

University of Central Florida

**STARS**

---

Honors Undergraduate Theses

UCF Theses and Dissertations


---

2016

## **Aptameric Sensors: In Vitro Selection of DNA that Binds Bromocresol Purple**

Derek B. Miller

*University of Central Florida*

 Part of the [Biochemistry Commons](#), [Diagnosis Commons](#), [Food Microbiology Commons](#), [Molecular Biology Commons](#), and the [Other Analytical, Diagnostic and Therapeutic Techniques and Equipment Commons](#)

Find similar works at: <https://stars.library.ucf.edu/honorsthesis>

University of Central Florida Libraries <http://library.ucf.edu>

This Open Access is brought to you for free and open access by the UCF Theses and Dissertations at STARS. It has been accepted for inclusion in Honors Undergraduate Theses by an authorized administrator of STARS. For more information, please contact [STARS@ucf.edu](mailto:STARS@ucf.edu).

---

### **Recommended Citation**

Miller, Derek B., "Aptameric Sensors: In Vitro Selection of DNA that Binds Bromocresol Purple" (2016). *Honors Undergraduate Theses*. 112.

<https://stars.library.ucf.edu/honorsthesis/112>

APTAMERIC SENSORS: IN VITRO SELECTION OF DNA THAT BINDS BROMOCRESOL  
PURPLE

by

Derek Miller

A thesis submitted in partial fulfillment of the requirements  
for the Honors in The Major program in Biomedical Sciences

in the College of Medicine

and in the Burnett Honors College

at the University of Central Florida

Orlando, Florida

Fall Term 2016

Thesis Chair: Dr. Kolpashchikov Ph.D.

## **Abstract**

Aptamers being used as sensors is an emerging field that has capabilities of being tomorrow's diagnostic tools. As aptameric sensors have become more popular, their visualization systems have been limited. The majority of today's aptameric sensors require expensive machinery such as a fluorometer in order to visualize results. We propose a system that will cut the need for instrumentation and be detected via the naked eye. With the selection of an aptamer to bind the pH indicating dye bromocresol purple (BCP) this may be achieved. When rendered active, the binding towards BCP will facilitate a color change from yellow to purple or vice versa. Previous studies have shown albumin contains the ability to facilitate this role and we now intend to use a DNA aptamer to achieve this as well. The BCP aptamer has the potential to serve as a signaling domain to any already selected aptamer thus making it a universal tool for both research and diagnostic measures. We have found that an alternative structure-switching systematic evolution of ligands by exponential enrichment (SELEX) method which left the dye unaltered was not sufficient for selecting an aptamer. We believe that a classical SELEX will enable us to select an aptamer that may be used to accomplish this role as a universal visual detector.

## **Dedication**

I would like to dedicate this thesis to Martin Osteen. Without his help and compassion in teaching me all the fundamental techniques used in this experiment I would have been a very lost puppy. I am extremely grateful for Martin's mentoring and genuine drive to help myself and others excel.

## **Acknowledgements**

I would first like to acknowledge Dr. Gerasimova due to the motivation she instilled and sparked in me during her biochemistry course. Without her I would have never been able to achieve this unforgettable learning experience throughout my stay at UCF. Secondly, Dr. Kolpashchikov served as my mentor and taught me the fundamental ethics behind why we do research. This opened up a new perspective on the importance of research for me. I want to thank Dr. Kolpashchikov for being the humble biochemist that he is and for the respect he has for each lab member. Through his guidance and trust, my journey while working in the Kolpashchikov lab will greatly enhance my future career no matter where I end up. I also want to thank all lab members. You all have given me the support necessary to continue with a project that seemed to never end. Lastly I want to thank my committee members Dr. Chai and Dr. Torres for their cooperation and patience throughout this process.

## Table of Contents

List of Figures .....	<b>Error! Bookmark not defined.</b>
Introduction: .....	1
Applications: .....	7
Results and Discussion .....	12
DNA Library Design: .....	12
Revised SELEX Design .....	20
Methodology:.....	24
Library Construction:.....	24
SELEX procedure: .....	24
PCR/Separation: .....	26
DNA Extraction: .....	27
Stringency:.....	29
Transformation:.....	30
Conclusions/Future Work: .....	32
References .....	33

## List of Figures

Figure 1M. Stojanovic and D. Kolpashchikov, Modular Aptameric Sensors. J. AM. CHEM. SOC. 2004, 126, 9266-9270 .....	4
Figure 2 D. Kolpachshikov, Binary Malachite Green Aptamer for Fluorescent Detection of Nucleic Acids. J. AM Chem. Soc., 2005, 127 (36), pp 12442–12443.....	5
Figure 3 BCP Albumin test: 40mM Citrate-Phosphate buffer used for 1-4, 50µM BCP for 1-4, 30µM HSA for 2 & 4. Showing that upon addition of HSA there is a color shift that is noticeable by the naked eye.....	9
Figure 4 Biocapture strand complimentary to the library with the green oval representing streptavidin/agarose beads in the column.....	13
Figure 5 Top represents the target conformation in different pH's. Bottom represents the competing complex formation on the streptavidin column when the target is presented.....	14
Figure 6 Library enrichment through comparing product yield from each round vs. target concentration from each round.....	15
Figure 7 A. Rd 14 product wash (library annealed with Biotynilated complimentary sequence), 10 washes done at 28°C, 3 target washes at 4 µM BCP. B. Rd 15 target concentration at 2 µM. C. Rd 18 target concentration at 1 µM BCP. D. Rd 19 target concentration at 1 µM BCP. ....	16
Figure 8 Library truncation at round 17 with 2 µM BCP. Labels correlate with Figure 6 descriptions.....	17
Figure 9 Osteen M. Library w/ 3 way junctions due to the pattern of purines(R) and pyrimidines(Y) .....	18
Figure 10 One of the aptamers that was sequenced using Fig 6. Library, example of self-folding .....	19
Figure 11 Adopted from Osteen M., Elution profile from aptamer ordered signifying that the selection was not successful. The Osteen 3 way junction library was used. ....	19
Figure 12 Round #21. 10 extra washes were implemented to see if the elution profile would clear up. Thus after doing so many rounds and still unable to greatly reduce the variability of the library we decided to start over with a revised protocol.....	20
Figure 13 Large scale amplification using dPAGE to retrieve DNA library. ....	21

Figure 14 Native Gels. A) Rd 1 Washes from left to right( 5<sup>th</sup>, 10<sup>th</sup>, and 15<sup>th</sup> buffer washes at 28°C, followed by the 20<sup>th</sup> wash at room temp.) Target washes were 100 µM BCP. B) Rd 3 Same labels as Rd 1. C) Rd 9 5 extra washes at 28°C (5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, and 20<sup>th</sup> washes) ..... 22

Figure 15 Osteon M. Excised band is circled. This pool of aptamers was used for the sequencing and enabled us to acquire the information mentioned above regarding issues with the structure switching method. .... 31



## **Introduction:**

Aptameric sensors are a growing area of research due to their certain advantages for diagnostic testing. An aptameric sensor can be designed to recognize signal analyte or target of interest. This approach is similar to that of antibody-based technologies which are the most common and widely used in bioanalytical assays. Antibodies are proteins obtained through in vivo processes and have high specificity towards their target. In doing so, they can be used as diagnostic tools which will give a detectable signal promptly such as ELISA's used in today's pregnancy tests. Aptamers can be designed and used for the same purpose while having numerous advantages over antibodies. For starters, the selection method is done in vitro as opposed to the in vivo process that antibodies require, thus eliminating the use of an animal to produce products. The antibody targets must also be able to cause an immunogenic response in the animal they are injected into. This inherently narrows the variety of targets to choose from. Aptamers are also relatively cheaper to make by the use of a nucleotide synthesizer once the aptameric sequence has been established. Furthermore, antibodies are proteins and are therefore fragile to work with and have the potential to aggregate or degrade easily. Nucleic acids on the other hand, especially DNA aptamers, are far more stable and thus will have a longer shelf life as diagnostic sensors.

Short single stranded nucleic acid based aptameric sensors can be obtained through systematic evolution of ligands by exponential enrichment (SELEX). SELEX is an in vitro selection

technique designed to obtain DNA with high binding selectivity towards a desired target molecule(1). A single stranded oligonucleotide is used, either DNA or RNA and is referred to as an “aptamer”, derived from the Latin term “*aptus*” meaning to fit. The SELEX process follows simple techniques in which oligonucleotides are eluted off from a pool of exponentially rich sequences referred to as a Library. Through numerous rounds this creates a survival of the fittest environment throughout the process. In doing so, a roughly 100-bp oligonucleotide library is generally chosen with known 5’ and 3’ terminal sequences to be used for PCR amplification along with a random region somewhere in between which is used to dictate the binding of the target molecule. This random region exponentially increases the variability of strands in the library pool with a 30 nucleotide random region giving  $4^{30}$  possible sequences. Theoretically with such a variety of aptamers in a library pool, the library can be systematically narrowed down to bind to a wide variety of targets. The process of continued rounds will systematically enrich the library by narrowing down the highly varied pool to only the aptamers that contain the desired affinity towards the selected target. Aptamers will interact with the target via stacking, hydrogen bonding, Van Der Waals, and electrostatic interactions. These interactions will facilitate the folding that the aptamers will engage in around the target thus forming a stable yet tight complex.

Some of the first aptamers that emerged were from the works of both the Gold and Szostak labs in 1990 where they published RNA aptamers that bound T4 DNA polymerase and organic dyes, respectively(1, 2). The Szostak lab coined the term aptamer while the Gold lab coined the

SELEX procedures. Aptamers can be selected to bind to a wide variety of targets such as small organic molecules (organic dyes), proteins (T4 DNA polymerase), cancer cells, and viruses (1-4). The field of aptamer research has grown tremendously since the work of both Gold and Szostak, and has provided us with numerous applications for these oligonucleotides. For example, aptamers are beneficial in applications where target information is not available or difficult to obtain. Therefore they can be designed with very little information of the target molecule. This allows for a selection of a wide range of desired targets.

Not only can aptamers provide high affinity but they also can display remarkably high specificity. RNA aptamers have been described to be able to differentiate between L and D isomers of adenosine, selecting for the L isomer up to 1700 fold(5). Molecules such as caffeine and theophylline which differ by only a methyl group are also highly distinguishable with existing aptamers that have 10,000 times more affinity towards theophylline over caffeine(6). In addition, aptamers such as the Malachite Green aptamer have been able to show both high affinity and specificity being used as a biosensor in both modular and binary designs (7, 8). Modular aptameric sensors are a two part system consisting of two aptamers joined together where one aptamer is used for recognition and the other for a signal display. An example of such is the malachite green aptamer attached to a Flavin mononucleotide (FMN) aptamer through a transducer module that acts as a bridge between the two aptamers [*Fig1.*].

Figure 1M. Stojanovic and D. Kolpashchikov, *Modular Aptameric Sensors*. J. AM. CHEM. SOC. 2004, 126, 9266-9270

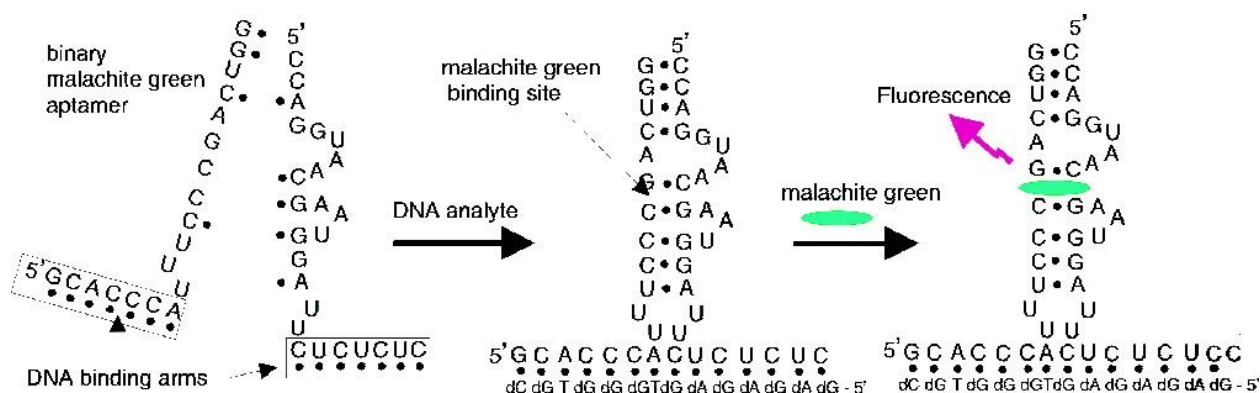


Here the FMN domain is the target recognition domain while the malachite green aptamer is the signaling domain. Only in the presence of FMN do both modules become active. Once the signaling domain (MGA) is activated it can readily bind malachite green and produce an increase in fluorescence which can then be measured. This MG-FMN modular aptamer proved to have a 30-50 fold increase in fluorescence when FMN is present. Similar modular aptamers have recognition domains that target theophylline and ATP, respectively(6). Another example of a similar design is the recently selected spinach aptamer that is now being used as a reporting module in the design of modular aptameric sensors (9). The spinach aptamer is the first RNA sequence that is able to mimic green fluorescent protein (GFP), making it possible to track in vivo processes via fluorescence. When the aptamer is in the presence of DHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone) a chromophore of GFP, the molecule produces a fluorescence that can be measured. With this, the aptameric sequence gene can be cloned into a pET-28c vector under the control of a T7 promoter and be expressed at high concentrations in vivo. Applications such as measuring ADP concentration in E.coli cells using a modular approach

where a recognition domain( ADP aptamer) is joined with the spinach aptamer via a transducer module and is able to fluoresce in vivo(10). This approach was used to visualize how ADP concentrations differ in cells when exposed to varying carbon sources.

Another approach used for the design of aptameric sensors is a binary system. For example, the MG aptamer can be split into two sequences with specific binding arms designed to bind the target analyte therefore now rendering the aptamer active(7). When the desired analyte is present, the aptamer reforms through complimentary base pairing and can now effectively bind to malachite green and produce an increase in fluorescence.

**Figure 2 D. Kolpachshikov, Binary Malachite Green Aptamer for Fluorescent Detection of Nucleic Acids. J. AM Chem. Soc., 2005, 127 (36), pp 12442–12443**



Aptameric sensors described above have an important role in today's diagnostic research and are the foundation of the Bromocresol Aptamer SELEX project. It is also important to note that aptamers have applications beyond being used as biosensors. Mucagen® for example, is the first FDA approved aptamer for the treatment of macular degeneration (11). There is a continued interest among researchers for biological based drugs such as Mucagen for minimal

toxicity treatments. Potential aptamer-based drugs for cancer treatments are now also in clinical phase trials (12). Aptamers can also be used as drug delivery systems. They can carry cytotoxic drugs specifically to cancer cells through their high specificity and save the normal cells from toxic drugs that are routinely used during cancer treatments (13).

### **Applications:**

With prior experimental knowledge from the various aptameric sensors mentioned above, we propose to select a DNA sequence that binds an organic dye, bromocresol purple. This aptamer will serve to not only bind BCP, but also facilitate its color change upon binding. This aptamer can then be used to design colorimetric sensors as it was described above for modular and binary designs. Thus far, the most common method to visualize target binding of an aptameric sensor has been through indirect measures that require expensive instrumentation such as a fluorometer. Expensive machinery has been a major disadvantage for underdeveloped countries that are in need of reliable and stable diagnostic tests. With the proposed bromocresol purple aptamer, a color change would be directly observed with the naked eye, thus canceling out the need for expensive instrumentation for visualization. With this application, the sensor could be used universally to test for a variety of targets, serving as the signaling domain.

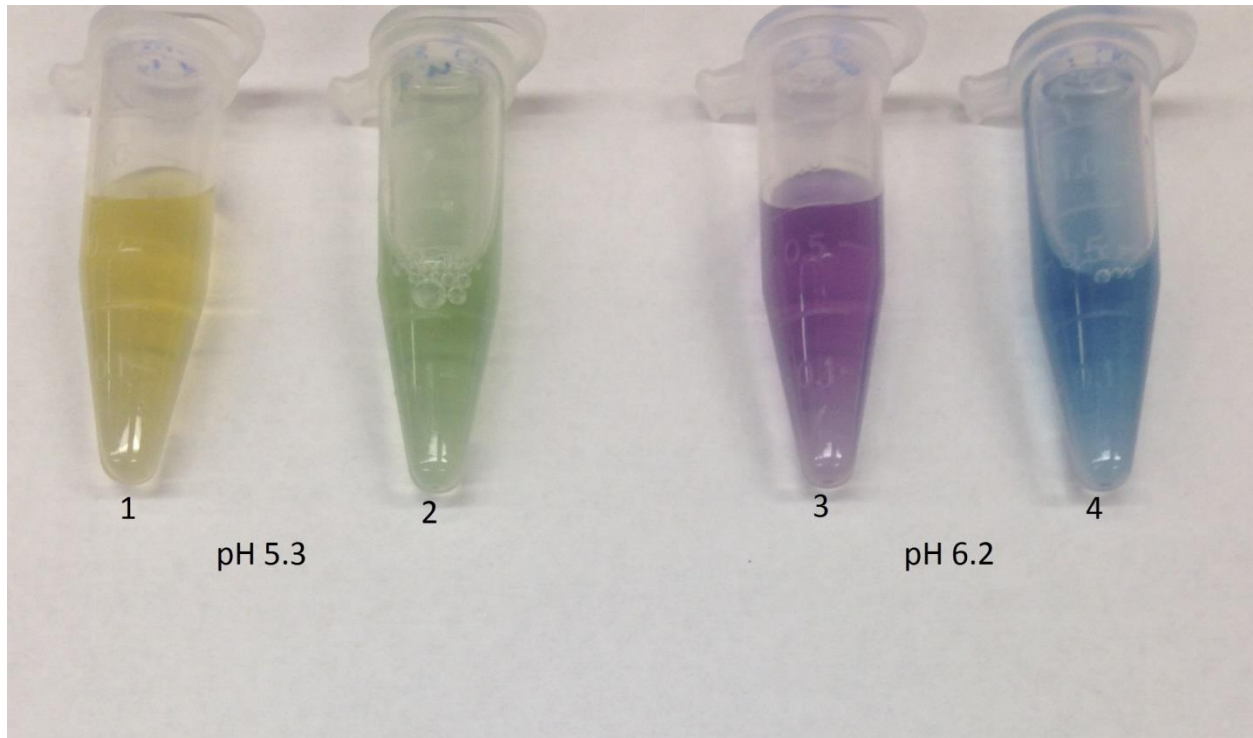
This would be a tremendous advancement for diagnostic testing throughout the world, cutting down visualization time and therefore decreasing cost. With the previous aptameric sensors mentioned above, the possibilities for this aptamer could change the way we visualize many experiments in the research world. The target, BCP, is a small organic dye which is a yellow color below pH 5.2 and a purple color in its planar form above pH 6.8. The intent is that when a selected aptamer effectively binds BCP at pH 5.2 in its yellow form it will bind in such a way that it facilitates it to change into its planar structure thus changing the color from yellow towards purple. This may be accomplished vice versa also. Starting from the purple planar conformation then changing it towards its yellow tetrahedral conformation. This will all be done

without changing the pH of the solution and occur only in the presence of the activated aptamer.

Based on literature, other groups and previous diagnostic measures have used human serum albumin (HSA) to bind BCP and create a similar color shift described above that can be used as a quantitative measure (14-16). For example, BCP has been used as a visualization tool for the measurement of albumin in the serum(17). There are two hydrophobic binding pockets for BCP on the HSA which facilitate the proton exchange from both planar and tetrahedral structures of bromocresol purple depending on the pH of the solution. Therefore, it seems possible that an aptamer can achieve the same role that HSA has towards BCP. If an aptamer is selected that can alter the structure of BCP similar to HSA then the possibilities for its use could be legitimate.



**Figure 3 BCP Albumin test: 40mM Citrate-Phosphate buffer used for 1-4, 50 $\mu$ M BCP for 1-4, 30 $\mu$ M HSA for 2 & 4. Showing that upon addition of HSA there is a color shift that is noticeable by the naked eye.**



If color results like shown above are possible with the BCP aptamer, then detection of gene fragments or single nucleotide polymorphisms from amplified DNA would yield results fairly quickly and, more importantly, visibly without instrumentation. The aptamer can then be designed to test for a large variety of sequences or gene fragments as long as the binding portion of the BCP aptamer is left intact. For example, if the amplified analyte is present, the BCP aptamer can be designed with flanking arms that are specific to the analyte therefore bringing the stem loop structure into play and increasing affinity towards BCP. This approach is similar to the split malachite green aptamer mentioned above (Figure 2). Other approaches

such as the split DNA enzyme (Deoxy ribozyme) for single nucleotide polymorphism (SNP) was used to form a colored product when the analyte was present have been documented(18).

Once again the most important application for the BCP aptamer that could prove to be universally accepted is the use of modularic systems (Figure 1) as described above in the introduction. By designing a recognition aptamer attached to the BCP aptamer, cooperativity can be achieved where if the recognition complex isn't formed then neither is the BCP aptamer complex. In doing so, virtually all selected aptamers could be potentially attached to the BCP aptamer and serve as its visual aid of reference. To enhance specificity, the splitting approach like described in the binary malachite green aptamer (Figure 2) can also be employed here as well. The designed modular or probe like aptamer could be split in half and have flanking arms attached that are specific to amplified genetic DNA or bacterial RNA. Another possible application could be the use of the aptamer in solution with the popular deoxy ribozymes. The BCP aptamer could be used as a detector for the resulting cleaved products from the deoxy ribozymes. This can be designed to where the DNA enzyme has cleaved its specific site and the cleaved portion can be detected visually with the BCP aptamer, thus canceling out the step for visualization via expensive machinery. This idea can be employed for many different areas of research where the deoxy ribozyme is used for diagnostic testing.

We intend to use DNA for this BCP aptameric sensor as it is easier to work with and more stable than RNA while still maintaining its high affinity and specificity for the target. This will also allow for use in extreme environments as the DNA can be easily renatured as opposed to

antibodies which must be in a stable environment for effectiveness due to their protein structure. Antibodies are used as disposable molecular sensors while DNA aptamers can be reused through the denaturation and renaturation process. This could prove to be beneficial in countries with limited resources. This reusability will save both time and money.

## **Results and Discussion:**

### DNA Library Design:

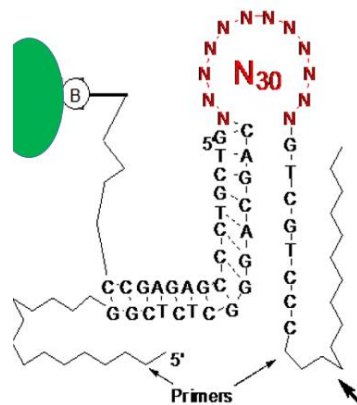
The approach we took was different from classical SELEX where the target is immobilized or allowed to interact with the DNA in solution for a period of time. The potential altered structure of BCP when attached to an agarose bead for example may not resemble how the dye interacts with the DNA in free solution along with affecting its color changing properties. With this in mind we decided to use a Structure Switching method based on literature in which some groups have accomplished selecting aptamers using this sort of method (19, 20). These methods published show results of aptamers differentiating glucose from galactose and proved to be an alternative method from classical SELEX geared towards targeting difficult small molecule targets such as BCP. This would also minimize the potential to select for aptamers that bind to the dye along with its conjugated complex as opposed to just the dye itself.

A single strand of DNA 94nt long was used as the starting library to select for BCP binding. The oligonucleotide was designed to form a predetermined hairpin loop in which the loop consists of a 30nt random region. There are also constant primer regions on each side of the stem to allow for PCR amplification of the oligonucleotide which can then be removed once selection is complete. This 30nt random region allows for  $4^{30}$  possible oligonucleotides in the starting DNA pool. These possibilities will be narrowed down in the course of SELEX procedure to the aptamers that have the highest affinity and specificity towards the dye using SELEX. Through multiple rounds of SELEX the pool of aptamers will become more enriched and have less

variation as the weak or unbound aptamers will be washed away keeping only the highest affinity aptamers to continue to the next round.

Our purpose here was to attempt a newer method that left the target unaltered in anyway. This method involves the DNA aptamer immobilized in the column through complimentary base pairing while the target is allowed to flow through. The idea is that the target will interact with the aptamer in such a way that it favors the stem reformation therefore outcompeting the binding it currently has while attached to the column. The Library contains a sequence within the constant region referred to as the BioCapture region. This Biocapture region is complementary to another sequence that is biotinylated and therefore attracted towards the streptavidin on the agarose beads.

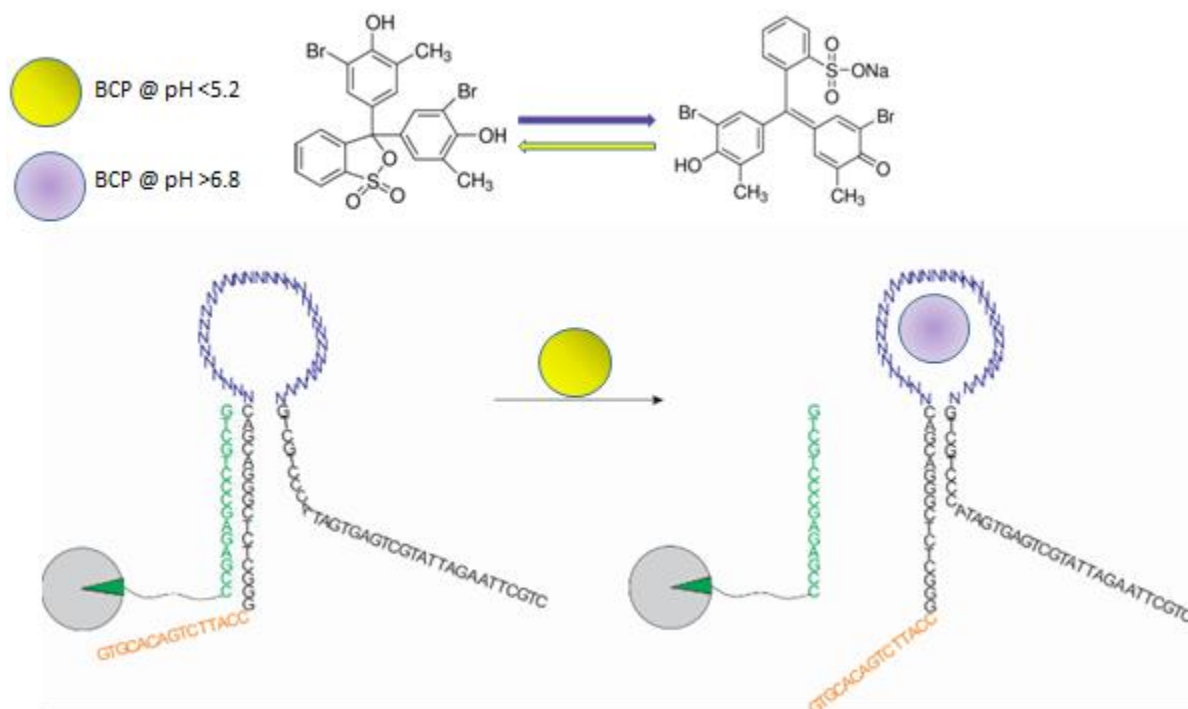
**Figure 4 Biocapture strand complimentary to the library with the green oval representing streptavidin/agarose beads in the column.**



Thus the aptamer and the complimentary Biocapture strand are heated and then allowed to anneal so they can interact with each other before being added to a streptavidin column

(streptavidin in Green). Once the target is presented, the structure switching will take place and favor the original stem loop structure along with the target bound somewhere in the random region, therefore eluting off the desired aptamers. (Fig. 4)

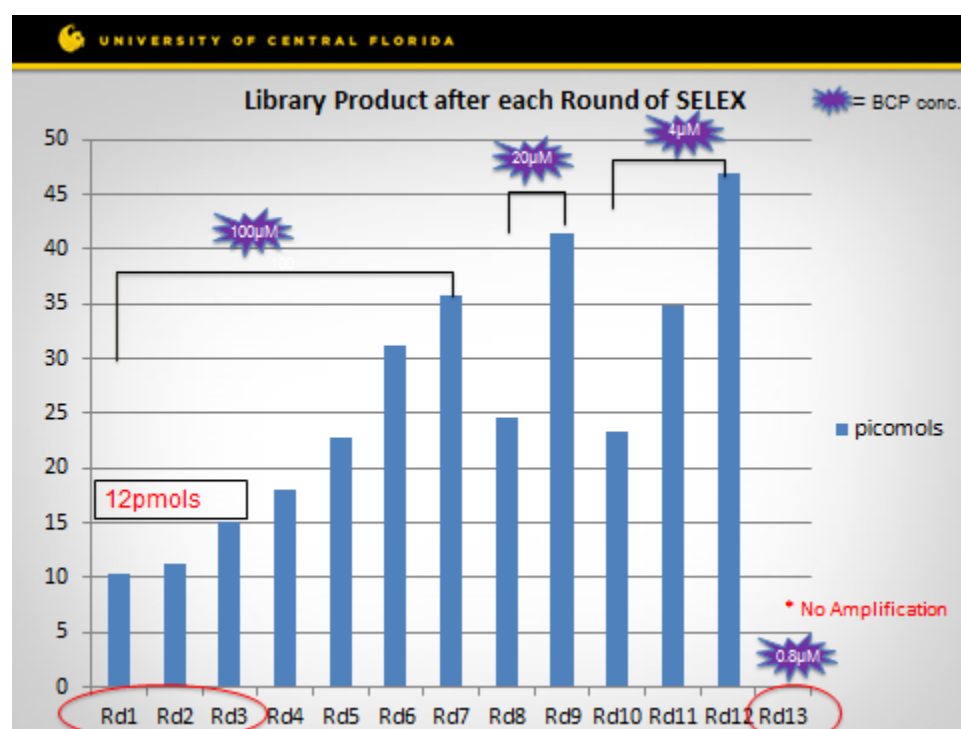
**Figure 5** Top represents the target conformation in different pH's. Bottom represents the competing complex formation on the streptavidin column when the target is presented.



To further narrow the library throughout the rounds, target stringency was used. In doing so the target concentration was systematically dropped after every 2 rounds. To monitor the enrichment of the library we averaged the product retrieval from the first 3 rounds and set it as a baseline for each consequent round. This meant that after round 3, the same amount of DNA was loaded in the column each round and the product retrieval was then compared. With the

library becoming more enriched with strands that bind BCP there will be a higher product yield with each round. After the BCP concentration was dropped we would see a drop in product yield. This would be followed up with an increase in product yield with the next round at the same target concentration. As the figure shows, there is a clear pattern in the product yields vs. the concentration of BCP. This was able to tell us that the library was becoming less and less diverse.

**Figure 6 Library enrichment through comparing product yield from each round vs. target concentration from each round.**



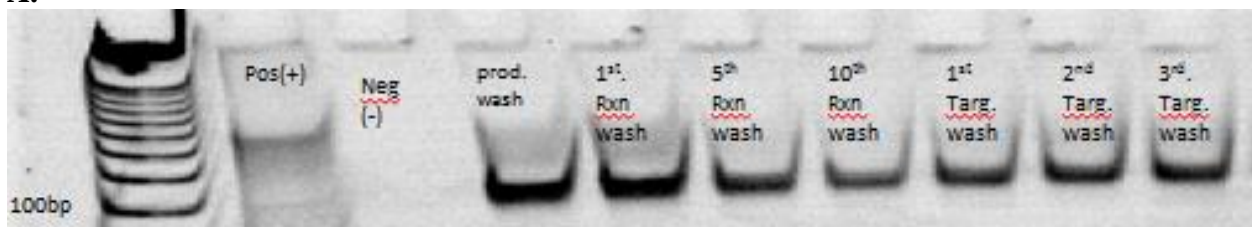
As we reduced BCP concentration down to 0.8 µM, there was no longer any amplification. This proved to be too drastic and low of a drop in target concentration. I was able to bring the concentration back to 4 µM and retrieve amplification in order to continue SELEX. Eventually I

was able to drop the target concentration to 2  $\mu\text{M}$  but this proved to be unnecessary due to other complications mentioned below (Figure 6, 7). Washes at elevated temperatures were also implemented in order to elute off any partially bound aptamers to the biotinylated column strand. These stringencies proved to be beneficial up until a certain point. The washes were done at 28  $^{\circ}\text{C}$  starting at round 10. At this part of the experiment the washes became very difficult to clean up and the library seemed to have been enriched in a way where there were always strands steadily flowing off the column whether the target was presented or not.

At round 14 we began analyzing each eluted fraction after PCR amplification by gel electrophoresis (elution profiles), which helped to visualize how the library was reacting throughout the washes without and with the target BCP. As shown from the elution profiles below there were always DNA strands steadily flowing off the column throughout all washes. This showed us that the aptamers coming off the column during the target washes were unfortunately not specific towards BCP. (FIG. 6)

**Figure 7** A. Rd 14 product wash (library annealed with Biotynilated complimentary sequence), 10 washes done at 28 $^{\circ}\text{C}$ , 3 target washes at 4  $\mu\text{M}$  BCP. B. Rd 15 target concentration at 2  $\mu\text{M}$ . C. Rd 18 target concentration at 1  $\mu\text{M}$  BCP. D. Rd 19 target concentration at 1  $\mu\text{M}$  BCP.

**A.**





**B.**



**C.**

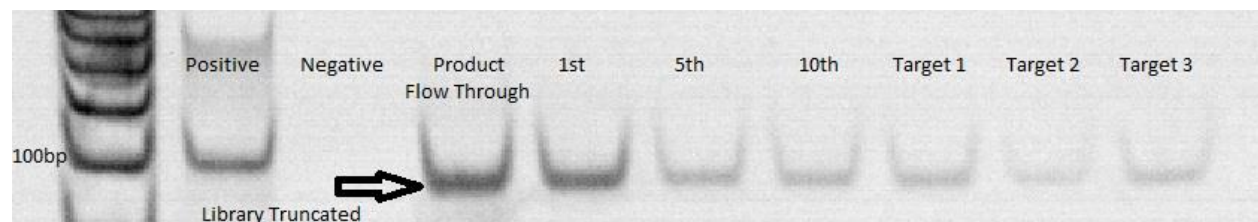


**D.**



This could be an issue with the structure switching method as the binding of the aptamers to the column may favor random detachments throughout the washes whether the target was present or not. We also noticed that the aptamer was truncated when visualizing the gel images. The library was initially 94nt and now has seemed to be shortened by several nucleotides.

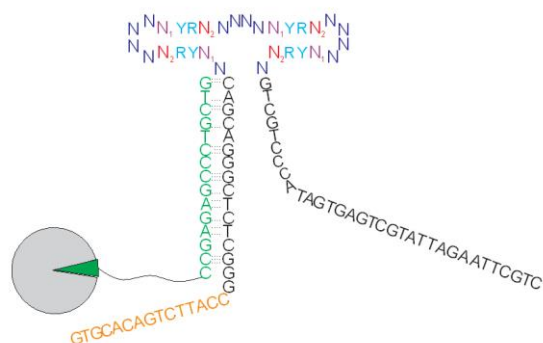
**Figure 8 Library truncation at round 17 with 2  $\mu$ M BCP. Labels correlate with Figure 6 descriptions.**



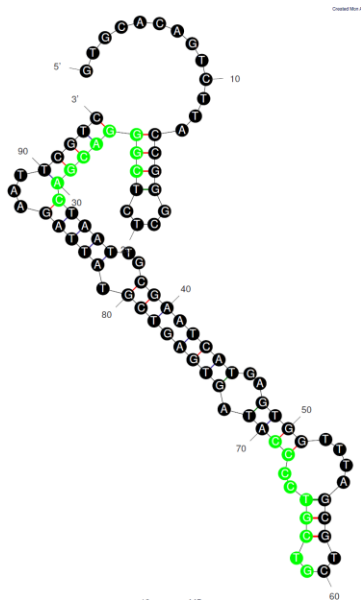
This truncation proved to be in the BioCapture region which is supposed to be the main player in adhering to the immobilized complementary strand in the column. This could explain the dirty washes in late rounds of the selection. The large number of random regions in the library could also be another factor why the library continued to be so diverse after so many

rounds. Although the library was adopted from the previous studies where the structure switching method proved to be successful in selecting aptamers towards sugars and a few amino acids, this experiment with BCP was not as successful (20). When we cloned a pool of the aptamers from a different library that has a similar design and was also experiencing truncation, we noticed that the Biocapture sequence had indeed been truncated by 2-3 nucleotides. The aptamer sequences also proved to be parasitic, meaning they would fold onto themselves and elute off at random times. The folding program used for each sequence was MFold. Each sequenced aptamer was ordered. The elution profiles of each showed no difference when the target was introduced, indicating the aptamers were not selective for the target. Fluorescence experiments were also done on the sequences with results coming back inconclusive as well.

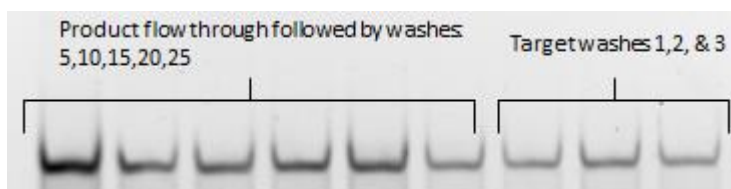
**Figure 9** Osteen M. Library w/ 3 way junctions due to the pattern of purines(R) and pyrimidines(Y)



**Figure 10** One of the aptamers that was sequenced using Fig 6. Library, example of self-folding

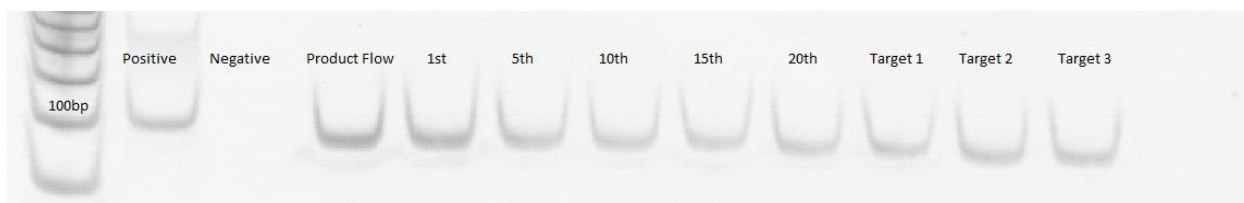


**Figure 11** Adopted from Osteen M., Elution profile from aptamer ordered signifying that the selection was not successful. The Osteen 3 way junction library was used.



Problems from this first attempt could have been from a variety of issues including the truncated bases and the design of the structure switching method used. These results were from a different library that used the same method described above (fig8). Once that result was obtained it, we decided to revise a new strategy.

**Figure 12 Round #21. 10 extra washes were implemented to see if the elution profile would clear up. Thus after doing so many rounds and still unable to greatly reduce the variability of the library we decided to start over with a revised protocol.**

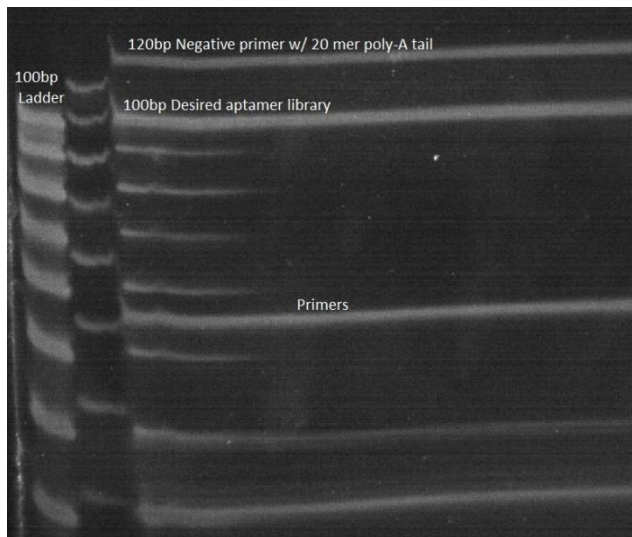


### Revised SELEX Design

The major reasons why we decided to revise a new SELEX design included the truncation of the biocapture region in the previous library and the continuous elution of strands from the column. These obstacles rendered it difficult to further narrow the library past a certain point. Revisions were made in regards to the Biocapture strand being truncated. With the previous design being truncated and therefore weakening its interaction with the column, a longer forward primer was used. This primer spans the whole length of the Biocapture region, eliminating the possibility of amplifying any truncated products. Also, the revised anti-sense biotinylated strand was elongated so that if it was not complimentary to the aptamer Biocapture strand then it would fold on to itself, rendering it inactive for nonspecific interactions. This was done to minimize any nonspecific interactions the library may have towards the immobilized anti-sense biotinylated strand attached to the column. Higher temperature washes were implemented starting from the first round as opposed to later rounds in our first attempt. We implemented 20 washes at 28 C° which was then followed by 5 room temperature washes to allow the column to equilibrate. The 5 room temperature washes

were done to ensure that the aptamers were not simply coming off the column due to the change in temperature. In addition, a poly Adenosine 20mer tail was attached to the negative primer so that the desired strand was able to be separated by dPAGE.

**Figure 13 Large scale amplification using dPAGE to retrieve DNA library.**

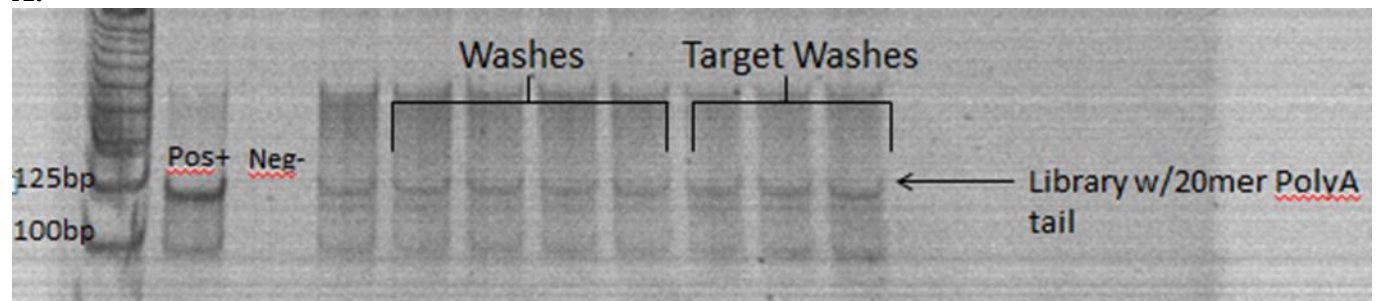


With this attempt we kept a close eye on how the library was reacting by performing elution profiles from the very start. We decided to keep the concentration of BCP stable throughout until there was clean lanes from the washes. Unfortunately after several rounds the elution profiles seemed to have the same problems as before (Figure 13). There were still strands coming off the column in a consistent manner, no matter how many washes we implemented. For round 9 we implemented an additional 5 washes to see if the wash lanes would clear up. As shown below in the elution profiles, it is apparent that the column was still eluting off random strands that were not selective for the target. At this point it was suggested to revise a complete new protocol of selection.

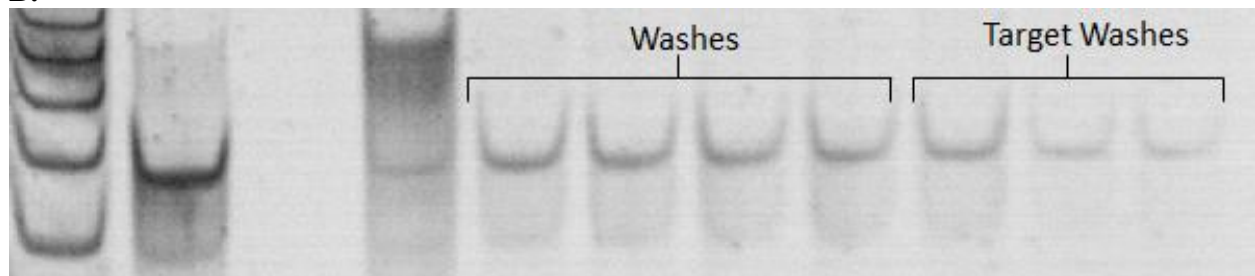
Again there may be a variety of reasons why we were not successful using the structure-switching method. It may be that the 30 mer random region was too diverse for this method of selection creating a variety of strands that come off the column consistently making it hard to isolate any particular aptamer that binds the target. At this point it seemed the structure switching methods described in this experiment were not working as intended and made a decision to revert to a more classical approach. For the next attempt, the dye will be modified in a way that allows it to be attached to an agarose bead in a classical SELEX style method. This method will allow the target to be immobilized in the column while the library can be washed through, essentially reversing roles in our previous method. This will eliminate the constant flow of DNA from the column creating a library that is still diverse.

**Figure 14 Native Gels.** A) Rd 1 Washes from left to right( 5<sup>th</sup>, 10<sup>th</sup>, and 15<sup>th</sup> buffer washes at 28°C, followed by the 20<sup>th</sup> wash at room temp.) Target washes were 100 µM BCP. B) Rd 3 Same labels as Rd 1. C) Rd 9 5 extra washes at 28°C (5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, and 20<sup>th</sup> washes)

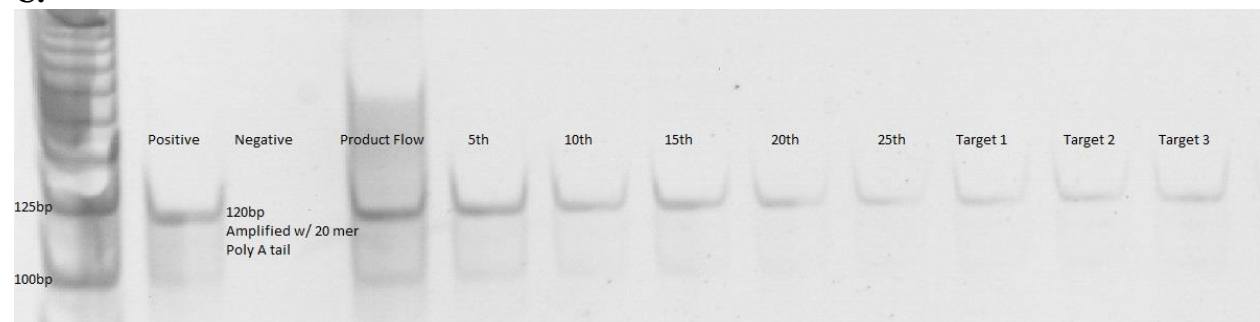
**A.**



**B.**



**C.**



## Methodology:

### Library Construction:

The ssDNA library was designed to form a hairpin loop structure with the loop being a 30nt random sequence meaning that the amount of variance in this initial pool is  $4^{30}$ . The library *P-lib3+* sequence used is : 5'-GTG CAC AGTC TTA CCG GGC TCT CGG GAC GAC NNN NNN NNN NNN NNN NNN NNN NNN NNN GTC GTC CC ATA GTG AGTC GTATT AGA ATT CGT C-3'. The underlined portions are primer regions for PCR amplification. The random region (Indicated by the 30 N's) is the loop portion of the secondary structure and is what will be enriched to correctly bind BCP.

### SELEX procedure:

A streptavidin agarose column will be used to immobilize the library until the target is introduced thus facilitating the elution of the enriched library through each round. Streptavidin consists of four identical subunits each of which has high affinity towards biotin. This tetrameric protein is isolated from actinobacterium *Streptomyces avidinii* and is covalently linked to agarose beads thus being immobilized in the column once the slurry is poured. Streptavidin has a surprisingly high affinity for biotin making it very effective in selecting structures with the biotin tag. In order for the DNA library to be immobilized in the column and be released only when target is presented, a biotinylated sequence of anti-sense DNA is used. The Bio-Capture sequence is complementary to the long arm of the library therefore competing with the stem from forming when it is annealed after denaturation. We used 0.1nmol of the library (P-lib3+) and incubated it at 95° C for 5 min with 0.125nmol of the Bio-Capture sequence all in 250µL of SELEX reaction buffer. The SELEX reaction buffer consists of 10mM



MgCl<sub>2</sub>, 150mM NaCl, 25mM Acetic Acid, 1% DMSO, and brought to a pH of 5.2 to maintain conditions where BCP is predominantly in its yellow form.

Once the mixture is heated and denatured for 5 min the solution will be allowed to cool down for 10 min to allow annealing to take place. The resulting structure will now have the Bio-Capture sequence base paired to the left binding arm as it out competes the base pairing between the stem of the library. This also leaves the random loop region open to target binding. A streptavidin agarose column is constructed using a micro biospin chromatography column in which 250µL (1-3mg biotinylated BSA/mL resin) is pipetted into the column. The column is then washed 5 times with reaction buffer to ensure it is equilibrated with the same buffer the oligonucleotide mixture is suspended in. The oligonucleotide mixture containing the Bio-Capture strand annealed to the library is allowed to flow through the column twice. This will help increase the amount of bound nucleotides to the column through streptavidin biotin binding. The column is then washed 10 times to elute off any unbound oligonucleotides or any remaining Bio-Capture strands that did not anneal to the library. Extra washes were implemented as needed depending on the outcome of elution profiles. Then 750µL of the target buffer separated in 250µL portions is allowed to flow through the column. The target buffer is identical to the reaction buffer but contains the target BCP. The initial target concentration was set at 100µM. Once BCP flows through the column different aptamers will bind BCP creating an altered conformation outcompeting the base pairing stability it had with the Bio-Capture sequence. These will be eluted off the column bound to BCP and collected in a micro centrifuge tube. The aptamers with sequences in the random region that don't participate in the binding of BCP will be left on the column bound to the streptavidin.

Through this process the library is enriched drastically lowering the variability of the aptamers in the library pool through each consecutive round. Once all 750µL of target buffer is captured, it is

concentrated. To concentrate the eluted product, an Amicon Ultra 30k centrifugal filter was used. The target wash is added to the filters and concentrated down to 50 $\mu$ L by centrifuging 500 $\mu$ L of the wash at 14,000g for 10 min and then 250 $\mu$ L of the remaining wash at 14,000g for 8 min due to the filter capacity being only 500 $\mu$ L. The concentrated elute can now be amplified via PCR in order to increase the concentration of aptamers so that this new pool is able to be used for the next round.

#### PCR/Separation:

Since the aptamer is single stranded it must be converted to double stranded DNA in order to be amplified. To start the next round, the aptamer must be retrieved from its complementary sequence created during PCR so the pool can be further selected for. To accomplish this, a biotinylated reverse primer (Bio-PR3) is used in conjunction with the forward primer (PR-3). The aptamers are therefore base paired to their complementary sequences with the undesired complementary strand having a biotin tag attached. This serves as a prelude for strand separation to obtain the amplified single stranded aptamer library. Several dilutions of the concentrated elute are made to identify the optimum dilution for a large scale PCR in order to get a large amount of DNA for consequent round. The dilution PCR samples consisted of the concentrated elute being diluted 25, 50, 100, and 200 times along with both negative and positive control. The Taq master mix used for all amplification consisted of 400nM Bio-PR3 and 400nM PR3 mixed with 2X Taq with GC buffer. The sample size used for dilution PCR is 25 $\mu$ L while the large scale PCR is 100 $\mu$ L.

After the dilution PCR products are obtained they are then analyzed on a 10% acrylamide native gel. A 25bp marker is also added to one of the wells as a molecular weight marker. Taking into account that the library consists of 100nt they will migrate accordingly with the 100bp in the marker along with the positive control. The resulting gel is then stained with Gel Red for 7 min before it is visualized under UV

light. The cleanest band visible for the given dilution is then chosen and up scaled for quantity. PCR is then performed again. The number of cycles ranged from 16-20.

#### DNA Extraction:

The gels obtained from the above step are then visualized under UV light and the bands at the 100bp mark are then excised out of the gel and minced into small pieces. The excised gel pieces are placed in a 2mL micro centrifuge tube and then raised in 500µL of diffusion buffer ( 0.1% SDS, 1mM EDTA, 500mM NaOAc)to facilitate the diffusion of DNA out of the gel. The sample is then placed on an orbital shaker overnight for the DNA to diffuse optimally.

The next day the sample is centrifuged at 14,000g for 10 min to move the gel pieces towards the bottom of the micro centrifuge tube leaving a nice clear supernatant. The supernatant is collected and concentrated down using the Amicon Ultra 30k centrifugal filters. They were then centrifuged at 14,000g for 15 min while an additional 500 µL of diffusion buffer is added to the diced gel pieces. This is to ensure there is less DNA left behind. The supernatant is removed again and added to the Amicon filters to be further concentrated at 14,000g for 15 min. The remaining solution in the filters ( approximately 50µL) is then washed 3 times with 450µL of washing buffer( 150mM NaCl, 25mM Acetic Acid, 1% DMSO, pH 5.2). The remaining solution containing the aptamer and its biotinylated complementary sequence are then allowed to flow through a clean streptavidin agarose column as discussed above for target binding. The column is first washed 3 times with washing buffer before the concentrated DNA is allowed to flow through. The DNA is captured and put through the column once more to ensure all the strands are immobilized. The column is then washed 5 more times with washing buffer to elute any unbound or unbiotinylated strands form the column. With the biotin being attached to the complementary sequence and the strong interaction between streptavidin and biotin; the desired

sequences (aptamer library) are eluted off by alkalosis. This will leave the unwanted sequence still immobilized on the column while the desired sequence is eluted off using 200 $\mu$ L 0.2M NaOH. The eluted aptamers are captured in a micro centrifuge tube containing 30 $\mu$ L 3M NaOAc to stabilize the now single stranded DNA. This is followed by an addition of 40 $\mu$ L HCl to neutralize the solution. During the second attempt we switched to the 20mer polyA tail method as described earlier using SDS-PAGE to distinguish the library from the anti-sense DNA. This was much more time and cost efficient.

The addition of 2 $\mu$ L of glycogen (10mg/mL) is also added to help carry the DNA into a pellet during ethanol precipitation. Ethanol (95%, 3x volume) is added after the addition of glycogen. The solution is then placed in -20° C freezer overnight. After incubation the sample is centrifuged at 14,000g for 15 min in which a pellet will form and the supernatant discarded. The pellet is allowed to dry for 5 min to let any excess ethanol evaporate. The pellet is then resolubilized in 100 $\mu$ L of reaction buffer and its absorbance is measured at 260nm via a spectrophotometer. At this point the next round can now be started using this product as the new library to be annealed to the biotinylated Bio-Capture strand and introduced to the target once more on the column. A set amount of DNA (12 pmols) is loaded into the next round as a means of setting a base line for comparison throughout the rounds. This amount was calculated to be the average of the first 3 rounds.

Like stated above for the revised protocol, the DNA extraction method was switched to using a polyA tail on the end of the negative primer instead of it being biotinylated. This allowed for a quicker separation method based on nucleotide length when the amplified products were run on an SDS-PAGE gel. The 100bp bands were excised and followed the ethanol precipitation method mentioned above.

### Stringency:

Stringency is necessary throughout the rounds to select for aptamers that have higher affinities towards BCP.

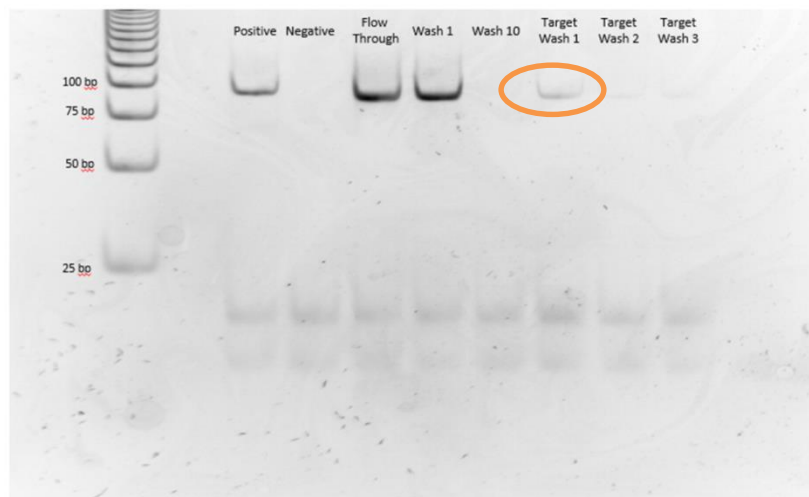
1. First and most important is to lower target concentration systematically which will further select for only aptamers that have high affinity towards the target. The target concentration is decreased systematically after every 2-3 rounds depending on the elution profile of each round. The first round is to select for the aptamers that bind at the lowered concentration initially which may not be very many, but through amplification we obtain a large amount once again. For the second and third round at the same target concentration, the library becomes more robust with these higher affinity aptamers. The starting concentration was 100uM of BCP and was systematically lowered to 20, 4, 1, 0.5  $\mu$ M.
2. The second way of increasing stringency was to use elevated temperature washes. During the first streptavidin agarose column the 10 washes are done in a 28°C water bath. These washes were increased to 20 for the revised protocol. This will elute off any partially bound strands towards the column, leaving only the aptamers that have the strongest affinity towards the anti-sense Biocapture strand.
3. Lastly, implementing 0.03% Triton X may also be used as a measure of stringency. Triton X-100 is used as a detergent and helps neutralize any interaction the DNA might have with the polypropylene glycol tubes which may hinder the correct elution of strands off the column. There have been reports of DNA adhering towards the walls of the micro centrifuge tube, thus making it difficult to detect small amounts of DNA (21). This also helps to clean the column before the target

is presented so that the only strands left on the column are ones with correct base pairing of the anti-sense Biocapture strand.

#### Transformation:

The library with the 3 way junction had what looked like to be a good elution profile with a clean wash followed by a more intense band in the following target wash [Fig. 14]. Therefore the target band was excised, purified, and used in a cloning kit. The cloning kit allows the sequences to be inserted into a plasmid via restrictive digestive enzymes. The plasmids are then transformed into E.Coli and the E.Coli will replicate and amplify the incorporated plasmids. Once sufficient replication is achieved the plasmids were purified from the cells then sent to a company that sequenced the plasmids. The data was then analyzed for sequences with similar bases in the random region known as families. These families of aptamers will be an indication that the random region in which they contain is important for the binding of BCP. The families were ordered and proved to be sequences that did not bind the target well enough to be visualized through elution profiles or fluorescent experiments. This was completed for both attempts with inconclusive results on both occasions.

**Figure 15 Osteon M. Excised band is circled. This pool of aptamers was used for the sequencing and enabled us to acquire the information mentioned above regarding issues with the structure switching method.**



## **Conclusions/Future Work:**

We conclude that there are a wide variety of factors that play a role in which why our selection did not go as planned. It is suggested from our results on these attempts and other related attempts not mentioned in this paper that the structure switching method has many possible points of error. For future selection attempts it may be beneficial to use a more widely used classical SELEX procedure with BCP such as magnetic streptavidin or simple streptavidin conjugated BCP affinity columns. The conjugation of BCP to an agarose bead is a fairly simple synthesis(22). Also, with the color changing properties that HSA has towards BCP it is possible to introduce these amino acid residues that are in the active site of HSA into the aptamer. Such an aptamer would bind BCP while the peptides facilitate the proton exchange exhibited in the hydrophobic pockets of HSA. There are specific DNA binding peptides as small as 5mer that may be able to accomplish this task(23). The peptides would bind the stem portion of the aptamer and hopefully interact with the dye. To date there is no published data that claims an aptamer/peptide complex being used to bind a target. It may be beneficial to start with an already known aptamer and observe the effects of incorporating DNA binding peptides towards the target. If it is concluded that affinity is enhanced than maybe it can be used for a BCP aptamer. For now, our goal is simply to achieve selecting an aptamer that binds BCP effectively. Once an aptamer is selected than other routes of experimentation to achieve the color changing properties of BCP may be pursued.



## References

1. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* (New York, NY). 1990;249(4968):505-10.
2. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature*. 1990;346(6287):818-22.
3. Shangguan D, Cao Z, Meng L, Mallikaratchy P, Sefah K, Wang H, et al. Cell-specific aptamer probes for membrane protein elucidation in cancer cells. *Journal of proteome research*. 2008;7(5):2133-9.
4. Negri P, Chen G, Kage A, Nitsche A, Naumann D, Xu B, et al. Direct optical detection of viral nucleoprotein binding to an anti-influenza aptamer. *Anal Chem*. 2012;84(13):5501-8.
5. Kleinjung F, Klussmann S, Erdmann VA, Scheller FW, Fürste JP, Bier FF. High-Affinity RNA as a Recognition Element in a Biosensor. *Analytical Chemistry*. 1998;70(2):328-31.
6. Ferapontova EE, Olsen EM, Gothelf KV. An RNA aptamer-based electrochemical biosensor for detection of theophylline in serum. *Journal of the American Chemical Society*. 2008;130(13):4256-8.
7. Kolpashchikov DM. Binary malachite green aptamer for fluorescent detection of nucleic acids. *Journal of the American Chemical Society*. 2005;127(36):12442-3.
8. Stojanovic MN, Kolpashchikov DM. Modular aptameric sensors. *Journal of the American Chemical Society*. 2004;126(30):9266-70.
9. Paige JS, Wu KY, Jaffrey SR. RNA mimics of green fluorescent protein. *Science* (New York, NY). 2011;333(6042):642-6.
10. Strack RL, Song W, Jaffrey SR. Using Spinach-based sensors for fluorescence imaging of intracellular metabolites and proteins in living bacteria. *Nature protocols*. 2014;9(1):146-55.
11. Ng EW, Shima DT, Calias P, Cunningham ET, Jr., Guyer DR, Adamis AP. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nature reviews Drug discovery*. 2006;5(2):123-32.
12. Bates PJ, Laber DA, Miller DM, Thomas SD, Trent JO. Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. *Experimental and molecular pathology*. 2009;86(3):151-64.
13. Liu Z, Duan JH, Song YM, Ma J, Wang FD, Lu X, et al. Novel HER2 aptamer selectively delivers cytotoxic drug to HER2-positive breast cancer cells in vitro. *Journal of translational medicine*. 2012;10:148.
14. Ito S, Yamamoto D. Identification of two bromocresol purple binding sites on human serum albumin. *Clinica chimica acta; international journal of clinical chemistry*. 2010;411(19-20):1536-8.

15. Ito S, Yamamoto D. Mechanism for the color change in bromocresol purple bound to human serum albumin. *Clinica chimica acta; international journal of clinical chemistry*. 2010;411(3-4):294-5.
16. Ito S. Structural features of bromocresol purple and its binding sites on human serum albumin for a proton-exchange reaction *International Journal of Pharmaceutical Chemistry*. 2016;IJPC3 (American Chemical Society).
17. Pinnell AE, Northam BE. New automated dye-binding method for serum albumin determination with bromocresol purple. *Clinical chemistry*. 1978;24(1):80-6.
18. Kolpashchikov DM. Split DNA enzyme for visual single nucleotide polymorphism typing. *Journal of the American Chemical Society*. 2008;130(10):2934-5.
19. Yang KA, Pei R, Stefanovic D, Stojanovic MN. Optimizing cross-reactivity with evolutionary search for sensors. *Journal of the American Chemical Society*. 2012;134(3):1642-7.
20. Nutiu R, Li Y. In vitro selection of structure-switching signaling aptamers. *Angewandte Chemie (International ed in English)*. 2005;44(7):1061-5.
21. Strauss CGaF. Avoiding adsorption of DNA to polypropylene tubes and denaturation of short DNA fragments. Elsevier. 1998;Technical Tips Online Vol.3:63-5.
22. Cho JK, Wong LS, Dean TW, Ichihara O, Muller C, Bradley M. pH indicating resins. *Chemical communications (Cambridge, England)*. 2004(13):1470-1.
23. Alam MR, Maeda M, Sasaki S. DNA-binding peptides searched from the solid-phase combinatorial library with the use of the magnetic beads attaching the target duplex DNA. *Bioorganic & medicinal chemistry*. 2000;8(2):465-73.