Modified DNA Aptamer Affinity Reagents for the Detection of Protein Tuberculosis Biomarkers in Urine

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Defended March 29, 2016

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Abstract

In 2015 the World Health Organization (WHO) determined that tuberculosis (TB) was the world’s most fatal infectious disease. Furthermore, Doctors Without Boarders (DWOB) published reports indicating that incidents of multi-drug resistant strains of TB (MDR-TB) infection have become rampant in resource-limited parts of the world. DWOB attributes the rapid increase in MDR-TB in part to a lack of accurate diagnostic techniques. The Feldheim group has been working towards developing aptamers to function as capture reagents in a new diagnostic platform for the detection of TB biomarkers in patient urine. Utilizing the SELEX process, an evolved pool of modified DNA aptamers were generated for the protein TB biomarker, MT2462, which is found in the urine of patients with active TB disease. Analysis of the evolved aptamer pool has revealed several aptamer sequences that could function as binders to MT2462. Specifically, an aptamer termed SEQ1, was selected for analysis. Determination of the aptamer affinity and specificity of SEQ1 for MT2462 will then create the potential for this sequence to be utilized in the development of a new diagnostic for TB.
Acknowledgments

I would like to thank Dr. Daniel Feldheim and postdoctoral fellow Dr. Rose Byrne for making my time in the lab one of the most enjoyable and rewarding experiences in my undergraduate career. Dr. Feldheim and Dr. Byrne were supportive throughout my time in the lab and great mentors for my thesis.

I would also like to give a special thank you to postdoctoral fellow Dr. Candice Smith, who took me under her wing and was both a mentor and a friend. Candice made it her personal goal to ensure that I understood every aspect of my research, and she gave me all the tools I needed to succeed. She was encouraging and patient as I struggled to grasp concepts, ultimately proving a wonderful teacher. My work in the lab, this thesis, and my growth as a scientist would not have been possible without Candice.

I would like to extended my gratitude to SomaLogic for providing the Feldheim Lab with access to the thymine modifications for use in isolating aptamers.

The Undergraduate Research Opportunity Program and Howard Hughes Medical Institute Individual Grant have allowed me to conduct my research given their financial support, for which I could not be more grateful.

Gilson Sanchez, a PhD candidate at the University of Colorado Boulder was a tremendous help in aiding with the analysis of my sequencing data. The second half of this thesis would not have been possible without his help.

Finally, I would like to thank my family and friends for their continued support in all my academic pursuits. They are my number one cheerleaders, and always motivate me to keep pursuing my goals. Thank you for all the love and support!
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Chapter 1: Introduction

1.1 Overview

The disease causing pathogen of Tuberculosis (TB) has infected approximately one third of the world’s population. In recent years, the bacteria have developed multiple mechanisms of drug resistance, making treatment difficult if not impossible. Doctors Without Borders has attributed the rampant spread of the disease in part to a lack of accurate diagnostic techniques, especially in resource-limited countries that lack the infrastructure necessary to make rapid and accurate diagnoses. Furthermore, the co-infection of tuberculosis with other immunocompromising diseases constrains the effectiveness of some diagnostics currently in place. It has become apparent that new diagnostics are necessary to overcome the current limitations in TB diagnosis.

Currently, there are several new diagnostics that have been approved by the World Health Organization (WHO) for the detection of TB. However, many of these diagnostics do not function well in resource-limited regions of the world, as they require greater infrastructure than is currently available. The ideal diagnostic would be portable, non-invasive, easy to administer, accurate in detection, cheap to produce, and robust despite environmental extremes.

Oligonucleotide aptamers, short strands of RNA or DNA that are capable of binding to molecules with high affinity and specificity, would serve well as a capture reagent in TB diagnostics, as they are not expensive to produce and are thermally stable. Ideally, a TB diagnostic that incorporates aptamers as a capture reagent would be designed to resemble a pregnancy test, resulting in a simple, portable, hand-held device which all patients or healthcare professionals could easily use. The goal of this thesis research project was to isolate aptamers for a TB protein that is known to be excreted in the urine of patients infected with the causative
agent of TB, *Mycobacterium tuberculosis* (*Mtb*). If successful, these aptamers could potentially be incorporated into a lateral flow urine test for TB to create an inexpensive and easy to operate point of care test for TB.

### 1.2 World Health Concerns Regarding Tuberculosis Diagnosis and Treatment

According to the WHO, TB is the deadliest infectious disease in the world. In 2013, 1.5 million people died of the disease, while 9 million contracted the infection. Over 95% of TB cases occur in developing countries, where Human Immunodeficiency Virus (HIV), malnutrition, and diabetes have been known to place individuals at higher risk of contracting the infection. Furthermore, Doctors Without Borders has determined that multi-drug resistant strains of tuberculosis are becoming more prevalent, partially due to the lack of accurate diagnostic techniques, increasing risk of infection amongst the population. The maps in Figures 1.1 and 1.2 show the dramatic increase in the incidence of drug resistant tuberculosis since 2005.

![Figure 1.1](image-url)  
Figure 1.1. Drug resistant cases of tuberculosis as recorded in 2005. The legend indicates the number of drug resistant cases of tuberculosis present in each of the represented countries.
A major part of the problem surrounding tuberculosis diagnosis and the dramatic increase in multiple drug resistant forms lies in what is known as the peripheral laboratory setting. This setting is the site for the diagnosis and treatment of tuberculosis, which is often found in some of the most remote regions of the world where tuberculosis infections are rampant. These laboratories are usually the simplest of facilities, often scarce in electricity. With the lack of a stable power source, the diagnostic techniques that can be implemented are fairly limited. In fact, most facilities only perform sputum smear microscopy, which is the simplest and most commonly employed method for TB detection. Even so, the accurate detection of tuberculosis via this technique requires well-trained and skilled laboratory staff, often a luxury not available in resource-poor settings. Furthermore, many of these laboratories are not equipped to handle high volumes of patient samples, and often have slow turn-around times, making rapid and accurate detection of TB difficult.
1.3 Current Diagnostics for Tuberculosis

Outside of the periphery setting, and especially in the United States the most commonly employed tests are serological diagnostics, which test patient immune responses. Serological diagnostics include the Tuberculin Skin Test (TST) and the Interferon Gamma Release Assay (IGRA). TST involves the injection of tuberculin (a purified protein derivative from $Mtb$) into the skin of the lower forearm, creating a small bump. Two days following the injection, patients return to clinicians to have the bump examined for swelling and tension on the skin, indicating an immune response. Depending on the size of the bump, a clinician will decide on a positive or negative result.\(^3\) One of the main limitations of using this test in the periphery is that patients often do not return to have the test site examined for swelling, potentially leaving the disease undiagnosed and untreated. Furthermore, the test can result in false-positives when diagnosing, especially if the patient has been vaccinated with the Bacillus Calmette-Guerin vaccine, or if they have been exposed to other mycobacteria in the environment that are related to $Mtb$.\(^{19}\)

As such, IGRAs are becoming an increasingly more popular and effective method for TB testing.\(^{19}\) In this technique, patient blood is drawn and tested to determine how strong of an immune response is seen once the blood is exposed to tuberculosis bacteria.\(^3\) Although a step up from TSTs, IGRAs lack the proper sensitivity to be able to accurately diagnose tuberculosis in all patients, especially those which are immunocompromised, a problem exhibited often in the periphery setting.\(^{11}\)

Although the limitations in serological tests are not prevalent in the United States, they are incredibly problematic in the periphery setting. In 2011, the director of the World Health Organization’s Stop TB Department published a statement regarding serological diagnostics, claiming: “Test results are inconsistent, imprecise and put patients’ lives in danger.”\(^{16}\) Since
then, the WHO has determined that these diagnostics should not be used in the periphery setting, and furthermore, that any new diagnostics produced must not be serological in nature.\textsuperscript{28}

As mentioned previously, the most common diagnostic for TB in resource-limited countries is sputum smear microscopy. Although the technique is ideal in that it does not require highly complex laboratory settings, it falls short with respect to sensitivity of detection, often leaving patients untreated, free to transmit the disease to others.\textsuperscript{18} Doctors Without Borders has confirmed that sensitivity of detection for smear microscopy has fallen to 45%, mostly due in part to the uncertainty regarding the amount of bacilli present in a sputum sample, and the inability to isolate a proper sample from all patients, including children and the elderly. More often than not, the bacterial cells lie inside of lung cavities, and are not always discharged in sputum. Furthermore, sputum microscopy cannot detect the presence of extra-pulmonary tuberculosis, meaning that patients with any form of tuberculosis infection existing outside the lungs can remain undiagnosed and untreated.\textsuperscript{9}

In recent years, the World Health Organization has endorsed alternative diagnostic techniques such as liquid cultures.\textsuperscript{18} Although these cultures are an improvement on smear microscopy in terms of sensitivity, the problem of turn-around time still exists. Liquid cultures can take up to a month to determine a positive result, which is not clinically effective especially in the face of multi-drug resistant (MDR) strains of TB.\textsuperscript{6} The table below provides a snapshot of several commercialized diagnostic techniques that have been outlined by Doctors Without Borders. The limitations of each test are also listed.
<table>
<thead>
<tr>
<th>Test Name</th>
<th>Test Type</th>
<th>Limitations of the Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>BACTEC MGIT 960 (Automated)</td>
<td>Liquid Culture</td>
<td>Expensive Machinery; Highly Trained Technicians</td>
</tr>
<tr>
<td>Rapid Liquid TB Culture Medium (Manual)</td>
<td>Liquid Culture</td>
<td>Expensive; Liquid Cultures Prone to Contamination</td>
</tr>
<tr>
<td>MB/Bact T System</td>
<td>CO₂ Detection</td>
<td>Expensive; Slow to Diagnose</td>
</tr>
<tr>
<td>MB Redox</td>
<td>Liquid Growth with Indicator</td>
<td>Slow to Diagnose</td>
</tr>
<tr>
<td>Phage-Based Tests</td>
<td>Phage Infection</td>
<td>Cannot be Used for HIV+ patients or children</td>
</tr>
<tr>
<td>Thin Layer Agar</td>
<td>Solid Culture</td>
<td>Requires Electricity (CO₂ Incubator)</td>
</tr>
<tr>
<td>LAM ELISA Urine Test</td>
<td>Antibody Test</td>
<td>Requires Electricity</td>
</tr>
<tr>
<td>AMPLICOR MTB Test</td>
<td>PCR of 16S rRNA</td>
<td>Expensive; Highly Trained Technicians</td>
</tr>
<tr>
<td>T Cell Based Test</td>
<td>Blood Test</td>
<td>Invasive; Requires Electricity (Refrigeration)</td>
</tr>
</tbody>
</table>

Table 1.1. Brief overview of a few of the diagnostics that are currently being used for the detection of tuberculosis and some of the limitations that have been discovered for each.⁹

A few common limitations stand out in most of the current diagnostics that are being tested in the periphery setting. Often the machinery needed for accurate completion of a test is expensive and requires skilled laboratory personnel. Another limiting factoring that restricts the types of tests conducted is electricity, a luxury that is often scarce in the periphery setting. Moreover, several of the current diagnostics do not have the proper sensitivity levels to be able to detect TB in all patients and often take extensive time to generate results.⁹

Based on the restrictive limitations of the current methods of diagnosis, it has become clear that a new diagnostic is needed for use in the periphery setting. The diagnostic must be one that can function without the use of electricity (either to administer the test or for storage), one which is easy to administer and does not require the use of skilled laboratory technicians, is relatively inexpensive to manufacture, and that can generate results in a timely manner. The Feldheim lab hypothesizes that oligonucleotide aptamers, short strands of RNA or DNA, could
function well as capture reagents in a diagnostic platform for TB that could overcome some of the above described limitations, as will be discussed in further detail in Chapter 2.

1.4 *Mycobacterium tuberculosis* (*Mtb*) and Tuberculosis

A more desirable diagnostic technique, especially in resource-limited countries, can stem from the very biological processes that govern a *Mtb* infection. *Mtb* is a Gram-positive, rod-shaped bacterium that is the causative agent for tuberculosis. The most common form of tuberculosis infection is pulmonary, although the bacteria can spread through the bloodstream to other parts of the body, producing abscesses in other organ systems. This dissemination of the bacteria through the body is called extra-pulmonary tuberculosis. Symptoms of tuberculosis usually present in as little as four to six weeks after infection, and include cough, fever, night sweats, and weight loss. The delayed onset of symptoms is due to distinctions between a latent pulmonary infection and an active pulmonary disease.

As the name suggests, a latent infection does not result in the manifestation of any symptoms, a phenomenon that is the result of the immune system’s ability to fight the bacteria to prevent growth and replication. As such, individuals infected with latent tuberculosis are not infectious and will not spread the disease. Tuberculosis is considered active when the immune system can no longer suppress the bacteria from multiplying in the body. Individuals with active forms of tuberculosis, by contrast, are considered contagious and can spread the infection to others through the air as they cough, sneeze or even speak. This ease of transmission is due to the aerobic nature of the bacteria, and thus active forms of pulmonary tuberculosis grow in the alveoli of the lungs. Furthermore, it should be noted that latent forms of tuberculosis can attack any part of the body including the brain, spine and kidney.
However, not every patient that contracts tuberculosis will move from the latent stage to the active stage of the infection. Depending on the individual’s overall health, tuberculosis symptoms could present within weeks of the initial infection or potentially even decades later. Generally, a healthy individual with a strong immune system could remain in the latent stage of the infection for decades. In fact, the Centers for Disease Control reported that only five to ten percent of patients who do not receive treatment for latent tuberculosis will ever see the disease manifest into an active infection. However, individuals that are immunocompromised in some way are at a higher risk of developing an active response immediately after contracting the infection. Other groups that are at risk for disease manifestation into the active stage include the elderly, babies, or any individual that is undergoing medical treatment that causes a decrease in immune response and function.\(^29\)

The reason tuberculosis exists in two forms is related to the physical manifestation of the disease in the form of a granuloma, or tubercle, a growth in the lining of the lungs. Droplets of \textit{Mtb} can reside in the atmosphere for several hours, awaiting inhalation by a human host. Upon entering the lung, the bacteria are engulfed by macrophages, which induce a pro-inflammatory response at the site of phagocytosis. This inflammatory response signaled by the infected macrophages allows for the recruitment of neutrophils, natural killer T cells, and CD4 T cells to the infected macrophage. These cells begin to surround the macrophage, forming the basis of the skeletal structure of the tubercle. As the inflammatory response continues, more immune cells are recruited to the tubercle site, and the granuloma begins to grow, confining the infected macrophages to the inside of the tubercle and inhibiting the bacteria’s ability to multiply and spread rapidly. This allows for the containment of the infection in what is considered the latent infectious stage of tuberculosis.\(^20\)
Furthermore, it is important to note that the bacteria have the ability to remain dormant within the body for decades before becoming active forms of tuberculosis. At some point during their life cycle, and as a result of different environmental stimuli, the granulomas become necrotic, allowing for caseation to occur. During this process, the granuloma decays, leaving behind cellular debris, and releasing bacteria into the airways. Once the airways become infected, the infection is considered to be in an active state, and transmission is now fully possible.

The *Mtb* cellular debris that can accompany the granuloma decay, as well as the death of *Mtb* throughout the course of an infection is of interesting diagnostic significance. Incorporated in this cellular debris are components of the cell wall, as well as many of the contents in the cellular cytoplasm. Among these contents are biomarkers that are unique to *Mtb*, allowing for their potential use as a diagnostic marker.

These diagnostic markers can be identified via the use of aptamers. Aptamers are generated by the process of the SELEX, as will be discussed in the next chapter. Our lab has hypothesized that modified oligonucleotide aptamers can be generated and specifically selected for their preferential binding to the biomarkers of TB. Upon the identification of several aptamers that bind with a high affinity to these biomarkers, a diagnostic platform can be generated that will incorporate these aptamers into a lateral flow assay using patient urine as a diagnostic medium.

The purpose of this thesis is to address the identification and analysis of aptamer sequences to the protein MT2462, a TB biomarker found in the urine of patients with TB disease. Chapter 2 provides background information on aptamers and the SELEX process. The results and methods for this research are addressed in Chapters 3 and 4, respectively.
Chapter 2: Modified-Oligonucleotide Aptamers and Selections

2.1 Modified-Oligonucleotide Aptamers

Oligonucleotide aptamers are RNA or DNA based single-stranded oligonucleotides that can be anywhere between 30 to 140 base pairs in length. Generally speaking, aptamers function as affinity reagents, meaning that they possess a high affinity binding constant for one specific target. Aptamer sequences bind to their targets of interest by allowing their nucleotide side chains to interact directly with their target, as seen in Figure 2.1 below.

![Figure 2.1](image.png)

Figure 2.1. “Arbitrary aptameric” molecular target (colored) interacting with an aptamer (green). The aptamer is single-stranded, but has folded upon itself in a way that allows specific binding sites to be present for the interaction with the molecular target.¹

Modified aptamers differ from conventional nucleic acid sequences and aptamers in that they contain modifications to the bases of one or more of their nucleic acids. Sometimes, these modifications are incorporated onto thymine nucleosides and resemble amino acid side chains, increasing the overall chemical diversity of the aptamer.⁵,⁷ Specifically, these modifications increase the valency of aptamers through more hydrophobic, hydrophilic, or charge-charge interaction sites on the aptamer, as seen in Figure 2.2, in hopes of generating strands that have an
increased affinity for their target molecules.\textsuperscript{5} In fact, SomaLogic®, a company in Boulder, Colorado, has shown that these modified aptamers bind with a high affinity to proteins and other small molecules with affinities that are often orders-of-magnitude greater than those observed in aptamers that do not contain modified nucleotides.\textsuperscript{7}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{aptamer_binding.png}
\caption{Illustration of an aptamer binding to its target. Although exaggerated, the binding sites on the target must match the chemical characteristics in the modified groups on the aptamer in order for binding to be possible. As shown in the figure, the interactions between target and aptamer are noncovalent, but rather due to hydrophobic, hydrophilic, or charge-charge interactions.\textsuperscript{1}}
\end{figure}

The mechanism that allows aptamers to interact with proteins or other molecular targets stems from the way the strand folds on itself, as demonstrated in Figure 2.3 below.\textsuperscript{7} The modification plays a crucial role in the folding of these aptamers. As discussed earlier, modifications function to increase the binding affinities for molecular targets, but they are also important in determining the way that the aptamer folds on itself. An aptamer modification that severely inhibits hydrogen bonding or the way that the nucleic acids interact is considered to be a poor modification, as it interferes with the primary role that the aptamer must play when coming into contact with molecular targets.\textsuperscript{5} The aptamer must be able to fold in such a way that the modified functional groups on the strand can bind to side-chains or functional groups on the protein or molecular surface of interest.\textsuperscript{5}
Figure 2.3. One possible structure of a folded aptamer. The modified nucleotides can be seen in the center of the figure, protruding out from the aptamer. These modifications function as the primary site for molecular contact with other agents in solution.

2.2 Specific Modifications to Aptamers

The specific modifications themselves are also a point of interest, as some modifications function more effectively than others. Over one hundred different modifications exist commercially, and many can be synthesized independently. Different protein or molecular targets will have variations in their affinities for an aptamer depending on the modification that is used. Although SomaLogic ® has manufactured several modifications that produce low dissociation constants in their binding to various protein targets, the choice of modification to use depends on individual experimental evidence. As discussed previously, a good modification should contain sites for hydrophobic, hydrophilic, or charge-charge interaction sites. However, the way that these sites interact with a target molecule is based on the chemistry of the molecule, as well as the chemistry of the aptamer.
Figure 2.4. Common modifications that are employed in the generation of aptamers. These modifications were selected for use by the Feldheim group based on their strong dissociation constants, as was demonstrated by SomaLogic ®. The R groups represent the functional modification that is placed on the nucleotide on the left.⁷

The Feldheim lab has incorporated several of the above modifications, as well as others not pictured here, in their aptamers. Attempting to elucidate the perfect combination of modification to Mtb biomarker is strictly subject to experimentation. The modification not only influences the way that the aptamer will bind to the biomarker, but also the efficacy of the aptamer itself, as the modification can influence the folded structure of the final aptamer, enhancing or hindering its use as an affinity reagent.⁵

2.3 Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

Aptamers are generated using a technique called the Systematic Evolution of Ligands by Exponential Enrichment (SELEX). SELEX begins with a pool of single-stranded biotinylated oligonucleotide templates (Figure 2.5) that contain two fixed primer regions flanking random nucleotide regions, which are typically about forty bases long. Together, all of the sequences in the pool comprise what is known as a library. The library is the most diverse pool of sequences that will exist throughout the selection, as all of the sequences should be different from one another, given the random nucleotide region that each sequence contains. This pool contains approximately $10^{14}$ unique sequences. The purpose of this randomization is to ensure that the
greatest variety of sequences is present prior to dwindling the pool down to a few sequences that are unique to a particular target.\textsuperscript{14}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Representation of the single-stranded oligonucleotides that constitute the pool of aptamers at the beginning of a selection.}
\end{figure}

The random template library of sequences is primer extended to incorporate a chemically modified uridine triphosphate (UTP) into the sequences. Following primer extension, the aptamer sequences are incubated with their target of interest. Sequences that contain some specificity for the target with which they are incubated with are termed specific binders. After incubation with the target, non-specific binder sequences are washed out of the pool, leaving behind sequences that bind preferentially to the target. When conducting the initial selection round, the concentration of target that is used for the selection bias is large to ensure that a variety of potential aptamers are selected. As the selection continues, the concentration of the target will decrease. The purpose of this is to increase the selective pressure on the library, attempting to ensure that the few sequences that are binding to the target are binding tightly and with high specificity for only that particular target.\textsuperscript{14}

Following each selection round, sequences that bind to the target must be amplified. In order to ensure their continued presence in solution, the sequences with specificity for the target undergo Polymerase Chain Reaction (PCR), which significantly amplifies the quantity of sequences that remain in the selection. These sequences are then primer extended yet again to incorporate the modification back into the sequences. The pool of aptamers has now become enriched in sequences that are specific to the target. Selection rounds continue and the target of interest is continually incubated with the pool at increasingly lower concentrations until
eventually an evolved pool is formed. This evolved pool should contain a high concentration of aptamers that are preferentially binding to the target of interest. Figure 2.6 below depicts a visual representation of the SELEX process.14

Figure 2.6. Schematic illustration of the selection and generation of an evolved pool of aptamers. Although many SELEX experiments follow the process diagramed, variations of this process exist based on the type of aptamer being generated.23

2.4 Advantages of Aptamers

Based on the limitations of existing diagnostics, as discussed previously, it is apparent that a new diagnostic which can cater to all patients as well as function in resource-limited regions of the world is necessary for overcoming the spread and potency of tuberculosis infection. The most effective way to accurately diagnose tuberculosis is through the use of molecular biomarkers. These biomarkers present themselves as part of the cellular debris that
results from tubercle disruption (or any kind of \textit{M. tuberculosis} cellular disruption). Therefore, an ideal diagnostic method would detect the presence of these biomarkers by utilizing molecules like aptamers. Aptamers are ideal for tuberculosis diagnosis due to the high specificity of aptamer sequences for their targets, the ability to reproducibly generate specific aptamers at a low cost, and the fact that aptamers are thermally stable.\textsuperscript{2}

For over thirty years, antibodies have been the preferred capture reagent for different diagnostic platforms utilized in the detection of pathogens. Although still useful in the medical realm, antibodies may be taking a back seat to aptamers in the near future. Unlike antibodies, aptamers are produced via a chemical synthesis, as described above. This \textit{in vitro} generation of aptamers means that they can be manipulated to function with high specificity under a plethora of conditions.\textsuperscript{14}

The high specificity of aptamers is the result of the selection technique used to generate them. The random library of aptamers is evolved \textit{in vitro}, eventually generating a pool of aptamers that is saturated with very specific sequences that bind their targets.\textsuperscript{7} Conversely, antibodies are produced \textit{in vivo} with the use of animal vectors, meaning that each batch of antibodies produced could potentially vary, depending on the conditions in which the host produces them. Based on this, the recognition of an antibody to its target, although decently specific, is not catered to the target as perfectly as an aptamer.\textsuperscript{14} Furthermore, aptamers can be produced in high quantity via various \textit{in vitro} techniques, which are significantly cheaper compared to the cost associated with the use of animal vectors for antibody production.\textsuperscript{2}

A huge advantage of aptamers, especially in resource-limited parts of the world, stems from the fact that aptamers undergo reversible denaturation in high temperature, unlike antibodies. Once an antibody is exposed to temperatures above the range in which it is capable of
functioning, it can denature and lose its binding capabilities. Aptamers, on the other hand, can re-nature following exposure to high temperatures, so long as the temperature is decreased slowly over a short period of time. Typically, the re-naturation only takes a few minutes. The advantage for resource-limited environments is that an aptamer-based diagnostic will be robust, even if exposed to higher temperatures, thus eliminating the need for refrigeration. Furthermore, the diagnostic can be transported outside of the laboratory setting, and will maintain a shelf life that is longer than that of antibodies.\textsuperscript{14}

In an ideal setting, an aptamer-based diagnostic will be generated to bind directly and with high affinity and specificity to \textit{Mtb} biomarkers. Immobilization of these aptamers to a diagnostic device resembling a pregnancy test will allow ease of transportation so that the diagnostic can be taken directly to patients without fear of the diagnostic becoming unusable. Although the Feldheim lab and their collaborators are still a ways off from generating the perfect diagnostic, the results thus far are promising.

\textbf{2.5 Protein MT2462}

One of the biomarkers that the Feldheim Group is working to isolate is a protein dubbed MT2462, or phosphoadenosine phosphosulfate reductase. A 2008 publication revealed four unique \textit{Mtb} proteins (including MT2462) are found in the urine of patients infected with active pulmonary TB.\textsuperscript{15} The prevailing hypothesis is that these proteins are produced in lung lesions, and filtered through the bloodstream into urine as the infection progresses. Furthermore, any bacterial cells from extra-pulmonary tuberculosis that undergo lysis in the body could also be filtered through the bloodstream and end up in the urine.\textsuperscript{15} Given its presence in urine, MT2462 is an ideal target for an aptamer-based diagnostic, and, more specifically, a urine diagnostic, as
patient urine samples are easy to obtain and non-invasive for the patient. This thesis addresses the development and characterization of the aptamers for MT2462.
Chapter 3: Results and Discussion

3.1 Selections

The round to round progress of the selection for aptamers to MT2462 was monitored using Real Time Polymerase Chain Reaction (RT-PCR). The graph shown in Figure 3.1 below represents the PCR results from round four of the selection. Each trace, or color, is indicative of the evolved pool sample, the background sample, or the controls being tested. The cycle at which each trace amplifies is most important for analysis of the selection round.

Although not quantitative, the amplification cycle of each trace can give a comparative measure regarding how much of the evolved pool of aptamers is binding to the MT2462 target, relative to the background. The background set of aptamers function as a control. The background pool should be nearly identical to the target set of aptamers, the only difference being that the background is never exposed to MT2462. By this logic, the quantity of aptamers that are present in the background should be fewer than those present in the target, given that the target should retain more sequences due to MT2462 binding; the binding to MT2462 is specifically designed using the variety of binding opportunities (by the modification) on the aptamer, as well as the counter-selection steps to the beads, as discussed in Chapter 4. Thus, it is expected that the target pool should have an earlier amplification relative to the background pool—the target trace should show an earlier cycle of amplification compared to the background trace.

The number of cycles that separate the target trace from the background is of importance as well. Only a few cycles of separation could mean that the target and the background pools have similar amounts of DNA, and that they most likely also contain a good bit of sequence similarity. However, larger amounts of cycle separation between the two pools not only indicate
a difference in the quantity of aptamers, but also lends to the thought that the aptamers in the target pool contain different sets of sequences.\(^7,^{25}\) The exact number of sequences in each pool is hard to determine. However, using the basic principles of PCR, and the understanding that PCR is an exponential process, an approximation can be estimated. The factor by which the target pool quantity differs from the background pool can be estimated using the equation

\[ R = 2^{C_T - C_B}. \]

Equation 3.1. Estimates the factor by which the target pool quantity of aptamers differs from the background. \( R \) stands for the ratio between the two pools. \( C_B \) is the cycle at which the background amplifies, and similarly \( C_T \) is the cycle in which the target amplifies.\(^25\)

Furthermore, as the supplemental material indicates (see Appendix) most of the target pools of sequences amplify around cycle eight during PCR. This delay in amplification (comparatively to amplifying at cycle one), alludes to the fact that there is a time delay between the start of PCR and when the primers and polymerases come into contact with the aptamer sequences. Thus, if the background amplifies a few cycles after the target, it can be concluded that it took more time for the primers and polymerase to come into contact with the aptamer sequences of the background pool. Given that the concentration of primers and polymerase are the same for both pools, the difference in the amplification cycles of both pools can be attributed to the quantity of aptamer templates in each pool. Using Equation 3.1 from above, it can be estimated that the quantity of aptamers that differ between the target and the background separated by four cycles, for example, is a factor of 16. Assuming there are even 10 picomoles (\(10^{-12}\)) of DNA in the target pool, a difference by a factor of 16 could result in a difference of \(10^{11}\) aptamer copies between the two pools.\(^7,^{25}\)

As can be seen from Figure 3.1 below, the target pool for the selection amplifies around cycle eight (blue), while the background comes up at cycle seventeen (pink). The cycle
separation observed for round four is nine cycles, indicating a 512 factor difference between the two pools.

**Round 4: 125 nM MT2462**

![PCR trace from round four of the selection, with 125 nM of protein MT2462 incubated with the target. The target (blue trace) amplified around cycle eight, while the background (pink trace) amplified at cycle seventeen, producing nine cycles of separation between the two aptamer pools. The green trace coming up around cycle 22 is the negative control, containing only the ingredients necessary for PCR, without any aptamers. The reason for its amplification could be due to primer dimer.]

The PCR traces for the other selection rounds can be found in the appendix of this thesis. The selection proceeded until the MT2462 concentration was down to 15 nM and the evolved pool enriched to six cycles of separation between the target and the background (round twelve). At this point in time, the selection was terminated. Given the low concentration of MT2462 exposed to the aptamers, and the fact that the target aptamers enriched during this round, it can be concluded that the aptamers in this evolved pool have an affinity for MT2462. Thus, the final evolved pool was sent for sequencing analysis to identify individual sequences in the pool that could be binding to MT2462.
3.2 Sequencing

The final evolved aptamer pool from round twelve was sent for Next-Generation Sequencing at the BioFrontiers Institute at the University of Colorado Boulder. The sequencing data returned for the evolved pool indicated that the pool, in its entirety, contained twenty-two million sequences. The data for the evolved pool was analyzed, and any sequences that were of poor quality and/or mutated were discarded from the data pool. Mutations can arise when aptamer sequences become longer or shorter due to indels (insertions or deletions) that result during the PCR rounds. Furthermore, some regions of a sequence do not return conclusive data regarding nucleotide identity following sequencing, and therefore are also discarded from analysis (Phred score less than 20). Following the removal of poor quality aptamer sequences, the evolved pool was left with twelve million sequences. Of the twelve million sequences remaining, there were approximately four million sequences that were unique, containing no duplicate sequences in the pool. This can be seen in Figure 3.2 A below.

From there, the data was organized based on cluster. A cluster is equivalent to a family of sequences, or sequences that contain regions of nucleotides that are identical. All the sequences within a cluster are very similar in terms of their individual sequences, meaning there is sequence homogeneity within each cluster. Based on the data analysis of the evolved pool, it was determined that there were 49 clusters present. Clusters were generated using a Levenshtein edit of 7 nucleotides. Figure 3.2 B shows how many total sequences exist within a cluster and then from there details how many of those total sequences are unique to that cluster.
Figure 3.2. A) The graph indicates how many total sequences existed in the evolved pool, how many of those sequences were of proper length, and how many sequences were both of proper length and unique to the evolved pool. B) The graph shows the total number of clusters (or families) of sequences that were in the evolved pool. It also details how many sequences were in each cluster, and how many of those sequences were unique to that cluster.

When reviewing the data, individual sequences were picked for analysis to determine if they were binding to MT2462 with high affinity. The first criteria used when selecting which sequence to analyze was its frequency of appearance in the evolved pool. A high population of one specific sequence could indicate that it is the sequence binding strongest to MT2462, as it was present in the highest quantity and amplified with the evolved pool during the final round. From there, high population sequences were analyzed to determine which ones had the highest
presence of the modified nucleotide. Recall that the modified nucleotide creates more binding surfaces from which the aptamer and protein can interact. Thus, a sequence that is both present in a high frequency within the evolved pool, as well as one that has several modified nucleotides in it is a good target for an aptamer that bound the protein it was selected for. In the case of MT2462, an aptamer sequence named SEQ1 by the Feldheim group, was selected as a potential candidate for specific binding to the protein.

SEQ1 belongs to cluster one, which had the highest presence within the evolved pool, totaling approximately 1 million out of the total 12 million sequences. Specifically, SEQ1 had a population of 533,529. This value indicates that SEQ1 populates approximately 50 percent of cluster one, and 4.5 percent of the total evolved pool. Furthermore, SEQ1 had thirteen modified nucleotides within its random region, further making it an excellent potential aptamer for MT2462 binding.

As noted, SEQ1 constitutes 4.5 percent of the total evolved pool. Based on this, it is reasonable to assume that there are other sequences within the other clusters that are strong binders for MT2462 as well. However, SEQ1 was selected for testing first given the high amounts of modification that it contains. All other individual sequences will be tested at a later time, and their results are therefore not included in this thesis. SEQ1 was used in characterization experiments to determine its binding affinity for MT2462, as is detailed below.

### 3.3 Characterization of the Evolved Pools

As the selection progressed, the evolved pools were analyzed using Fluorescence Polarization Anisotropy (FPA) to help narrow down the range in which the dissociation constant for MT2462 to aptamer binding lies. FPA tracks the anisotropy (rotation) of a fluorophore tagged
to an aptamer. A free aptamer in solution will have a higher anisotropy (rotating quickly) compared to an aptamer that is bound to a protein (rotating slowly). These changes in anisotropy are indicative of a protein binding to an aptamer.\textsuperscript{27} Due to the fact that the evolved pools are composed of millions of different aptamer sequences, an accurate dissociation constant for one specific aptamer cannot be determined from such an analysis. However, operating under the assumption that at least one, if not more, of the sequences in that evolved pool are specific binders for MT2462, such as SEQ1, an approximate range of dissociation constants can be determined. This range is then used when testing the individual sequences determined from the sequencing data of the final evolved pool (SEQ1) in order to determine a more precise dissociation constant.\textsuperscript{21}

Figure 3.3 details the overall change in anisotropy for the aptamers of the evolved pool in round four, as MT2462 was titrated into the pool. The data indicates that after the addition of 150 nM of MT2462, the aptamers in solution that bind MT2462 are saturated with protein. This is evident from the fact that there is very minor fluctuation in the anisotropy values after the addition of approximately 150 nM of MT2462. Due to the lack on anisotropy change, the assay was terminated and a non-linear regression was conducted for this set of data. Based on the regression, a dissociation constant of 79 nM ± 16 was determined for the evolved pool. Thus, the assays conducted on the individual sequences being tested from the final evolved pool use MT2462 concentrations less than 79 nM in order to determine a more accurate dissociation constant for these individual sequences.\textsuperscript{21}
26

Figure 3.3. Change in anisotropy of the Round 4 evolved pool upon the addition of MT2462 to the aptamers in the pool. A non-linear regression for the data is also displayed.

3.4 Characterization of Individual Sequences

Bead Binding Assay

Sequence 1 (SEQ1) from the final evolved pool was selected for analysis. A bead binding assay was conducted in which SEQ1 was exposed to 10 nM of MT2462 (bound to His-Talon beads). SEQ1 was also exposed to the beads alone, as a control for this assay. Sequences that bound the protein or the bead were eluted and amplified via PCR to qualitatively determine if SEQ1 has a preference for MT2462. The PCR results are shown in Figure 3.4 below. Based on the results, there are 6 cycles of separation between the SEQ1 aptamers that were exposed to 10 nM of MT2462 (blue trace), and those that were not exposed to the protein (green trace). Given the low concentration of protein used, and the cycle separation between the two samples, it can be hypothesized that SEQ1 has an affinity for MT2462. FPA analysis (see below) was used in an attempt to determine a more accurate dissociation constant for the aptamer.\textsuperscript{7,25}
Figure 3.4 PCR trace for the bead assay with 10 nM of protein MT2462 incubated with SEQ1 (blue trace). The green trace is SEQ1, which was not exposed to any MT2462. The negative control (lime green) showed amplification that is due to primer dimer, while the blank (red) showed no amplification.

**Fluorescence Polarization Anisotropy (FPA)**

SEQ1 was subjected to FPA analysis, with MT2462 being titrated into solution. The FPA data for the sequence is shown in Figure 3.5 below.

![Figure 3.5](image)

Figure 3.5. Plots of the change in anisotropy of SEQ1 as various concentrations of MT2462 are titrated into the aptamer solution. A shows the change in anisotropy without the large fluctuations in anisotropy factored in. B accounts for these large fluctuations as the error bars indicate.

Unfortunately, the FPA data for SEQ1 does not yield an accurate dissociation constant. As the error bars in Figure 3.5B indicate, the large fluctuations in anisotropy produced by the aptamer overpowered the assay. These fluctuations mean that the changes in anisotropy that were
seen cannot be attributed to the addition of the MT2462 protein. Due to time constraints, a solution to this problem has not yet been elucidated. However, outlined below are several methods that could be implemented to attempt and decrease these large fluctuations so as to determine an accurate dissociation constant for this aptamer.

It is known that the fluorescent counts per second (CPS) needed in order for the Fluorolog ® to be able to accurately interpret the fluorescence of the aptamer is between 100,000 and 2,000,000 CPS without the polarizers in place. Thus, once the polarizers are in place, as they are for these FPA assays, the limit decreases. Experimentally it has been determined that the new linear range of detection is between 100,000 to 1,000,000 CPS with the polarizers in place. The evolved pool assays indicated that the dissociation constant for the evolved pool was 79 nM. Furthermore, the bead binding assay eluded to the fact that SEQ1 has an affinity for MT2462 at a concentration of 10 nM, further narrowing down the range in which the dissociation constant for the individual sequences could lie. In order to generate data for a dissociation constant based on a low concentration of protein, the aptamer concentration must also be low. However, it has been determined experimentally that lowering the aptamer concentration decreases the CPS reading from the fluorophore to a range that is lower than that which can be detected by the Fluorolog ®, which could be contributing to the fluctuations in anisotropy that are seen in the SEQ1 FPA assay.

One possible solution to this is to increase the concentration of SEQ1 in solution to attempt and raise the CPS to a detectable level for the Fluorolog ®. The primary limitation in this method would be that the dissociation constant generated from the data may not be very accurate due to the high concentration of aptamer comparatively to the protein. However, it would be beneficial to experiment with various concentrations of aptamer in solution to attempt and
elucidate an ideal concentration of aptamer that generates CPS measurements that are detectable by the Fluorolog®, yet also is a concentration that is low enough to yield an accurate dissociation constant.

Another solution would be to adjust the excitation and emission slit widths in the Fluorolog®. Ideally, the excitation slit width should be open to maximum capacity (10 nm) so that all the light can be absorbed by the sample. The emission slit width could then be altered to still allow enough light to pass through to produce high CPS, but limit the amount of extra fluctuations that are detected by the Fluorolog®.

More than likely, a combination of the above mentioned solutions would fix the problem currently exhibited in FPA analysis. However, if none of the mentioned solutions yield tangible data, another method for determining the dissociation constant of SEQ1 can be implemented. These experiments will be conducted over the course of the next few months.

3.5 Conclusions

The SELEX process was utilized to generate an evolved pool of aptamers that have an affinity for the Mtb protein MT2462,7 which is found in the urine of patients that have confirmed active TB disease.15 The evolved pool of aptamers were sent for Next-Generation Sequencing at the BioFrontiers Institute at the University of Colorado at Boulder. The sequencing data revealed that 12 million sequences were present in the evolved pool. Upon analysis of the data, an aptamer termed SEQ1 was selected to test binding specificity to protein MT2462. SEQ1 was analyzed using a Bead Binding Assay as well as Fluorescence Polarization Anisotropy (FPA) to determine a dissociation constant for the aptamer to the protein. Although a dissociation constant has not been determined at this time, the PCR results from the bead binding assay detect binding
of SEQ1 to MT2462 at 10 nM of protein, suggesting that the dissociation constant for the aptamer could lie within this range, or within an order of magnitude above and below this concentration. Further experimentation is still required to determine an accurate value.

Complete analysis of the evolved aptamer pool involves not only determining a dissociation constant for SEQ1 to MT2462, but also analyzing other sequences in the evolved pool that could be specific binders to MT2462. Completion of the analysis of the evolved pool would then open the door for generation of a TB diagnostic that utilizes these aptamers as a capture reagent for the protein MT2462. As mentioned previously, the diagnostic platform would resemble a pregnancy test, utilizing the concept of lateral urine flow to yield a diagnostic result. Accomplishing these goals, however, does not just generate a new diagnostic for TB—it generates a urine-based diagnostic that is thermally stable, cheap to produce, accurate, portable, rapid in generating results, and convenient for both patients and clinicians. A diagnostic of this nature is exactly what the WHO could utilize to combat MDR-TB in resource-limited parts of the world.
Chapter 4: Methods

4.1 Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

Original Template Library

The synthesis of a random library of aptamers begins from a random template library. The sequences comprising this library were synthesized by Integrated DNA Technologies (IDT). Each sequence in the template library contains a variable forty nucleotide random region that is flanked by two fixed primer regions. The five prime primer is biotinylated for primer extension purposes during the selection rounds.17

Generating a Random Aptamer Library

MyOne Streptavidin Dynabeads (magnetic, 0.25 mg/mL—50 µl total) were washed three times with binding and wash buffer (1M NaCl, 10 mM Tris, pH 7.5). 1 nmol of random template library (10 µL in 1x IDTE Buffer pH 8.0) was added to the beads with 100 µl binding and wash buffer and incubated at room temperature for twenty minutes on a rotisserie. Any unbound sequences were washed away using three washes of binding and wash buffer. Primer extension was performed by adding the following solution to the beads: 15.15 µM forward primer, 1.0X Primer Extension Buffer (99.0 mM Tris, 10.0 mM KCl, 0.1044 mg/mL BSA, 6.0 mM NH₄SO₄, 7.0 mM MgSO₄, 10% Triton, and dH₂O up to 1 mL) 0.5 mM each dNTP, 0.1 U/µl Kod XL Polymerase, in a total volume of 150 µl. The beads and solution were incubated for 60 minutes on a heater/shaker at 72°C and shook at 40 rpm. After primer extension, beads were washed four times with binding and wash buffer and once with 16 mM NaCl. The modified nucleotide aptamer library was then eluted from the template-bead complex using 80 µl of 20 mM NaOH.
The eluted product was neutralized with 20 µl of 80 mM HCl and beads were discarded. The random aptamer library was then used for round one of the selection.

Selection Round Assays

Round 1

First the library was refolded by placing 80 µl of the modified-DNA aptamer library in to 20 µl of 5X selection buffer (200 mM HEPES, 25 mM KCl, 25 mM MgCl₂, 0.05% Tween-20 (100%), dH₂O up to 10 mL) and heating to 95°C for 3.5 minutes. The library was then cooled to 22°C in the course of 12 minutes. Two sets of cobalt His-Talon beads (0.002 mg/mL, 20 µl total) were each washed with 1X selection buffer three times. The heat/cooled library solution was added to one set of beads for a counter-selection and incubated at room temperature on the rotisserie for 15 minutes. The supernatant containing the unbound aptamers was removed and transferred to a tube containing the hexa-Histidine tagged protein, MT2462, bound to the cobalt His-Talon beads (protein MT2462 was bound to the beads at a concentration of 250 nM for a final volume of 200 µl). This solution was then incubated on the rotisserie for 24 hours at room temperature.

Following the 24 hour incubation, the beads were washed five times with 1X selection buffer. The beads were incubated in the buffer for 2 minutes between washes, and then transferred to a new tube during the fifth wash, as a preventative measure to avoid plastic binding aptamers. Upon completion of the washes, the aptamers were eluted from the protein with 80 µl of 2 mM NaOH and a five minute incubation on the rotisserie at room temperature. The eluted product was then neutralized with 20 µl of 8 mM HCl and the beads were discarded.
All eluted and neutralized aptamers were then subjected to PCR. A 5X PCR Mix was generated as follows: 5 µM forward primer (biotinylated), 5 µM reverse primer, 25 mM MgCl₂, 5X KOD XL Polymerase Buffer, 2 mM of each dNTP, 0.0875 U/µl of Kød XL Polymerase, 5X SYBR Green, and dH₂O to a final volume of 50 µL. The PCR Mix was then diluted to a concentration of 1X for each sample by adding 10 µl of PCR Mix to 33 µl of aptamer and 7 µl of dH₂O. The aptamers were amplified using the following PCR protocol: Tubes were heated at 95°C for 30 seconds, 55°C for 15 seconds and 71°C for 30 minutes. Afterwards, tubes underwent 30 cycles of heating to 96°C for 15 seconds and then 71°C for 1 minute, followed again by 96°C for 15 seconds.⁷

Following amplification of the aptamers, 50 µl of the PCR product was captured onto MyOne Dynabeads (washed 3 times with binding and wash buffer) via a 30 minute incubation on the rotisserie with 50 µl of binding and wash buffer at room temperature. After the incubation, the beads were again washed three times with binding and wash buffer. One of the strands of the double stranded product was eluted and neutralized using 80 µl of 20 mM NaOH and 20 µl of 80 mM HCl, respectively. This eluted strand was archived as a back up in case of unforeseen needs in the future. The beads were then washed three times with a 1X concentration of the Primer Extension Buffer. Upon completion of the washes, the following Primer Extension Mix was added to the beads: 1X Primer Extension Buffer (same ingredients as listed above), 2.5 µM of forward primer, 0.5 mM of each dNTP, 0.015 U/µl of Kød XL Polymerase and dH₂O up to a final volume of 40 µl. The beads were incubated at 72°C for 30 minutes while shaking at 40 rpm. After the incubation, the beads were washed four times with binding and wash buffer and once with 16 mM NaCl. Finally, the aptamers were eluted using 80 µl of 20 mM of NaOH (pipet
mixing for one minute) and neutralized with 20 µl of 80 mM HCl. The aptamers generated were then subjected to selection round assays as the selection proceeded into round two.⁷

**Round 2**

The aptamers generated in Round 1 were refolded by placing 40 µl of the modified-DNA aptamer into 20 µl of 5X selection buffer (200 mM HEPES, 25 mM KCl, 25 mM MgCl₂, 0.05% Tween-20 (100%), dH₂O up to 10 mL) and 40 µl of dH₂O. As in Round 1, the sequences were heated to 95°C for 3.5 minutes. The aptamers were then cooled to 22°C in the course of 12 minutes. The heat/cooled aptamers were incubated on the rotisserie at room temperature for 15 minutes with a set of washed (three times with selection buffer) cobalt His-Talon beads for a counter-selection. The supernatant from the counter-selection was then split into two tubes, one for the background and one for the target. The background tube contained 50 µl of heat/cooled and counter-selected aptamers along with 50 µl of 1X selection buffer. The target tube also contained 50 µl of the heat/cooled, counter-selected aptamers as well as 41 µl of 1X selection buffer and 250 nM of protein MT2462 (final volume of 100 µl). These tubes were then incubated on the rotisserie for 24 hours at room temperature.⁷

Following the 24 hour incubation, the background and target solutions were incubated with their own set of cobalt His-Talon beads (washed three times with selection buffer) for 15 minutes at room temperature on the rotisserie. The beads were then washed five times with 1X selection buffer and incubated in the buffer for 2 minutes between washes. At the completion of the fifth wash, the solution was transferred to a new tube as a preventative measure to avoid plastic binding aptamers. Upon completion of the washes, the aptamers were eluted from the protein with 80 µl of 2 mM NaOH and a five minute incubation on the rotisserie at room
temperature. The eluted product was then neutralized with 20 µl of 8 mM HCl and the beads were discarded.

All eluted and neutralized aptamers were then subjected to PCR. A 5X PCR Mix was generated as follows: 5 µM forward primer (biotinylated), 5 µM reverse primer, 25 mM MgCl₂, 5X KOD XL Polymerase Buffer, 2 mM of each dNTP, 0.0875 U/µl of Kod XL Polymerase, 5X SYBR Green, and dH₂O to a final volume of 50 µL. The PCR Mix was then diluted to a concentration of 1X for each sample by adding 10 µl of PCR Mix to 33 µl of aptamer and 7 µl of dH₂O. The aptamers were amplified using the following PCR protocol: Tubes were heated at 95°C for 30 seconds, 55°C for 15 seconds and 71°C for 30 minutes. Afterwards, tubes underwent 30 cycles of heating to 96°C for 15 seconds and then 71°C for 1 minute, followed again by 96°C for 15 seconds.

Following amplification of the aptamers, 50 µl of the PCR product was captured onto MyOne Dynabeads (washed 3 times with binding and wash buffer) via a 30 minute incubation on the rotisserie with 50 µl of binding and wash buffer at room temperature. After the incubation, the beads were again washed three times with binding and wash buffer. One of the strands of the double stranded product was eluted and neutralized using 80 µl of 20 mM NaOH and 20 µl of 80 mM HCl, respectively. This eluted strand was archived in the event that the round needed to be re-conducted. The beads were then washed three times with a 1X concentration of the Primer Extension Buffer. Upon completion of the washes, the following Primer Extension Mix was added to the beads: 1X Primer Extension Buffer (same ingredients as in previous rounds), 2.5 µM of forward primer, 0.5 mM of each dNTP, 0.015 U/µl of Kod XL Polymerase and dH₂O up to a final volume of 40 µl. The beads were incubated at 72°C for 30 minutes while shaking at 40 rpm. After the incubation, the beads were washed four times with
binding and wash buffer and once with 16 mM NaCl. Finally, the aptamers were eluted using 80 µl of 20 mM of NaOH (pipet mixing for one minute) and neutralized with 20 µl of 80 mM HCl. The aptamers generated were then subjected to selection round assays as the selection proceeded into round three.7

**Round 3**

The aptamers generated in Round 2 were refolded by placing 20 µl of the modified-DNA aptamer in to 10 µl of 5X selection buffer and 20 µl of dH2O. As in Rounds 1 and 2, the sequences were heated to 95ºC for 3.5 minutes. The aptamers were then cooled to 22ºC in the course of 12 minutes. The heat/cooled aptamers were incubated on the rotisserie at room temperature for 15 minutes with a set of washed (three times with selection buffer) cobalt His-Talon beads for a counter-selection. The supernatant from the counter-selection was then split into two tubes, one for the background and one for the target. The background tube contained 20 µl of heat/cooled and counter-selected aptamers along with 80 µl of 1X selection buffer. The target tube also contained 20 µl of the heat/cooled, counter-selected aptamers as well as 80 µl of 1X selection buffer and 250 nM of protein MT2462 (final volume slightly over 100 µl). These tubes were then incubated on the rotisserie for 24 hours at room temperature.7

Just as in Rounds 1 and 2 following the 24 hour incubation, the target and background solutions were captured on to the cobalt His-Talon beads, the beads were washed with selection buffer, and the aptamers were eluted, amplified, and regenerated in primer extension following the same protocols detailed above.7

**Round 4**
The Round 4 protocol is identical to that of Round 3, with one minor discrepancy. Rather than using 250 nM of protein MT2462 for the target selection assay, 125 nM of the protein were used instead, so as to cut the amount of target present and increase the selective pressure on the assay.  

*Round 4 with Fluorescent Tag*

At this point in the selection, the Cal-Fluor 610 fluorescent tag was added to the forward primer used during Primer Extension to generate the aptamer pool. The purpose of this addition was so that the evolving pools of aptamers could be monitored via Fluorescence Polarization Anisotropy (see below) to determine the strength of binding between the evolving aptamers and protein MT2462.  

40 µl of PCR product from Round 4 was incubated for 30 minutes at room temperature on the rotisserie with 50 µl of Pierce Streptavidin UltraLink Resin beads (washed three times with binding and wash buffer). After the incubation, the beads were again washed three times with binding and wash buffer. One of the strands of the double stranded product was eluted and neutralized using 80 µl of 20 mM NaOH and 20 µl of 80 mM HCl, respectively. This eluted strand was archived in the event that the round needed to be re-conducted. The beads were then washed three times with a 1X concentration of the Primer Extension Buffer. Upon completion of the washes, the following Primer Extension Mix was added to the beads: 1X Primer Extension Buffer (same ingredients as in previous rounds), 15 µM of the Cal-Fluor 610 forward primer, 0.5 mM of each dNTP, 0.1 U/µl of Kod XL Polymerase and dH₂O up to a final volume of 50 µl. The beads were incubated at 72°C for 30 minutes while shaking at 40 rpm. After the incubation, the beads were washed four times with binding and wash buffer and once with 16 mM NaCl.
Finally, the aptamers were eluted using 80 µl of 20 mM of NaOH (pipet mixing for one minute) and neutralized with 20 µl of 80 mM HCl. The aptamers generated were then subjected to selection round assays as the selection proceeded into round five.  

**Rounds 5 through 12 with Fluorescent Tag**

Rounds 5 through 12 all followed the protocol detailed below. The only variations in protocol were the result of differences in concentration of protein MT2462, as is detailed in Table 5.1, following the protocols below.

The aptamers generated in each round were refolded by placing 20 µl of the modified-DNA aptamer in to 10 µl of 5X selection buffer (200 mM HEPES, 25 mM KCl, 25 mM MgCl$_2$, 0.05% Tween-20 (100%), dH$_2$O up to 10 mL) and 20 µl of dH$_2$O. The sequences were heated to 95 ºC for 3.5 minutes. The aptamers were then cooled to 22 ºC in the course of 12 minutes. The heat/cooled aptamers were incubated on the rotisserie at room temperature for 15 minutes with a set of washed (three times with selection buffer) cobalt His-Talon beads for a counter-selection. The supernatant from the counter-selection was then split into two tubes, one for the background and one for the target. The background tube contained 20 µl of heat/cooled and counter-selected aptamers along with 80 µl of 1X selection buffer. The target tube also contained 20 µl of the heat/cooled, counter-selected aptamers as well as 80 µl of 1X selection buffer and a variable concentration of protein MT2462 (final volume slightly over 100 µl). These tubes were then incubated on the rotisserie for 30 minutes at room temperature; the decrease in the incubation time was important for increasing the selective pressure on the selection.  

Following the 30 minute incubation the background and target solutions were incubated with their own sets of cobalt His-Talon beads (washed three times with selection buffer) for 15
minutes at room temperature on the rotisserie. The beads were then washed five times with 1X selection buffer and incubated in the buffer for 2 minutes between washes. At the completion of the fifth wash, the solution was transferred to a new tube as a preventative measure to avoid plastic binding aptamers. Upon completion of the washes, the aptamers were eluted from the protein with 80 µl of 2 mM NaOH and a five minute incubation on the rotisserie at room temperature. The eluted product was then neutralized with 20 µl of 8 mM HCl and the beads were discarded.7

All eluted and neutralized aptamers were then subjected to PCR. A 5X PCR Mix was generated as follows: 5 µM forward primer (biotinylated), 5 µM reverse primer, 25 mM MgCl₂, 5X KOD XL Polymerase Buffer, 2 mM of each dNTP, 0.0875 U/µl of Kod XL Polymerase, 5X SYBR Green, and dH₂O to a final volume of 50 µL. The PCR Mix was then diluted to a concentration of 1X for each sample by adding 10 µl of PCR Mix to 33 µl of aptamer and 7 µl of dH₂O. The aptamers were then amplified using the following PCR protocol: Tubes were heated at 95ºC for 30 seconds, 55ºC for 15 seconds and 71ºC for 30 minutes. Afterwards, tubes underwent 30 cycles of heating to 96ºC for 15 seconds and then 71ºC for 1 minute, followed again by 96ºC for 15 seconds.7

Following amplification of the aptamers, 40 µl of the PCR product was captured onto Pierce Streptavidin UltraLink Resin beads (washed 3 times with binding and wash buffer) via a 30 minute incubation on the rotisserie at room temperature. After the incubation, the beads were again washed three times with binding and wash buffer. One of the strands of the double stranded product was eluted and neutralized using 80 µl of 20 mM NaOH and 20 µl of 80 mM HCl, respectively. This eluted strand was archived in the event that the round needed to be re-conducted. The beads were then washed three times with a 1X concentration of the Primer
Extension Buffer. Upon completion of the washes, the following Primer Extension Mix was added to the beads: 1X Primer Extension Buffer (same ingredients as in previous rounds), 15 μM of the Cal-Fluor 610 forward primer, 0.5 mM of each dNTP, 0.1 U/μl of Kod XL Polymerase and dH2O up to a final volume of 50 μl. The beads were incubated at 72°C for 30 minutes while shaking at 40 rpm. After the incubation, the beads were washed four times with binding and wash buffer and once with 16 mM NaCl. Finally, the aptamers were eluted using 80 μl 20 mM of NaOH (pipet mixing for one minute) and neutralized with 20 μl of 80 mM HCl. The aptamers generated were then subjected to selection round assays in subsequent selection rounds.  

<table>
<thead>
<tr>
<th>Round Number</th>
<th>Concentration of Protein MT2462</th>
</tr>
</thead>
<tbody>
<tr>
<td>Round 5</td>
<td>62.5 nM</td>
</tr>
<tr>
<td>Round 6</td>
<td>62.5 nM</td>
</tr>
<tr>
<td>Round 7</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Round 11</td>
<td>15.0 nM</td>
</tr>
<tr>
<td>Round 12</td>
<td>15.0 nM</td>
</tr>
</tbody>
</table>

Table 4.1. Concentration of protein MT2462 that was used in each round of the selection. Refer to Chapter 3 for an explanation on the concentrations used in each round.
At the conclusion of Round 12, the selection was terminated and the aptamer sequences of the evolved pool were subjected to sequencing analysis (below FPA). All of the selection rounds described above were reproducible.

4.2 Fluorescence Polarization Anisotropy (FPA)

Evolved Pool Assays

Beginning with Round 4, all aptamers generated in rounds with the Cal-Fluor 610 fluorescent tag were subjected to FPA analysis. The purpose of this was to determine if aptamer sequences within the evolved pool were binding specifically to MT2462. 100 nM of each aptamer pool generated was heat/cooled with 1X selection buffer at a final volume of 125 µl. The same heat/cool protocol used in the selection rounds was used here as well. FPA analysis was done in triplicate, using three quartz cuvettes.21

A baseline read of the anisotropy (rotation of a molecule) for each evolved aptamer pool was obtained on the Fluorolog ®. Following this reading, protein MT2462 was added to the evolved aptamer pool in solution and the anisotropy of the fluorescent tag was measured again to determine if the binding of the protein to the aptamers would alter the anisotropy of the sequences. Over the course of four hours, increasing concentrations of protein were added to the sequences and changes in the anisotropy of the sequences in the evolved pools were compared to their respective baseline reads. In order for an anisotropy change to be deemed significant, the change must be greater than three times the standard deviation of the baseline signal, plus the anisotropy of the baseline. Experiments were conducted in triplicate.

Control Assays
Several control assays were conducted for FPA to ensure that the changes in anisotropy that resulted upon addition of protein to the aptamers were in fact due to the protein binding the aptamer.

**Negative Control**

The buffer solution in which protein MT2462 resides is composed of 150 mM NaCl and 100 mM Tris. A solution of this buffer was generated and tested via FPA so as to ensure that the changes in anisotropy that were observed in the aptamer sequence pools were not the result of the addition of the buffer, but were in fact due to the protein binding the aptamer.  

The same protocol for the analysis of the evolved pools detailed above was conducted using the buffer. A baseline anisotropy of the evolved pools was obtained on the Fluorolog® after which the protein buffer was added to the pools in 4 µl increments (total of 20 µl) and the anisotropy was determined.  

**“Randomer” Control**

A modified random aptamer with the Cal-Fluor 610 primer was selected for use in this assay. A baseline read of the anisotropy for the aptamer was obtained on the Fluorolog®. Following this reading, protein MT2462 was added to the aptamer pool in solution and the anisotropy was measured again to determine if MT2462 was binding to the aptamer. Over the course of four hours, increasing concentrations of protein were added to the aptamer and changes in the anisotropy of the sequences in the pool were compared to their respective baseline reads.  

**4.3 Characterization and Testing of the Evolved Pool**
Aptamer Sequencing

The final evolved aptamer pool from Round 12 was subjected to PCR amplification prior to sequencing. A 5X PCR Mix was generated as follows: 5 µM forward primer (biotinylated), 5 µM reverse primer, 25 mM MgCl₂, 5X KOD XL Polymerase Buffer, 2 mM of each dNTP, 0.0875 U/µl of Kod XL Polymerase, 5X SYBR Green, and dH₂O to a final volume of 50 µL. The PCR mix was then diluted to a concentration of 1X by adding 10 µl of PCR Mix to 2 µl of aptamer and 38 µl of dH₂O. This setup was replicated in triplicate. The aptamers were then amplified using the following PCR protocol: Tubes were heated at 96°C for 15 seconds, 55°C for 10 seconds and 71°C for 30 minutes. Afterwards, tubes underwent 4 cycles of heating to 96°C for 15 seconds and then 71°C for 1 minute, followed again by 96°C for 15 seconds.

Following PCR, all amplified samples of the evolved pool were combined and run through a 6% polyacrylamide gel electrophoresis (PAGE) gel for purification. The target band containing the aptamers was excised from the gel and incubated with RNA/DNA gel purification buffer (50 mM Tris, 4 mM EDTA, 200 mM NaOAc, and 0.2% SDS to a final volume of 200 mL) overnight on a rotisserie at room temperature. Aliquots from the buffer solution were removed, and the DNA was ethanol precipitated with 100% ethanol at -80°C for one hour. DNA samples were then recombined and a second ethanol precipitation was conducted to purify the samples further, following the aforementioned protocol.

Once a purified sample was attained, a NanoDrop ® was used to determine the concentration of the double-stranded product. Samples were then sent to BioFrontiers at the University of Colorado at Boulder for NextGeneration Sequencing.

Aptamer Sequencing Analysis
Sequencing data was returned from NextGeneration Sequencing for analysis. The FASTA file was cleaned using the online program Galaxy. First, the reverse primers were clipped from the sequences, followed by the forward primers. The end result left behind the sequences of the random nucleotide regions that are the functional unit of the aptamers, i.e. the random forty nucleotide region. It should be noted that the removal of the primers also removed aptamers that were mutated, as the lengths of mutated aptamers were longer than the standard 95 nucleotides that the aptamers should be.

Once the sequences were cleaved, the data was processed through FASTAptamer, and the sequences were aligned and screened for families. Families are groups of sequences that share many similarities, often varying in less than three nucleotides. The family analysis revealed the presence of 49 different families, each with unique aptamers within the family. Sequences from specific families were selected for further analysis. The sequences were selected based on their population within the evolved pool, and based on the number of modified nucleotides present in the aptamer, as aptamers with more modifications should interact with higher specificity with MT2462. Six total aptamer sequences were selected for analysis, as described in Chapter 3.

4.4 Characterization and Testing of Individual Sequences

Bead Binding Assay

Sequence 1 (SEQ1) was the first sequence to be tested, as it existed in the highest population in the evolved pool, and also contained the most modifications within its sequence. The sequence was primer extended using the same protocol described in the selection rounds, only TAMRA-5 Fluorophore was incorporated into the primer, rather than Cal-Fluor 610. Following primer extension, the sequences were refolded by placing 10 µl of the modified-DNA
aptamer in to 10 µl of 5X selection buffer (200 mM HEPES, 25 mM KCl, 25 mM MgCl₂, 0.05% Tween-20 (100%), dH₂O up to 10 mL) and 30 µl of dH₂O. The sequences were heated to 95°C for 3.5 minutes. The aptamers were then cooled to 22°C in the course of 12 minutes. The heat/cooled aptamers were incubated on the rotisserie at room temperature for 15 minutes with a set of washed (three times with selection buffer) cobalt His-Talon beads for a counter-selection. The counter-selection step was repeated a second time to attempt and remove any miss-folded sequences from the assay. Another set of 20 µl of His-Talon beads were washed (three times with selection buffer) and then incubated with 10 nM MT2462 on the rotisserie for 15 minutes at room temperature, so as to bind the protein to the beads. The supernatant from the counter-selections was then split into two tubes that each contained washed (three times with selection buffer) His-Talon beads; one set was exposed to 10 nM MT2462, and one the other had no exposure to MT2462. These tubes were then incubated on the rotisserie for 30 minutes at room temperature.⁷

Following the 30 minute incubation the beads were washed five times with selection buffer and incubated in the buffer for 2 minutes between washes. At the completion of the fifth wash, the solution was transferred to a new tube as a preventative measure to avoid plastic binding aptamers. Upon completion of the washes, the aptamers were eluted from the protein with 2 mM NaOH and a five minute incubation on the rotisserie at room temperature. The eluted product was then neutralized with 8 mM HCl and the beads were discarded.⁷

All eluted and neutralized aptamers were then subjected to PCR. A 5X PCR Mix was generated as follows: 5 µM forward primer (biotinylated), 5 µM reverse primer, 25 mM MgCl₂, 5X KOD XL Polymerase Buffer, 2 mM of each dNTP, 0.0875 U/µl of Kdo XL Polymerase, 5X SYBR Green, and dH₂O to a final volume of 50 µL. The PCR Mix was then diluted to a
concentration of 1X for each sample by adding 10 µl of PCR Mix to 33 µl of aptamer and 7 µl of dH₂O. The aptamers were then amplified using the following PCR protocol: Tubes were heated at 95°C for 30 seconds, 55°C for 15 seconds and 71°C for 30 minutes. Afterwards, tubes underwent 30 cycles of heating to 96°C for 15 seconds and then 71°C for 1 minute, followed again by 96°C for 15 seconds. Similarly to the selection rounds, the bead binding assay was reproducible.

**Fluorescence Polarization Anisotropy (FPA)**

10 nM of the SEQ1 aptamer was heat/cooled with 1X selection buffer at a final volume of 125 µl. The same heat/cool protocol used in the selection rounds was used here as well. FPA analysis was done in triplicate, using three quartz cuvettes. A baseline read of the anisotropy for the aptamer was obtained on the Fluorolog®. Following this reading, protein MT2462 was added to the evolved aptamer pool in solution and the anisotropy was measured again to determine if the binding of the protein to the aptamer would alter the anisotropy of the sequences. Over the course of four hours, increasing concentrations of protein were added to the sequences and changes in the anisotropy of the sequences were compared to their respective baseline reads. Based on these results, a dissociation constant for the aptamer to the protein was determined.
Appendix: Supplemental Information and Figures

A.1 Selections

Figure A.1, shown below, represents selection rounds one through four. During the first round, the aptamer pool generated was exposed to 250 nM of protein MT2462. There is no background used in this round, as it is the first round of the selection. This is done to ensure that any aptamers that could be potential binders to MT2462 are selected for, without immediately weeding some of them out via the introduction of a background. Amplification is seen at around cycle eight.

During round two, the background is introduced. At this point, it is necessary to ensure that there is a difference between the pools of sequences exposed to MT2462 (target) and those not exposed to the protein (background). It is acceptable to introduce the background beginning in round two, because it is assumed that there is now an even distribution of aptamers following the first selection round and PCR amplification. As Figure A.1 shows, the target pool amplified at cycle eight, while the background pool amplified at cycle thirteen, indicating five cycles of separation between the two pools. At this point, it is necessary to conduct what is known as an enrichment round. An enrichment round uses the target aptamers from the current round, splits them into a new set of target and background pools, but still only exposes the target pool to the same concentration of protein as was used in the previous round. The purpose of this is to ensure that the target sequences that were just amplified can bind again to the protein, but this time only sequences with a higher affinity for the protein will bind, given that there should be more copies of the selected target sequences.

Round three of the selection is the enrichment round at a concentration of 250 nM of protein MT2462. As is evidenced by the PCR trace in Figure A.1, the target still amplified at
cycle eight, while the background amplified at cycle fourteen, yielding six cycles of separation. Based on this, it is clear that there is a greater amount of aptamers that are binding to the target, comparatively to the background.

Given that the selection enriched, it is possible to begin increasing the selective pressure on the pool of aptamers, meaning decreasing the concentration of protein MT2462 that the aptamers are exposed to, so only those aptamers that bind strongly to the protein are maintained in future rounds. During round four of the selection, the protein concentration was cut to 125 nM. As is seen in the trace, the target amplified around cycle eight, while the background amplified at cycle seventeen, yielding nine cycles of separation. The drastic difference between the amplification cycles of these two pools (target and background) indicates that there is also a significant difference in the amount of aptamers in each pool. The target pool can be assumed to have a higher number of aptamers, as they are most likely binding MT2462 and being carried on in the selection. Conversely, the background sequences are not exposed to MT2462 for binding, and therefore are fewer in number than those of the target. Again, because PCR traces are not quantitative, proof that binding is occurring is shown via bead binding assays and FPA. However, the qualitative results that the PCR traces provide are enough evidence for monitoring the progress of the selection.
Figure A.1. PCR traces for rounds one through four of the selection. The amount of protein used in each round is also noted.

Round four indicated great separation between the target and background pools of aptamers, as was discussed above. Based on this, it was decided that the evolving pools of aptamers should be analyzed for quantitative data on MT2462 to aptamer binding. A fluorescent tag, Cal-Fluor 610, was added to the forward primer for use during the selection rounds. The purpose of the fluorescent tag was so that the evolved pools could be analyzed by FPA.

In order to ensure that the fluorescent tag was not skewing the results of the selection, as monitored by the PCR traces, round four was conducted again, using the target pool from round three. The results of the round are indicated in Figure A.2 below. As can be seen, the PCR trace
for this round appears identical to that of round four without the fluorescent tag. The target amplified at cycle eight and the background at cycle seventeen.

The concentration of MT2462 that the target aptamers are exposed to was cut in half to 62.5 nM for round five. (Given the strong cycle separation in round four, followed by the reproducibility of the results once the fluorescent tag was added, an enrichment round was not necessary.) The target amplified at cycle eight, while the background amplified at cycle fourteen, yielding six cycles of separation. The background did amplify slightly earlier from the previous round, as round four had a background amplification at cycle seventeen. However, this is not necessarily concerning for the selection. The most likely reason for the increase in background amplification by three cycles is due to the selective pressure on the evolved pool. Recall that the target pool from the previous round is split in half to yield a new target and background pool of aptamers for the subsequent round. Thus, as the selective pressure on the evolved pools increases, the sequences should also be more and more identical. Thus, the background and target pools will initially appear to be slightly more homogenous than their counterparts in the previous round. However, an enrichment round should further amplify sequences that bind with high affinity to MT2462, decreasing the similarity between the two pools. It should also be noted that the round five PCR trace indicated some production of primer dimer that amplified around cycle seventeen. Given that this amplification was after the background amplification, it can be assumed that the background pool does have aptamer sequences in it, and its amplification is not due to primer dimer.

Round six of the selection was the enrichment round for 62.5 nM MT246. The target again amplified at cycle eight, while the background came up at cycle sixteen, yielding eight
cycles of separation. The selection enriched two cycles between rounds five and six, indicating that the difference between the two pools of aptamers grew.

Figure A.2. PCR traces for rounds four through six of the selection following the addition of Cal-Fluor 610 to the forward primer for the selection.

During round seven, the protein concentration of MT2462 was cut to 30 nM, further increasing the selective pressure on the evolved pools. This cut in concentration was shocking for the aptamers, as can be seen in the PCR traces in Figure A.3 below. Round seven shows the target amplifying at cycle twelve, while the background is amplifying at cycle fourteen, only yielding two cycles of separation. The most likely explanation for the vast decrease in aptamers in the evolved pools is due to the cut in the concentration of protein. There are now significantly
fewer proteins for the aptamers in the target pool to bind to, meaning that only the sequences that are potentially strong binders are sticking around. This drastic decrease in aptamer quantity is expected when the evolved pools are put under stress of this nature.

Round eight was used as an enrichment round to boost the evolved pool of aptamers, and increase the differences between the target and background aptamers. As is seen in Figure A.3, the target amplified at cycle fifteen while the background amplified at cycle eighteen. This round increased the separation between the target and background to three cycles. However, the inconsistency between the rounds in which the target is amplifying comparatively to round seven is of interesting note. It is important to remember that these selection rounds depend on the ability of the aptamers to come into contact with their target of interest. Although experimentally everything was kept the same between rounds seven and eight, the aptamers used for round eight were significantly diminished coming out of round seven and following the harsh cut in MT2462 concentration. Thus, it is expected that there would be some variability in the rounds at which the target amplifies, at least until the selection balances itself out, and the aptamers that are potentially strong binders for MT2462 begin to predominate the evolved pool again. Another thing to keep in mind is that the background has never exceed cycle fourteen, in this round, or any other round, indicating that the pool of aptamers in the background are not enriching overall throughout the selection. An enrichment of the background would indicate that the aptamers in each round have a higher affinity for some other reagent in the selection solution, rather than for the protein target itself, which has not been seen in this selection.

Round nine is yet another enrichment round for the 30 nM MT2462 concentration. The target sequences amplified at cycle ten while the background amplified at cycle sixteen, indicating six rounds of separation. At this point, it is clear that not only is the background not
evolving, but the target sequences are beginning to enrich, as the evolved pool amplifies earlier on in the cycles. Originally, in earlier selection rounds, the target pools were amplifying around cycle eight. Given round nine amplified at cycle ten, another enrichment round was conducted to not only further enrich the target sequences, but to restore the selection to a state similar to that of its original state prior to the increased selective pressure.

The enrichment round in ten shows the target amplifying at cycle eight again, while the background is amplifying at cycle sixteen, which enriches the selection by two rounds of cycle separation, for a total separation of eight rounds. At this point, it is clear that not only are the target sequences of the evolved pool preferentially binding to MT2462, but the background pool is staying consistent, with no enrichment evident.

Figure A.3. PCR traces for rounds seven through ten with the Cal-Fluor 610 tag on the forward primer.
Given the success of round ten, the protein concentration of MT2462 was again cut in half to 15 nM. As Figure A.4 indicates, the round eleven targets amplified at cycle ten, while the background pool amplified at cycle fourteen, yielding four cycles of separation. As was seen in rounds seven through ten, the background did jump up slightly (although did not exceed cycle fourteen), while the target did decrease slightly. Again, this is normal to see during a round where the selective pressure is increased on the evolved pools.

In order to restore the selection, an enrichment round was conducted for round twelve. As Figure A.4 shows, the target amplified at cycle eight, while the background amplified at cycle fourteen, yielding six rounds of separation. Notably, the background showed no enrichment, while the target sequences showed enrichment as the selection stabilized. At this point, the selection was terminated.

![PCR traces for rounds eleven and twelve of the selection with the Cal-Fluor 610 tag on the forward primer.](image)

**A.2 FPA Controls**

**Negative Controls**

Figure A.5 below is the negative control for the MT2462 buffer. In order to ensure that the changes observed in anisotropy were in fact due to the protein binding to the aptamers, rather
than the salts from the buffer altering the fluorescence of the Cal-Fluor and TAMRA-5 fluorophores, a control experiment was conducted in which only the MT2462 buffer was added to the aptamer pool, and changes in fluorescence were monitored. As is evident in Figure A.5, there are no notable changes or major fluctuations between the fluorescence readings as the buffer is titrated into the aptamers.

Figure A.5. Anisotropy values produced by the evolved pool of aptamers as the MT2462 buffer was titrated in at increasing concentrations.

Figure A.6 displays data for what is known as the “Randomer” control. In this control, a random aptamer with the Cal-Fluor 610 tag on the primer was exposed to increasing concentrations of MT2462. The data indicates that there was no significant variation in the anisotropy for this aptamer, indicating that MT2462 was not binding to the aptamer. This data lends credit to the idea that the evolved pool and the individual sequences being tested are not simply binding to MT2462 randomly, but have in fact been selected throughout the course of the experimentation to have a preferential affinity for MT2462.
Figure A.6. Anisotropy values produced by a random aptamer as MT2462 was titrated in at increasing concentrations.
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