCR instrument was used to dramatically reduce amplification time by utilizing extremely quick temperature ramp times and novel reaction tubes designed for more efficient heat transfer [70]. The rapid thermal cycler is used with a PhilisaFAST™ DNA polymerase, an enzyme designed to facilitate fast extension times.
Each 25 μL reaction contained 1.0 μL of CDC1551 chromosomal Mtb DNA (20 ng/μL), 12 μL of 10 μM Primer Stock, 5.0 μL of FASTBuffer, 4.0 μL of 2.5 μM dNTPs, 0.5 μL of PhilisaFAST™ Polymerase, and 2.5 μL of diH₂O. This 10 μM primer stock contained 1.0 μL of each of the twelve forward and reverse primers (10 μg/μL) needed for the amplification of the 23S, 16S, inhA, rpoB, gyrA, and katG genes, making 400 nM the final concentration of each primer within the 25 μL PCR reaction. The multiplex PCR products were verified for clear differentiation and size on a 2% agarose gel, also using a “no-DNA” control to determine contamination.

The PCR was optimized to obtain maximum specificity and yield. Switching from conventional PCR methods to the rapid Philisa PCR strategy required optimization of the thermal cycler parameters to determine the most efficient duration for annealing and number of cycles. To determine the most efficient annealing temperature that provides a balance between maximum yield and minimum mis-priming, the annealing temperature was tested in 2 degree intervals. Then the number of PCR cycles that would yield the most DNA was determined by running reactions with 30, 40, and 50 cycles. To develop the most time efficient protocol, the timing of each step of the PCR reaction was adjusted to the fastest time that would still amplify the most DNA. The PCR conditions shown in Table 6 were determined to be the most optimal, producing an efficient protocol that is just over 30 minutes long. The PCR products were ran on a 2% agarose gel to verify that they were present at the correct size and roughly equivalent amounts.
Table 6: Philisa PCR Reaction Settings

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
<th>PCR Step</th>
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<td>30</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>(50X)</td>
<td>98</td>
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<td>Secondary denaturation</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>10</td>
<td>Annealing Phase – based in the primers’ melting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>temperature.</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>10</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

DNAzyme Sensor Assay

**General Assay Design**

The assay was designed to identify the presence of TB and for the detection of MDR SNPs. The assay designed for gyrA, shown in Figure 4, consists of a substrate, analyte, and two sensor strands, each containing an analyte binding arm, half of the catalytic core, and a substrate binding arm. The substrate is a dual-labeled synthetic oligonucleotide with a FAM fluorophore on the 5’ end and a quencher (Blackhole Quencher 1 (BHQ1)) at the 3’ end.

![Figure 4: Schematic diagram of the gyrA 94 wild-type DNAzyme sensor](image)

Binary DNAzymes specific for the WT Mtb gyrA 94 and MT Mtb gyrA D94G analytes were designed. The WT Mtb gyrA 94 analyte strand is shown with the SNP of the MT strand above it. The DNAzyme consists of a substrate (orange), specific analyte (black), and two sensor strands. Two A strands (Dz_a) were designed, one complementary to the WT analyte and the other complementary to the MT, each containing an analyte binding arm (pink), half of the catalytic core (purple), and a substrate binding arm (purple). Only one version of the B strand (Dz_b), complementary to the WT sequence, needed to be designed, also consisting of an analyte binding arm (red), half of the catalytic core (green), and a substrate binding arm (green). The Mz substrate is a dual-labeled synthetic oligonucleotide with a FAM fluorophore on the 5’ end and a quencher (BHQ1) at the 3’ end. When the specific gyrA analytes are present, the sensor strands will bind adjacent to the analyte. After binding the analyte, the sensors will then bind the substrate, reforming the catalytic core, and subsequently cleaving the substrate. This cleavage separates the quencher and the fluorophore resulting in the production of a fluorescent signal/output.
The substrate is a dual-labeled synthetic oligonucleotide with a FAM fluorophore on the 5’ end and a quencher (Blackhole Quencher 1 (BHQ1)) at the 3’ end. This project specifically focuses on designing an assay to detect drug\textsuperscript{R} conferring SNPs within the \textit{gyrA} gene. Sensors for targeted regions of 16S and 23S ribosomal RNA were chosen for the species-specific identification of \textit{Mtb} because their sequences are conserved in both TB and BCG, yet not conserved in other strains of Mycobacteria. Multiple sensors will increase the specificity of the assay by identifying both the 16S and 23S rRNA regions in the mycobacteria [15, 71]. The sensors were tested against multiple templates: a negative control to detect contamination, synthetic analytes that are a perfect match to the sensors, CDC1551 \textit{Mtb} DNA, DNA from the \textit{gyrA} D94G BCG constructed in Objective 1, and purified DNA containing the \textit{gyr94} SNP from XDR-TB, provided by Dr. Susanne Homolka and Dr. Stefan Niemann at the National Mycobacteria Reference Center in Borstel, Germany.

Sensors were designed to detect the \textit{gyrA} D94G (GAC\textsuperscript{\rightarrow}GGC) SNP and tested for optimal balance of selectivity and sensitivity. This was accomplished by constructing the short and long analyte binding arms with different lengths and target Tm’s to allow for sensitivity and selectivity. The short arm is designed to hybridize to the region overlapping the SNP. The binary DNAzyme sensors were tested against a matched analyte and a mismatched analyte to determine specificity. The specificity was optimized by testing the sensors with short binding arms of differing lengths and Tm’s. After designing sensors for the identification of the \textit{gyrA} D94G SNP, a LOD assay of the \textit{gyrA} WT and MT sensors was completed to determine the sensitivity of the assay. A serial dilution of the synthetic analytes ranging from 1.0 nM to 12.5 pM, was used in multiple trials. The data was plotted to calculate a linear trend line and the equation used to determine the LOD was based on the average of 7 background samples + 3x(the Standard deviation of the 7 background samples). An
optimized assay combining multiplex PCR with DNAzyme sensors will enable the rapid, POC detection of drug resistance.

Table 7: Assay Sensors
The color coding and bolding refers to the following, Red: Analyte Binding Arm B. Blue: Analyte Binding Arm A. Green: Substrate Binding Arm B. Blue: Substrate Binding Arm A. Underline: catalytic core sequence. Bold: location of SNP. C=MT. T=WT.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>GC Content</th>
<th>#/nts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate:</strong></td>
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<td>19</td>
</tr>
<tr>
<td>A_16s_rRNA</td>
<td>CACAAAGACATGCATCCCGTACAACGGAAGGAAACCTT</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>B_16s_rRNA</td>
<td>TGGCCAGGGAAGCTAGCTGTCCTATCCGGATATTAGACCC</td>
<td>72</td>
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Experimental Setup

The assay was first designed to test the designed sensors using a background control, WT synthetic analyte, and MT synthetic analyte. The assay was conducted in a 384-well plate. The analytes and RNAse free H₂O were added directly to the wells first and a master mix containing the appropriate sensor strands was added last. The total volume of the assay was 60 μL per well. First, water was added to the wells. 15 μL of water was added to the sensor only (15 nM) background column and 9.0 μL of water was added to the analyte (1.0 nM) columns. Then 6.0 μL of each 10 nM synthetic analyte was added to the corresponding well.

Then a master mix containing the substrate and specific adaptor strands was created, comprising 75% of the total volume of the assay. First, half the total volume in COL 2X Buffer (consisting of 50 mM HEPES, 50 mM MgCl₂, 20 mM KCl, 120 mM NaCl, 1% DMSO, and 0.03% Titon-X-100 reagents dissolved in sterile water) added. Then the amount of water needed to make up 75% of the total volume is calculated by subtracting the volumes of COL 2X Buffer, substrate, and sensor strands. The volume of substrate (200 nM) and adaptor strands (15 nM) necessary for the assay is dependent on the total volume of samples. The master mix is then thoroughly mixed and 45 μL is pipetted to each sample well. The assay is then sealed to prevent evaporation and incubated for 1 hour at 55°C. Once incubated, the assay is measured in the Biotek Synergy 4 multimode plate reader using specific filter plus dichroic mirror optics at an excitation wavelength of 485/20 and an emission wavelength of 528/20.

Five multiplex PCR reactions were prepared, each utilizing a different DNA template: a negative “no DNA” control, WT CDC1551 DNA, WT gyrA 94 DNA, gyrA D94G MT BCG, and the gyrA D94G XDR-TB DNA. These multiplex PCR reactions were tested in an assay with gyrA 94
and 23S sensors. The \textit{gyrA} 94 sensors were also assayed with WT and MT analytes and the 23S species specific sensors were assayed with \textit{Mtb}, \textit{Msmeg}, and \textit{Mabs} synthetic analytes. Based on preliminary assays using the WT sensor, 25 \( \mu \text{L} \) was determined to be the amount of PCR mixture needed to yield robust signals. By aliquotting 25 \( \mu \text{L} \) and 19 \( \mu \text{L} \) of sterile H\(_2\)O respectively, the background and analyze wells were also brought up to a total volume of 25 \( \mu \text{L} \). The master mix concentrations were adjusted to a volume of 35 \( \mu \text{L} \) to keep the final volume at 60\( \mu \text{L} \).

**Data Analysis – S/B ratios**

The data was analyzed to determine the performance of the sensors. This was accomplished by comparing the fluorescent output of the substrate to the fluorescent output of the background in a signal to background (S/B) ratio. The ability of the WT and MT sensors to differentiate between the WT and MT analytes, as well as the 5 multiplex reactions: negative “no DNA” control, WT CDC1551 DNA, WT \textit{gyrA} 94 DNA, \textit{gyrA} D94G MT BCG, and \textit{gyrA} D94G XDR-TB DNA were analyzed. An optimized assay combining multiplex PCR with DNAzyme sensors will enable the rapid, POC detection of drug resistance.

**Alternative Approaches**

Many potential problems involved with designing the panel of drug\( ^8 \) BCG strains have been resolved through the use of the TOPO cloning kit. However, the transformation efficiency of \textit{Mtb} and BCG is lower than that of \textit{E. coli} so introducing an insert with the correct drug resistant mutation was difficult. The phenotype of the insertion is strong so if the transformation did not proceed successfully then the plasmid did not survive on the drug resistant selective plate. If we were unable to design the mutant of interest then we could have selected for the correct
spontaneous drug\textsuperscript{R} mutation by plating on medium containing the corresponding antibiotic. Then, the colonies that were able to grow could be sequenced for the target loci to find the drug\textsuperscript{R} strain.

The specificity, equal amplification efficiency, and optimization of the PCR conditions were challenging due to a number of different variables being involved. Through trial and error it took multiple optimizations to determine the correct number of cycles, annealing conditions, reagent concentrations needed for efficient amplification. Contamination of the negative PCR control also became problematic. This was attempted to be resolved through the use of a biosafety cabinet as well as DNase and RNAse free reagents and materials. Further multiplex PCR problems that may present in the future include how well the reaction will amplify DNA directly from clinical sputum samples and whether it will be sensitive enough.

Designing the DNAzyme sensor assay as well as combining it with the multiplex PCR proved to be very challenging. Multiple optimization trials were required to determine the most efficient reagent volumes, assay temperature, and incubation time. Many trials were necessary to design sensors with optimal signal to background ratios while maintaining high specificity. The specificity was optimized by designing sensors with different TM’s, lengths, and SNP locations.
RESULTS

The development of a novel diagnostic involved constructing a surrogate panel of safe drug\textsuperscript{R} \textit{M. bovis} BCG strains, designing a 12 primer multiplex PCR, and combining the multiplex PCR with a DNAzyme assay that can be used to identify TB and detect drug\textsuperscript{R}.

Construction of Safe Surrogate Panel of DrugR \textit{M. bovis} BCG

In order to safely test the DNAzyme diagnostic assay, a nonpathogenic \textit{M. bovis} BCG strain was engineered to contain the drug\textsuperscript{R}-conferring \textit{gyrA} SNP as detailed in the methods section. Primer design and PCR amplification of the WT \textit{gyrA} gene was necessary before the MT strain could be constructed. To verify the size of the WT \textit{gyrA} PCR product on an agarose gel, a 1 kb Gene Ruler was used \cite{footnote}. 3.0 μL of this standard was loaded into the first lane before the gel was run. An example of a gel that was imaged, after being stained in Gel Red dye, to verify the presence of the 2000 bp WT \textit{gyrA} insert is shown in Figure 5. The M13 primers in the second lane were used to confirm the correct size and the \textit{gyrA} primers in the third lane show the insert. The upper bands are likely supercoiled plasmid from the template DNA.
Figure 5: gyrA PCR Insert
Example of the verification of the WT gyrA fragment PCR product DNA size on an agarose gel using both M13L and gyrA primers.

After confirming the amplified DNA by running the plasmid on an agarose gel, the PCR products were TOPO cloned to insert the fragment into the pCR4Blunt-TOPO plasmid. The plasmid contained a Kan\textsuperscript{R} gene that enabled clones which incorporated the vector to be selected for upon plating on LB-Kan agar. Colony PCR was performed on the colonies that grew to screen for clones containing the pTOPO-gyrA plasmid. Once colony PCR screened, the positive colonies were grown overnight in an LB broth containing Kan to amplify the DNA and miniprepped to purify the DNA. After determining the DNA concentration using a Nanodrop spectrophotometer, the purified \textit{E. coli} DNA was sent for sequencing to confirm that the WT gyrA fragment was inserted into the TOPO plasmid (data not shown).

After successful cloning of the intended gyrA fragment was confirmed, QuikChange Site-Directed Mutagenesis was used to insert the D94G mutation. First, specific primers containing the SNP in the center of their sequences were designed. Then, the WT DNA was amplified using the QC primers to amplify the mutant. The bacteria that incorporated the resulting plasmid were
selected for using the appropriate antibiotic. Colonies that grew were screened and verified on a gel to ensure that the correct plasmid was present (data not shown). In addition to visualization of the DNA on a gel, the plasmid was sequenced to confirm that the D94G mutant SNP was inserted into the WT plasmid. The sequence confirmation of gyrA D94G – TOPO plasmid is shown in Figure 6.

Figure 6: Sequence confirmation of gyrA D94G – TOPO plasmid
The gyr94 – TOPO plasmid was sent for sequencing and then aligned with the WT gyrA 94 sequence (top) to ensure that the correct mutation was present. The GAC→GGC mutation is shown in the red box.

After the MT was verified, the PCR amplified and linear DNA fragment transformed into BCG using electroporation. The BCG that took up the MT fragment was selected for drugR on Ciprofloxacin antibiotic plates. The colonies that grew were colony PCR screened and verified on a gel to ensure that the PCR product was successfully double crossed into the BCG chromosome (data not shown). In addition to visualization of the DNA on a gel, the PCR product was purified using the PCR clean-up protocol discussed in the methods section and sent for sequencing to confirm that the drugR SNP was present. The sequence confirmation of the gyrA D94G BCG strain is shown in Figure 7.

Figure 7: Sequence confirmation of gyrA D94G BCG strain
WT gyrA (top) aligned with MT gyrA D94G BCG (bottom) to verify that the mutation was inserted correctly. The GAC→GGC mutation is shown in the red box.
Optimization of 12 Primer Multiplex PCR

In order to develop an assay to specifically detect TB and identify first and second line drug-resistant (DR) organisms in a single sample, a multiplex PCR with the ability to simultaneously amplify particular targets including Mycobacterium tuberculosis (Mtb)-specific loci and drug-resistant (DR) loci for multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, was designed in a single reaction. Nucleic Acid Amplification, like PCR is needed to increase the sensitivity of the diagnostic assay, because the current limit of detection is not sensitive enough to detect a single copy on the chromosome. Then the multiplex PCR product was combined with DNAzyme sensors in an assay to specifically detect TB and identify first and second line drug-resistant in a single sample.

First, 6 primer pairs were designed to specifically amplify the regions of interest. 2 of these primers were designed to target the hypervariable regions of 16S and 23S rRNA that are only present in Mtb, enabling the assay to distinguish Mtb and the M. bovis BCG vaccine strain from other members of the MTC. The other 4 primer pairs were designed to target the gyrA, katG, rpoB, and inhA genes which have been shown to contain the most prevalent SNP mutations that lead to first and second line drug-resistant. The amplicons were designed to be approximately 50 base pairs apart to allow for gel resolution.

Once designed, the PCR conditions were optimized to achieve optimal specificity, yield, and speed. This was accomplished using the Philisa thermocycler which facilitates rapid amplification through implementation of extremely fast ramp times and novel tubes allowing quick heat transfer. Multiple PCR trials were conducted to determine the most efficient primer concentration and cycling conditions. The optimized 10 uM primer stock contained 1.0 μL of each of the 12 forward and reverse primers (10 μg/μL) needed for the amplification of the 23S, 16S, inhA, rpoB, gyrA, and katG genes, making 400 nM the final concentration of each primer within the 25 μL PCR reaction.
The annealing temperature was tested in 2° intervals and 65°C was determined to yield the most PCR product. The number of denaturation-annealing-extension cycles was optimized through 10 cycle interval trials and 50 cycles was shown to yield the most robust results. The multiplex PCR products were verified for clear differentiation and size on a 2% agarose gel, also using a “no-DNA” control to determine contamination. Figure 9 is an example gel image of the optimized 12 primer multiplex using each individual primer pair.

![Figure 9: Example gel image of the optimized 12 primer multiplex using each individual primer pair.]

**Figure 8: 12 Primer Multiplex PCR for drug detection with DNAzyme assay**
Agarose gel verification of the 12 Primer Multiplex PCR product compared to the PCR products of the 6 individual primer pairs.

**Creation of DNAzyme Sensor Assay**

To enable the rapid POC detection of drug resistant TB, a DNAzyme sensor assay of PCR analytes for the detection and DST of Mtb was designed and optimized. The sensors, shown in Table 7 of the methods, were designed to detect the gyrA D94G (GAC→GGC) that this project specifically focuses on and then tested for optimal balance of selectivity and sensitivity. This was done by constructing the short and long analyte binding arms with different lengths and target Tm’s to allow for optimal sensitivity and selectivity. Once designed, the binary DNAzyme sensors were first tested against a matched analyte and a mismatched analyte to determine specificity. The
specificity was optimized by testing the sensors with short binding arms of the DNAzyme with differing lengths and Tm’s.

The first round of \textit{gyrA} sensor strands (A1 WT and A1 MT, and WT B1) tested were designed with analyte binding arms of about 16-18 nucleotides in length. The SNP was located in the sequence hybridized by the A analyte binding arm. Since the A strand was used for SNP discrimination, both a WT and MT A strand were designed and only a single WT B strand was needed. The A1-MT analyte binding arm was designed to contain the SNP near its center. The efficacy of the sensors was determined by comparing the signal of the specific strand to that of the nonspecific strand, this is referred to as the specificity ratio. A sensor is deemed efficient if it can yield a high specific signal and keep the nonspecific signal under 2. Through several assay trials, the A1-MT sensor strand, shown in Figure 10, proved to yield a high specificity ratio of about 4.3. This is found by dividing the average specific MT signal, 6.26, by the average nonspecific WT background signal, 1.45. However, new strands needed to be designed because the A1-WT sensor strand was not specific, having a low specificity ratio (data not shown). The specificity was optimized by shifting the sequences of the A and B analyte binding arms down 3 nucleotides and then 3 specific A strands, now referred to as A2, were designed by trimming the length of the shifted analyte binding arm. The newly designed A2 analyte binding arms were 18, 17, and 16 nucleotides long and had corresponding Tm’s of 64, 62, and 61°C, shown in Figure 10. The A2 WT sensor with the highest Tm’s, 64°C, had the worst specificity, yielding a specificity ratio of approximately 1.56, found by dividing the average specific WT sensor signal, 8.02, but the average nonspecific MT signal, 5.12. The A2 WT 62 sensor had an average specific WT signal of 6.91 and an average nonspecific MT signal of 3.09, yielding a specificity ratio of 2.2. The sensor with the shortest
analyte binding arm and lowest Tm yielded the best specificity ratio of approximately 3.2: found by dividing the average specific WT signal, 6.28, by the average nonspecific MT signal, 1.98. This shows that shortening the arm, and therefore lowering the Tm, gave better specificity with only minimal loss of signal.

The A1-MT and B1 strands were used in the assay to identify the MT and the A2-WT and B2 strands were used in the assay to identify the WT. The combination of these two sensor sets yielded the most efficient assay with the highest signal.

![Figure 9: Optimization of gyrA 94 WT sensor](image)

The specificity of the sensor strands were optimized by testing different analyte binding arm lengths and Tm’s. A1 refers to the first set of sensors developed and, through multiple trials, the A1-MT yielded a high, match to mismatch specificity ratio of approximately 6: 6.26(MT)/1.45(WT). The A1-WT sensor (not shown) proved to be nonspecific, with a low specificity ratio so a new set of sensors were designed by shifting the sequences by 3 nucleotides. New WT sensors, referred to as A2 were designed and optimized by trimming the analyte binding arm from 18 to, 17, and 16 with corresponding Tm’s of 64, 62, and 61°C (as shown above). A trend appeared in which the sensor's specificity ratio increases as the length and therefore Tm, decreases. From the 8.02/5.13 specificity ratio of WT A2-64, 6.91/3.09 of WT A2-62, to the highest specificity ratio: 6.28/1.96 of the WT A2-61 sensor.

After designing and optimizing sensors for the identification of the gyrA 94 SNP, a LOD assay of the gyrA WT and MT sensors was conducted using synthetic analytes to determine the sensitivity of the assay. A serial dilution of the synthetic analytes ranging from 1.0 nM to 3.125 pM,
was tested in multiple trials. The data was plotted to calculate the linear trend line, shown in Figure 10, and the equation used to determine the LOD was based on the average of 7 background samples +3 (the Standard deviation of the 7 background samples).

The calculated LOD is higher than expected. This may be due to variability in the S/B ratios of the 7 background samples (Data not shown). The LOD for the WT 61 sensor was calculated using the equation above to be 170 pM with an $R^2$ value of 0.97. The signal of each reaction compared to the no analyte background control, known as the substrate to background (S/B) ratio, was used to analyze the data. At a concentration of 1 nM, the WT 61 sensor produced an average S/B ratio 3.95. At 500 pM, the S/B ratio decreased to an average of 2.8 and then decreased to 1.75 at 250 nM. The S/B decreased to 1, unable to distinguish the analyte signal from the background, at an analyte concentration of 50 pM. The LOD for the WT 62 sensor was calculated to be 212 pM with an $R^2$ value of 0.98. At a concentration of 1 nM, the WT 62 sensor produced an average S/B ratio 4.0. At 500 pM, the S/B ratio decreased to an average of 2.7 and to 1.9 at 250 nM. The S/B decreased to 1 at an analyte concentration of 50 pM. Further optimization of the WT sensors is needed for a more specific assay.

The LOD for the MT sensor was calculated to be 108 pM with an $R^2$ value of 0.98. At a concentration of 1 nM, the MT sensor produced an average S/B ratio 6.3. At 500 pM, the S/B ratio decreased to an average of 4.5. At analyte concentrations of 250 and 50 pM, the S/B remained at 2.9 and then plateaued to 1 at an analyte concentration of 50 pM.
Figure 10: *gyrA* 94 WT LOD

Serial dilutions of the *gyrA* WT 61, WT 62, and MT synthetic analytes, from 1 nM to 3.125 pM (x axis), were ran with WT and MT-specific binary sensors and detected by the binary DNAzyme assay. The Average S/B ratio (y axis) shows that the MT DNAzyme sensor can detect as low as 108 pM of analyte and the WT 61 and WT 62 DNAzyme sensors can detect as low as 170 and 212 pM of analyte, respectively.

The binary DNAzyme assay was tested with the multiplex PCR after verifying their successful PCR amplification on a 2% gel (data not shown). To determine the efficacy of our multiplex PCR-DNAzyme DST assay, ability to detect drug\(^8\) SNPs in the multiplex PCR, the *gyrA* 94 Multiplex PCR analytes from WT CDC1551, *gyrA* 94 BCG MT DNA, and *gyrA* 94 XDR-TB DNA was analyzed using WT and MT *gyrA* 94 sensors and 23s rRNA sensors. A negative “no DNA” multiplex PCR analyte was also used to verify contamination. The results are shown in Figures 11-13.

Figure 11 shows the S/B ratios of the *gyrA* WT sensor, specifically the optimized A2-61°C WT sensor (background data not shown). The negative control, which was expected to have a low S/B ratio of approximately 2, was higher than expected due to contamination of the multiplex “no DNA” control. The WT sensor reacted with the *gyrA* 94 Multiplex PCR analytes from WT
CDC1551 as predicted, yielding a high S/B ratio of 7.1. This was expected because the WT sensors are specific for the WT template. The WT sensor did not produce as much signal when combined with the Multiplex PCR analytes from the \textit{gyrA} 94 BCG MT DNA, however it still produced an average S/B ratio of 5.3. This nonspecific signal is higher than expected but may be due, in part, to the contamination shown in the negative control. The WT sensor produced a lower signal when combined in the assay with the \textit{gyrA} 94 XDR-TB DNA Multiplex PCR analytes. The lower signal was expected because the WT sensor is not specific for the \textit{gyrA} MT. The S/B ratio of 4.04 may also be due to the contamination that is shown in the negative control. When assayed with the WT synthetic analyte (1 nM), the WT sensor produced a moderate S/B ratio of 4.7. Since the sensor was designed to be specific for the WT analyte, the S/B ratio was expected to be higher. The WT sensor produced little signal when combined with the MT synthetic analyte. The low S/B ratio of 2.2 was expected because the WT sensor is not specific for the MT analyte.

The difference in the S/B ratio of the WT and Mt analytes indicates that the sensor can successfully differentiate between the pure analytes. However, the contamination of the negative “no DNA” is problematic and the high signal from the \textit{gyrA} D94G BCG MT and \textit{gyrA} D94G XDR-TB multiplex PCR analytes indicate that further optimization of the WT sensor is necessary to distinguish the multiplex PCR analytes.
**Figure 11: gyrA WT Sensor S/B Ratios**

Negative Control: “no DNA” Multiplex PCR control. WT CDC: *gyrA* 94 Multiplex PCR analytes from WT CDC1551. 
*gyrA* D94G BCG: *gyrA* 94 Multiplex PCR analytes from *gyrA* D94G BCG MT DNA. *gyrA* D94G XDR-TB: *gyrA* D94G XDR-TB DNA Multiplex PCR analytes. WT syn (1 nM): WT synthetic analyte with a final concentration of 1 nM. MT syn (1 nM): MT synthetic analyte with a final concentration of 1 nM.

Figure 12 shows the S/B ratios of the optimized A1-MT *gyrA* sensor. The negative multiplex PCR analyte, produced a low S/B ratio of 1.36. This was expected because no signal should have been seen from the “no DNA” control. The MT sensor also produced a low signal when assayed with the *gyrA* 94 Multiplex PCR analytes amplified from WT CDC1551. The S/B ratio of 1.54 was expected because the sensor is not specific for the WT template. When assayed with the multiplex PCR analytes amplified from the *gyrA* D94G BCG MT DNA, the MT sensor produced a S/B ratio of 7.42. This high signal was expected as the MT sensor was designed to be specific for the SNP. The MT sensor also produced a high signal when assayed with the *gyrA* D94G XDR-TB DNA Multiplex PCR analytes. The S/B ratio of 7.43 was expected because the *gyrA* D94G XDR-TB DNA also contains the SNP that the MT sensor is specific for.

When assayed with the WT synthetic analyte (1 nM), the MT sensor produced a S/B ratio of 1.18. The low S/B ratio was expected because the sensor is not specific for the WT analyte. The MT
sensor produced a high signal when combined with the MT synthetic analyte. The high S/B ratio of 5.35 was expected because the MT sensor was designed to be specific for the MT analyte. The high S/B ratios of the WT (both CDC1551 and synthetic) multiplex’s and the low S/B ratios of the MT (5.1, 1598, and synthetic) MT multiplex analytes indicate that the sensor can successfully distinguish the SNP.

Figure 12: gyrA MT Sensor S/B Ratios
Negative Control: “no DNA” Multiplex PCR control. WT CDC: gyrA 94 Multiplex PCR analytes from WT CDC1551. gyrA D94G BCG: gyrA 94 Multiplex PCR analytes from gyrA D94G BCG MT DNA. gyrA D94G XDR-TB: gyrA D94G XDR-TB DNA Multiplex PCR analytes. WT syn (1 nM): WT synthetic analyte with a final concentration of 1 nM. MT syn (1 nM): MT synthetic analyte with a final concentration of 1 nM.

Figure 13 shows the S/B ratios of the species specific 23S rRNA sensor. The negative control, which was expected to have a low S/B ratio below 2, was slightly higher than expected due to contamination of the multiplex “no DNA” control. The S/B ratios of the species specific 23s rRNA are expected to be similar and high amongst the WT gyrA 94 CDC1551, gyrA D94G BCG MT, and gyrA D94G XDR-TB Multiplex PCR products, and Mtb synthetic (1 nM) analytes because they are all members of Mtb and contain identical 23S rRNA sequences. This similarity is seen in the
results with the S/B ratios of 4.25, 4.89, 3.82, and 6.06. The slight variability that is present may be due to differences in PCR amplification. The slightly higher *Mtb* synthetic analyte S/B ratio of 6.06 is because the sensors were designed specifically for that analyte therefore should produce the highest signal. *M. abscessus* (*Mabs*) and *M. smegmatis* (*Msmeg*) are not members of *Mtb* and lack the species identifying 23S rRNA, therefore they were expected to yield a low S/B ratio. When assayed with the *Mabs* and *Msmeg* synthetic analyte (1 nM), the WT sensor produced very low S/B ratios of 1.15 and 1.02, respectively.

The difference in the S/B ratio of *Mtb* versus Non-*Mtb* (NTM) indicates that the sensor can successfully differentiate between the species. However, the contamination of the negative “no DNA” is problematic and further optimization is necessary to yield a more accurate control.

![23S rRNA Sensor](image)

**Figure 13: 23S rRNA Sensor S/B Ratios**

*Mtb* syn (1 nM): *Mtb* synthetic analyte with a final concentration of 1 nM. *Mab* syn (1 nM): *Mabs* synthetic analyte with a final concentration of 1 nM. *Msm* syn (1 nM): *Msmeg* synthetic analyte with a final concentration of 1 nM.
DISCUSSION

Due to the rise of MDR-TB and XDR-TB worldwide, the need for new diagnostic assays that are able to efficiently detect *Mtb* while synonymously identifying drug resistance has increased. One-third of the world’s population is currently living with TB and about 9 million more people became infected annually. The TB infection causes over 1.3 million deaths each year. TB’s ability to be transmitted through breathing in the aerosolized droplets coughed up by someone with active disease has given it the potential to unknowingly spread to others at a disastrous rate. In order to try to address this problem, this project was aimed to develop a novel diagnostic technique combines DNAzyme sensor assays with multiplex PCR analytes to enable the rapid, POC detection of tuberculosis and identification of multi-drug resistance.

The aims of this project were to construct a BCG strain with *gyrA* D94G drug resistant SNPs, develop a multiplex PCR that amplifies chromosomal regions necessary for TB detection and DST, and design SNP specific binary sensors to enable the identification of any drug resistant *Mtb* mutations present. PCR amplification and TOPO cloning allowed the amplification and insertion of the WT *gyrA* gene, before it was able to be mutated to contain the SNP of interest using a QuikChange mutagenesis method. Then the *gyrA* gene containing the resistance-conferring SNP was transformed into the *M. bovis* BCG vaccine strain using electroporation. Construction of a validated panel of BCG with the *gyrA* D94G drug resistant SNP allowed us to have safe template strains to test the Multiplex assay. A 12 primer multiplex PCR that amplifies 6 chromosomal regions necessary for TB detection and DST was designed. After multiple trials, a 30 minute PCR reaction with 50 cycles provided the most efficient amplification. While contamination of the negative “no DNA” proved to be problematic, the multiplex was successfully optimized for the amplification of 6 targets.
in a single reaction for use in the DNAzyme Assay. The development of \textit{gyrA} D94G SNP specific binary sensors that are able to identify the drug resistant \textit{Mtb} were designed for use in the DNAzyme assay. The sensors were optimized for specificity and high signal to background ratios and then the LOD and their ability to detect multiplex PCR analytes were tested in multiple assays. While the MT sensors proved to be specific while still maintaining a high signal, the WT sensors require further optimization.

Future work for this project includes the development of BCG strains containing other drug\textsuperscript{r} conferring SNPs. This will enable us to safely optimize the DNAzyme diagnostic assay. Further testing of SNP specific sensors that will be able to discriminate between the SNPs and still produce a high signal. Other work includes the development of a multi-color assay with specific colors detecting specific resistances in a single tube. This way we might be able to give a molecular profile of the specific drug\textsuperscript{r} present.

The final outcome of this project has led to the development of a novel method for detecting TB and identifying the drug\textsuperscript{r} present. With the emergence of MDR-TB, this project has furthered the development of future drug\textsuperscript{r} diagnostic assays that will enable the prompt and specific diagnosis of TB allowing for the implementation of an effective treatment regimen that will ultimately lessen transmission and control the emerging global threat of TB.
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