Selecting Mutant Specific DNA Aptamers against Live *E.coli* Cells

By

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Abstract

DNA aptamers consist of short strands of oligonuclotides that bind to a specific target molecule based on their charge and their adapted conformation. Aptamers are similar to antibodies in their binding affinity and specificity to the target molecule yet they possess a variety of advantages over antibodies. They are more stable in various environmental conditions and can be synthesized more easily at lower costs. More importantly, aptamers are not immunogenic and they have a small size, which allows them for more tissue penetration. This study focuses on selecting aptamers for live K12MG1655 *E.coli* cells. Live bacterial cells have not been a common target in aptamer selection, yet this project has proved that with some modifications in overall selection techniques we can select aptamers for live bacterial cells. Two strains of K12MG1655 have been chosen: wild type strain (BRL02) and its mutated strain (BRL25/26) that lacks poly-N-acetyl glucosamine on its outer membrane. A modified Systematic Evolution of Ligands via Exponential enrichment (SELEX) procedure was used to find aptamers with affinity and selectivity to potential target (sugar motif) on *E.coli* surface. Hence, having a sugar molecule as a potential target adds to the novelty of this research since up to now, majority of aptamers were generated against proteins and peptides (mostly proteins) but not sugar molecules. Additionally, we have evidenced that selecting aptamers for the absence of a target molecule is possible as well by selecting aptamers against mutated *E.coli* cells.

Aptamer pools obtained after 10 rounds of selection showed high affinity and selectivity to targeted *E.coli* cells when tested by fluorescent spectrophotometer by monitoring all collected aptamer pools. Out of 10 DNA pools collected for BRL25/26, one individual pool, the 10^{th} one, was selected for EC_{50} analysis. Obtained EC_{50} of candidate aptamer pool was significantly lower than DNA library. Candidate aptamer pool can be used for further selection, cloning and possibly sequencing. Furthermore selected aptamer can be used to isolate known biomarkers from the cells or to identify new ones. They can also be used as a selective drug-delivery agent or a bioassay tool in diagnostics, food analysis, and anti-bioterrorism.

Dedications;

I dedicate this paper to my mother and father for their tremendious support and help.

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Statement of Contribution

Genetically engineered *E.coli*, K-12 MG1655 BRL25/26, was kindly provided by Dr. Benjamin R. Lundgren. In addition Dr. Christopher Boddy was immensely elaborative in guiding me how to prepare, culture, and restore *E.coli* cells. Additionally, Dr. Boddy was kind to provide me with the comfort of utilizing his lab materials whenever necessary. As far as literature contributions are concerned, I am greatly thankful to the authors of the following text for providing me with the guidance I needed to pursue my research goals and conducts:*"Selection of Aptamers against Live Bacterial Cells"* by Camille L. A. Hamula, Hongquan Zhang, Le Luo Guan, Xing-Fang Li, and X. Chris Le.

Furthermore, Ms. Darija Muharemagic kindly contributed in different aspect(s) of the research; this included a tutorial on how to operate the following; PCR cycler, imaging machine, spectrophotometer, as well as nano drop. She was indeed quite helpful in teaching me how to prepare PCR master-mix, and TAE Buffer as well.

Last but not least, Dr. Maxime Berezovski's tremendous contributory list includes guiding me within each phase of the research by reviewing and revising my research proposal in different rounds of aptamers selections.

Introduction

Aptamers are single-stranded small (typically 40-100mers) oligonucleotides that bind to a target molecule with high affinity and specificity (1-4). Aptamers often possess high affinity to their target due to their ability to fold into unique three-dimensional structures. Aptamers have a flexible method of interaction with their target molecules. They are able to incorporate small molecules into their three-dimensional nucleic acid structures or to be integrated into the structure of their targets (4). They can also interact with a specific region of the target molecule (26). Aptamers are similar to antibodies in their binding affinity and specificity to the target molecule yet they possess a variety of advantages over antibodies. They are more stable in various environmental conditions and can be synthesized more easily at lower costs. More importantly, aptamers have immunogenicity and they have a small size, which allows them for better tissue penetration. These oligonucleotides have a lot of applications in affinity-based separation and detection processes (2). Aptamers can be used to isolate known biomarkers from the cells or to identify new biomarkers. They are also great candidates for novel bioassay tool covering areas such as diagnostics, environmental and food analysis, and anti-bioterrorism (20).

In this experiment, deoxyribonucleic acid (DNA) aptamers were selected for live *E.coli* cells. Although the majority of bacteria are non-pathogenic, there are some that cause infections in humans such as *Mycobacterium tuberculosis* that kills about 2 million people a year. Inherently, *E.coli* bacterium is commonly found in the lower intestine of warm-blooded organisms usually without causing any infection. However, there are some, such as serotype

O157:H7, that can cause Hemolytic-Uremic Syndrome which is a saviour urinary tract infection. Pathogenic *E.coli* mainly causes food poisoning and is responsible for majority of product recalls. Therefore, having aptamers as a novel bioassay tool can significantly help in bacterial infection diagnostics and in environmental and food poisoning analysis.

In this research target bacteria is a live *E.coli* (K-12 MG1655) that was derived from a stab-culture descendant of the original K-12 strain (4). K-12MG1655 is a non-pathogenic, rod-shaped, gram-negative bacterium with a rich variety of potential targets for aptamers on its surface. Potential targets are peptidoglycans, transport proteins, polysaccharides, lectins, and mucus binding proteins. Amongst all, N-acetyl glucosamine, sugar units of K12 MG1655 cell wall's peptidoglycans (PG) was targeted molecule for aptamer selection. This proposes a novelty in aptamer selection since majority of aptamers were selected for proteins and not sugar molecules. These polysaccharide-selective aptamers could have considerable functionalities and would contribute towards the development of polysaccharide-based vaccines for bacterial infections.

Two strains of K12MG1655 have been chosen: wild type strain (BRL02), and its mutated strain (BRL25/26) that lack poly-N-acetyl glucosamine on its outer membrane. Trying to generate aptamers with high selectivity toward the target molecule, mutated strain was used as negative target while selecting for wild type *E.coli* cells. Similarly, wild type strain was used as negative target while selecting for mutated *E.coli* cells. In more details, collected DNA strands that bound to BRL02 were incubated with BRL26/25. Amongst these DNA strands, those that possessed any interactions with BRL26/25 were eliminated from the pool (note that same processes were

applied when selecting DNAs for mutated strand). This way, a more selective DNA aptamers would be generated. This proposes another novelty of this research since the presence and absence of a structure is being detected by selected aptamer. Hence, selected aptamer is able to detect the absence of poly-N-acetyl glucosamine in case of BRL26/25, or the presence of poly-N-acetyl glucosamine in case of BRL02. Absence of ploy-N-acetyl glucosamine from the surface of bacteria reveals or reforms some structures on the surface that were otherwise different or masked in the presence of poly-N-acetyl glucosamine. Selected aptamer detects these changes on the surface and that's how an absence of poly-N-acetyl glucosamine can be detected with a high selectivity.

Aptamers are typically discovered using in vitro selection technique (2) which is predominantly via Systematic Evolution of Ligands via Exponential Enrichment (SELEX). Aptamers are generated by enriching a population of random-sequence DNAs or ribonucleic acids (RNA) for sequences that can bind to target molecules with high affinity and selectivity. Up to very recent, *in vitro* generated aptamers were counted as the only existing aptamers, while recent findings have revealed that RNA aptamers exist naturally as a form of gene control element (2). These RNA aptamers are called riboswitches (7) that regulate the expression of some metabolic genes in some eukaryotes (11) and in many eubacteria (8-10). Both engineered and naturally occurring aptamers exhibit fastidious specificity and exquisite affinity for the target molecules. However, naturally occurring aptamers (riboswiches) show greater specificity and affinity compared to their engineered counterparts (14). These disparities between these two kinds of aptamers can be minimized by using a larger nucleic acid constructs and by performing the procedure in a more native state of the target molecules.

Aptamers are usually selected for purified target molecules that are commonly affixed to a solid support such as a column. These solid supports provide benefits of immobilizing target molecules and easing separation of bound and unbound aptamers (1). However, a solid support does not represent a native environment of the target molecules and hence the target molecule won't reveal its native state and conformation. Providentially, recent developments in SELEX techniques' using capillary electrophoresis (CE) and flow cytometry (FCM) separation allows the target molecule and the DNA library to be suspended in solutions (3). This provides a medium in which target molecules will be more likely to be in their native state and hence improve the efficiency of aptamer selection. Therefore, this technique was adapted for selecting aptamers for targeted *E.coli* cells. Targeted cells were suspended in a solution and no solid support of any kind was used. This allows for separation of bound from unbound DNA via simple centrifugation and it keeps target molecules in a more native state. Moreover, SELEX has rarely been used for live bacterial cells (1) hence, selecting mutant specific aptamers for live *E.coli* cells using CELL-SELEX is a breakthrough in aptamer selection.

Historically, *in vitro* selection of aptamers is dated back to 1990's; where two groups independently developed RNA sequences that could specifically bind to target molecules (5). Later, these RNA oligonucleotides were termed *aptamers,* derived from Latin *aptus*, which means, "to fit" (6). DNA-based aptamers were discovered later, and after almost 20 years' endeavour, a lot of DNA and RNA aptamers have been identified for a broad range of targets(4). Untill now, aptamers have been selected for metal ions (e.g.,K^+, Hg^{2+} and Pb^{2+}) and small organic molecules such as amino acids, antibiotics, and vitamins . Moreover, a lot of aptamers have been selected for peptides and proteins such as HIV-associated peptides, thrombin and

growth factors (12-19). Hence, as mentioned earlier they were rarely selected for sugar molecules. While the most likely target for aptamers on wild type *E.coli* is poly-N-Acetyl glucosamine, the aptamers been most likely selected against sugar molecules. This can add to the broad spectrum of potential target molecules for aptamers. Therefore, availability of such a broad range of these oligonuvleotide makes them a great candidate as a novel bioassay tools covering areas such as diagnostics, environmental and food analysis, and anti-bioterrorism(20).

Diagnostics of deep-seated bacterial infection is a challenging problem. Common imaging techniques such as X ray, computed tomography (CT), magnetic resonance imaging (MRI), and ultra sonography can be helpful but they are not specific for infection. Consequently, a technique that relies on the demonstration of patho-physiological changes, rather than gross changes in structures would be a better alternative. Therefore, radiolabelled leucocyte imaging (WBC imaging) is currently the chief method used for imaging infections (21). In WBC imaging, labelled neutrophil migrates to the site of infection via chemotaxis and diapedesis (22). Even though this technique is very selective for detecting inflammatory sites it is unable to distinguish between infective and non-infective inflammatory conditions. Another method for diagnosing bacterial infection is analyzing a specimen taken from the patient. Therefore, biopsy or similar difficult or painful sampling procedure is sometimes required .

Imaging with labelled antibodies or aptamers might be a better alternative imaging technique for detecting bacterial infections offering more specificity toward infective inflammatory conditions (21). Unlike radiolabelled leukocytes, antibodies can selectively bind to bacterial cells hence enhancing the detecting processes. This diagnostic procedure determines

interactions between specific antibodies or aptamers and antigens previously found to be associated with the infecting organisms. However, aptamers exhibit many advantages compared to traditional antibodies as detecting agent in bacterial infection. Labelled aptamers can be injected to infected host and get dispersed by blood throughout the body. Labelled aptamers will accumulate in the site of infection since they exhibit higher binding affinity to target bacterial cells rather than host cells. Aptamers, particularly DNA aptamers, possess remarkable chemical and thermal stability compared to antibodies (1-4,24).They can be synthesized more easily compared to their antibodies counterpart (24) since they do not require animals for synthesis (1). More importantly, aptamers have low immunogenicity therefore they would be considered safer to be used as imaging agents. Additionally, aptamers are small in size (10-20,000 Da versus 150,000 Da for antibodies), which allows for increased tissue penetration. Furthermore, these characteristics of aptamers have merit antibodies not only in detecting but also in therapeutic applications. For example, antibodies are sometimes unable to diffuse extravascularly or to penetrate into large solid tumours due to their large size. However, aptamers are able to penetrate more easily toward the target due to their small size.

In this experiment, aptamers were selected using modified SELEX procedure. Throughout the procedure the condition were kept optimal for *E.coli* cells. Hence any rupture in bacterial cell wall could expose the interior of the *E.coli* and hence disrupt the aptamer selection. Temperature was kept mainly at 32˚C for two reasons: First to have an optimal temperature for *E.coli* cells to stay active and alive. The second reason was to closely resemble the conditions where aptamers would be applied for diagnostic or therapeutic purposes. Target molecules and DNA aptamers can adapt different conformation upon temperature changes.

Therefore selecting aptamers in conditions, which they are more likely to be applied in is crucial.

To address all these concerns and to produce mutant-specific DNA aptamers, with high selectivity and affinity against live bacterial cells, 10 rounds of selection for both mutated and wild type *E.coli* cells have been performed. In both cases, higher binding affinity was observed for positive binders compared to their negative binders. Binding affinity improved with each successive round of selection and by-passed the DNA library's binding affinity.

Materials and Methods

Preparing *E.coli* cultures

Both K-12 MG1655 BRL02 and BRL 26/25 were provided by Dr. Christopher N.Boddy and Dr.Benjamin R. Lundgren. Mutation in BRL 26 has been introduced by recombinant genetic modification and hence BRL26 is kanamycin resistant. Both strains were grown in aerobic conditions at 37°C . BRL 02 was grown in Luria-Bertani (LB) agar dishes while BRL 26 was grown in LB+ kanamycin antibiotic agar dishes purchased from Fisher. For liquid culture both strains were grown in LB only. Both strains were harvested in their logarithmic phase of growth.

DNA Library and primers

The naive DNA library contained a central randomized sequence of 40 nucleotides flanked by 20-nt primer hybridization sites (5'-CTC CTC TGA CTG TAA CCA CG-(N)40-GCATAG GTA GTC CAG AAG CC-3')was used . The 5'-primer labelled with Alexa Fluor® 488 NHS Ester (5'-/5Alex488N/CTC CTC TGA CTG TAA CCA CG-3) and the 3'-primer (5'-GGC TTCTGG ACT ACCTAT GC-3') were used in PCR reactions for the synthesis of single-stranded DNA molecules. All DNA molecules were custom synthesized by Integrated DNA Technologies (Coraville, IA). Aexa647 for FACS.

PCR Amplification and Gel Electrophoresis

Fifteen cycles of symmetric PCR, followed by 20 cycles of asymmetric PCR were used for the amplification of single-stranded DNA. PCR was carried out in a Mastercycler pro S thermal cycler (Eppendorf, Mississauga, ON, Canada). In addition to the DNA sequence template, the PCR reaction mixture contained Green GoTaq Flexi Buffer, 2.5 mM MgCl2, 10 mM dNTP Mix,

and 0.025 U/µl of Taq DNA Polymerase. For the symmetric amplification, 300 nM of Alexa 488-labelled forward primer and unlabelled reverse primer was used, and for the asymmetric amplification, the concentration of the forward primer was 20 times higher than the concentration of the reverse primer (1 µM and 50 nM, respectively). Thermocycling parameters were 94 ˚C for 2 min denaturation, followed by 15 or 20 cycles of denaturation at 94 ˚C for 30 sec, annealing at 56 ˚C for 15 sec, and extension at 72 ˚C for 15 sec. A final extension step of 72 ˚C for 5 min was carried out following the last cycle.

After PCR, the reaction products were tested on 3.0% agar gel in 1X TAE buffer. All gels were imaged by Alpha Innotech imaging system (multiImage III-Fluro Chem Q).

Aptamer selection

Preparing E.coli Cells for Aptamer Selection. Both *E.coli* were grown overnight on appropriate agar dishes. Then their liquid culture was grown over night. Cells were pelleted at 5000g for 5 min at 32 ˚C for 5 min then washed twice in 1X PBS+$MgCl_2$ (3.2mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3mM KCl, 1.35mM NaCl, 4.0mM $MgCl_2$)(PH 7.4) at room temperature.

Preparing Enriched DNA Library. Initially DNA library was denatured by heating them at 95 ˚C for 5 min in incubation buffer (1X PBS+$MgCl_2$). Then they were renatured on ice for 10 min. 5x10^9 *E.coli* cells were incubated with 5 µl DNA library (stock) [1 µM (5 nmol or 3x10^15 sequences)] and 495µl incubation buffer for 45 minutes at 37 ˚C (or room temperature). *E.coli* cells were then centrifuged at 5000 g for 5 min at 32 ˚C to separate bound and unbound ssDNA sequences. E.coli cells were wahsed two times using 500 µl of incubation buffer at 32 ˚C to remove unbound DNAs. *E.coli* pellets were resuspended in 50 µl of incubation buffer and were

heated at 95 ºC for 10 min to release DNA bound to target cells. Finally *E.coli* cells were centrifuged at 10,000 g for 15 min at 32 C and then supernatant was collected.

DNA Amplification. Collected DNA was amplified by symmetric and asymmetric PCR as explained above.

DNA Purification. Amplified DNA were transferred onto 30/10 kDa PALL nanosep molecular cut off filters. Solution was centrifuged at 5000 rpm for 15 min at 15 ˚C. Solution was washed two times with incubation buffer (50 mM Tris-Acetate, 50 mM NaCl, 5mM MgCl2) at 5000 rpm at 15 ˚C for 8 min. Nanodrop was used to measure concentration of selected DNA. Final enriched ssDNA was diluted in 150 µl of incubation buffer. The enriched amplified pool was stored at -20 ˚C (-80˚C).

Negative Selection of Aptamers. 10^9 of E.coli cells were incubated with 250nM masking DNA(DNA from Salmon Testes purchased from Rockland) in 250 µl of incubation buffer for 15 min at 37˚C. Then mixture was incubated with 250 µl of 100nM enriched library in incubation buffer for 30 min at 37˚C. Cells were centrifuged at 5,000g for 5 min at 37˚C and supernatant containing unbound DNA aptamers was collected.

Positive Selection of Aptamers. Supernatant was transferred to another 1.5 ml eppendorf tube containing 5x10^9 E.coli cells in 500 µl of incubation buffer. Then the solution was incubated for 30 min at 37˚C.Target cells were centrifuged at 300g for 5 min at 37˚C to separate bound and unbound ssDNA sequence.Ttarget cells were washed two times using 500 µl incubation

buffers at 5000g for 3.5 min at 37 ˚C to remove unbound DNAs. Cell pellet was resuspend in 50 µl of incubation buffer and it was heated at 95˚C for 5 min to release DNA bound to target cells. *E.coli* cells and cell debris were removed by centrifugation at 5000 g for 15 min at 37˚C.

Candidate aptamers were amplified using symmetric and asymmetric PCR as described previously.

These steps were repeated for 10 rounds to generate an E.coli specific pool of DNA aptamers.

Results and Discussion

Selection of DNA Aptamer against Live k12 MG1655 *E.coli* Cells.

There are six main steps in the selection of aptamers against live *E.coli* cells: (1) incubating DNA library with the target live *E.coli* cells—positive selection, (2) separating bound and unbound DNA via centrifugation (3) denaturing the bound DNA from the cell surface by heat(4) Incubating collected DNA strands with negative *E.coli*target cells—counter selection, (5) removing the bound DNA from the unbound DNA by centrifugation, (6) Amplifying the collected DNA aptamerby PCR—new library for the subsequent round of selection (figure 1).

Random-sequence DNA oligonucleotides had been subjected to 10 rounds of selection to favour molecules that bind effectively and selectively to targeted *E.coli* cells. Prior to selection , enriched DNA library was prepared. Initially DNA library was denatured by heat (95˚C) for 5 min and then was renatured on ice for 15 min to restore their native 3D structure. Then, positive selection followed by negative selection was performed with renatured DNA library. Enriched DNA library was successfully prepared for both *E.coli* strain (figure 2).

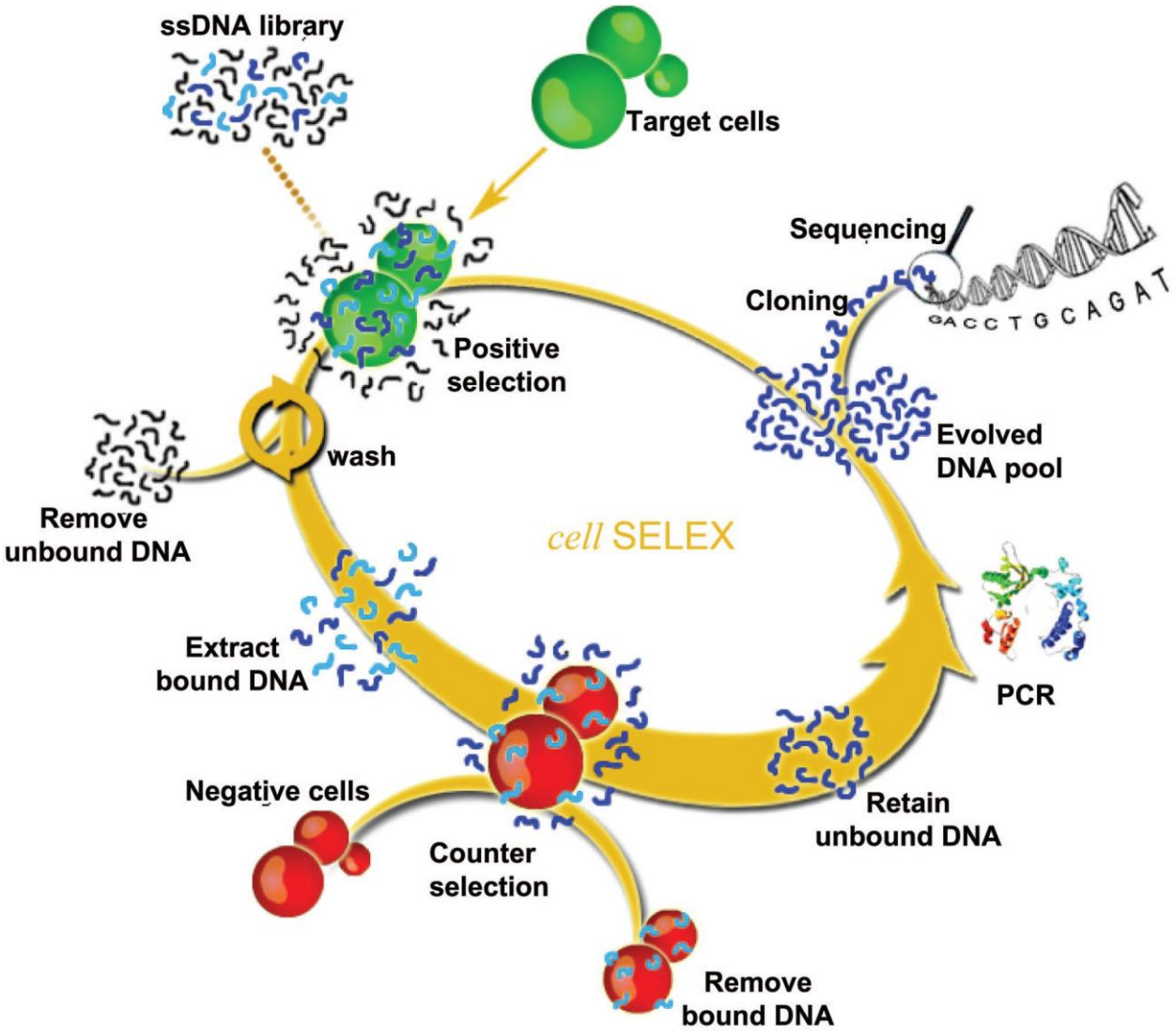

Figure 1. Schematic representation of the CELL-SELEX technique for DNA aptamer generation. Picture was retrieved from; http://www.chem.ufl.edu/~tan/group/chemicalbiology.html.

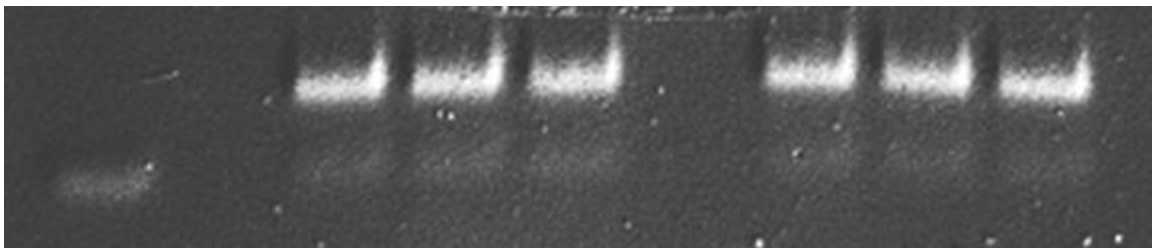

Figure 2.PCR amplified oligonucleotide fractions after the first round of SELEX (Enriched DNA library). A randomized single-stranded DNA library was incubated with BRL02 and BRL26 *E.coli* cells. After incubation, the cells were centrifuged to separate bound and un-bounds DNA strands. Un-bound DNA strands were removed by disregarding supernatant followed by two washings to remove DNA sequences that were non-specifically or weakly bound. Bound DNA strands were separated from targeted *E.coli* using heat at 94˚C for 15 minutes. The suspension was centrifuged, and the supernatant was collected contacting the enriched DNA library. Collected DNA strands were amplified by symmetric PCR amplification followed by asymmetric amplifications. –ve control band on the gel represents PCR master mix without any DNA.

Second round of selection was performed using collected DNA from the first round.collected DNA was amplified and purified prior to second round to generate enough DNA strands for aptamer selection.Since PCR optimization was not performed in this experiment , other precautions was take in place to make sure that the concentration of collected DNA from each round is sufficient for proceeding to the next round.Therefore, prior to succeeding to consecutive round the concentration of collected aptamer was routinely measured by nanodrop. Another important step to make sure that amplified DNA is the DNA that has been collected in each round PCR master mix was tested with each amplification. (note: PCR master mix for each set of rounds is loaded on the gel as a negative control). If the PCR master mix gets contaminated with DNA it would amplify it and DNA band would appear. If contamination was observed, the selected round, which contaminated PCR solution was used for its amplification was disregarded. The selection was repeated with a new PCR master mix to make sure that selected aptamer is being amplified not the contamination. If we leave the contamination in our master mix very gradual selection, if any, will take place since the concentration of bounders would be slightly higher than non-bounders since we always would have a lot of non bounders in our master mix.

Three more round of selection was performed successfully. All the mentioned precautions were applied in each step individually.

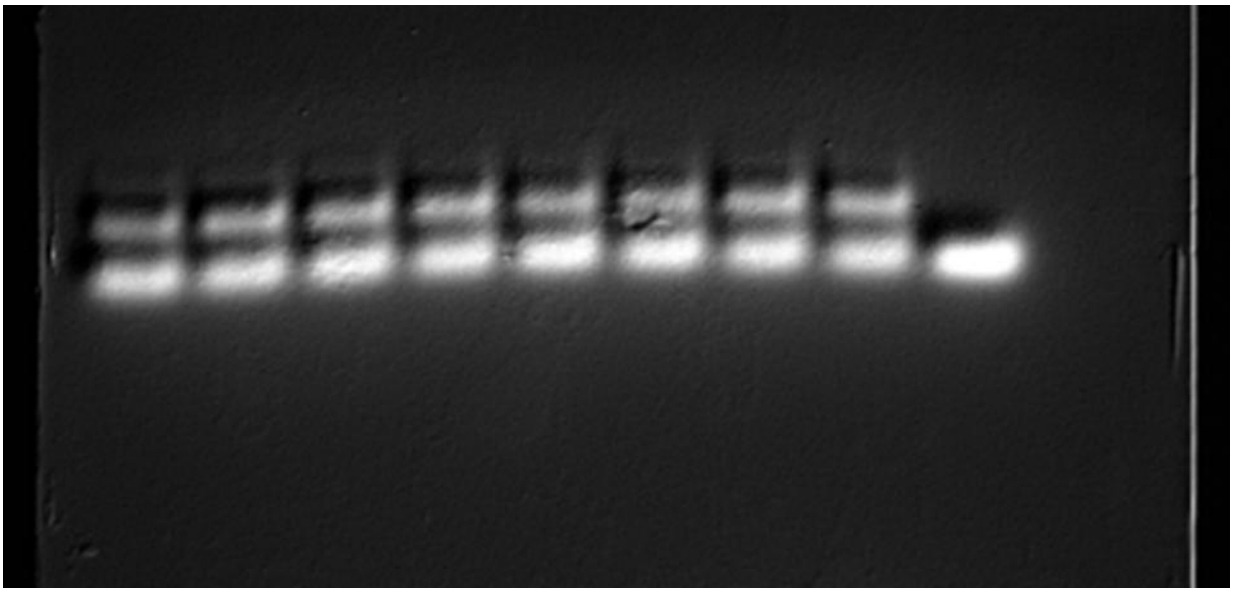

Figure 3. PCR amplified oligonucleotide fractions after the 2^{nd},3^{rd},4^{th}, and 5^{th} round of SELEX . Initially, the enriched DNA library was incubated with BRL02 and BRL26 *E.coli* cells. After incubation, cells were centrifuged to separate bound and un-bounds DNA strands. Un-bound DNA strands were removed by disregarding supernatant followed by two washes to remove DNA sequences that were non-specifically or weakly bound. Bound DNA strands were separated from targeted *E.coli* using heat at 94˚C for 15 minutes. The suspension was centrifuged, and the supernatant was collected. A SELEX negative selection was carried out in which collected DNA strands were incubated with the other *E.coli* cells. Un-bounders were separated by centrifugation. Collected DNA strands were amplified by symmetric PCR amplification followed by asymmetric amplifications. –ve control band on the gel represents PCR master mix without any DNA. Indetical procedure was applied for successive rounds.

Next three successive rounds of selection were performed identically (figure 4). 6^{th} round of selection was performed successfully. After amplifying and purifying DNA pool obtained from 6^{th} round its DNA concentration was checked with Nanodrp. After confirming its concentration, following rounds of selection was performed. 7^{th} round was performed successfully as well however the amplified DNA concentration was very low according to band fluorescent intensity. Therefore DNA obtained from 7^{th} round was amplified again to adjust the concentration (figure5). The dramatic increase in DNA concentration after second purification is observed. The reason for low amplification rate in frirst round of amplification can be due to low concentration of templates since DNA template collected from each round varies.

Last two rounds were performed successfully as well (figure 6). However more PCR solution were used for last two rounds since the template concentrations were very low. This is simply due to increased specificity of aptamer pools due to elimination of huge number of non-binders. In figure 6final amplified results of last two rounds is shown.

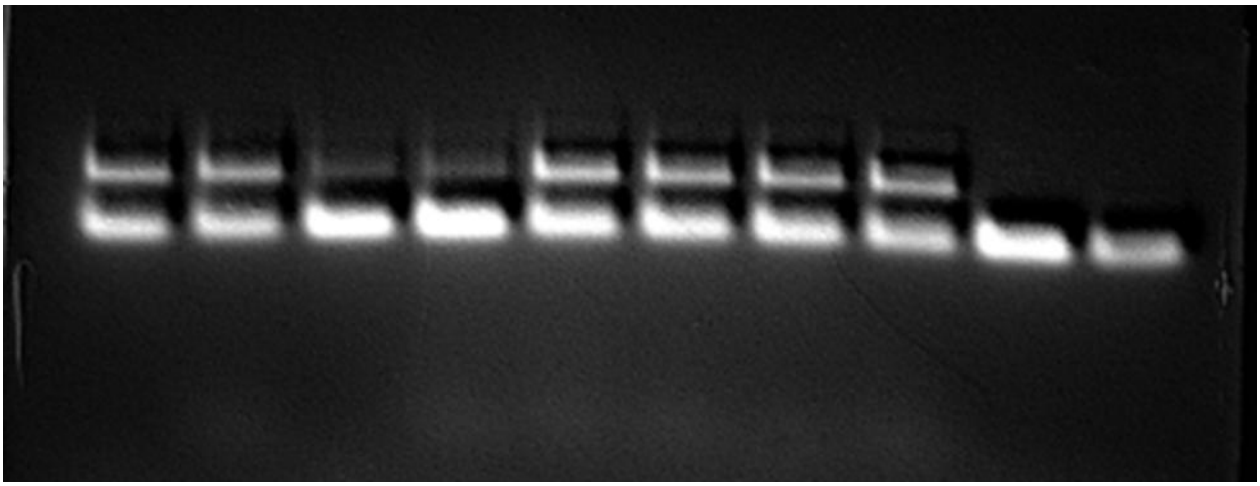

Figure 4. PCR amplified oligonucleotide fractions after the 6^{th}, 7^{th}, 8^{th} round of SELEX . Collected DNA library from previous round was incubated with BRL02 and BRL26 *E.coli* cells. After incubation, cells were centrifuged to separate bound and un-bounds DNA strands. Un-bound DNA strands were removed by disregarding supernatant followed by two washes. Bound DNA strands were separated from targeted *E.coli* using heat at 94˚C for 15 minutes. The suspension was centrifuged, and the supernatant was collected. A SELEX negative selection was carried out in which collected DNA strands were incubated with the other *E.coli* cells. Un-bounders were separated by centrifugation. Collected DNA strands were amplified by symmetric PCR amplification followed by asymmetric amplifications. 7^{th} round was amplified two times to obtain optimal DNA concentration. –ve control band on the gel represents PCR master mix without any DNA. Identical procedure was applied for successive rounds.

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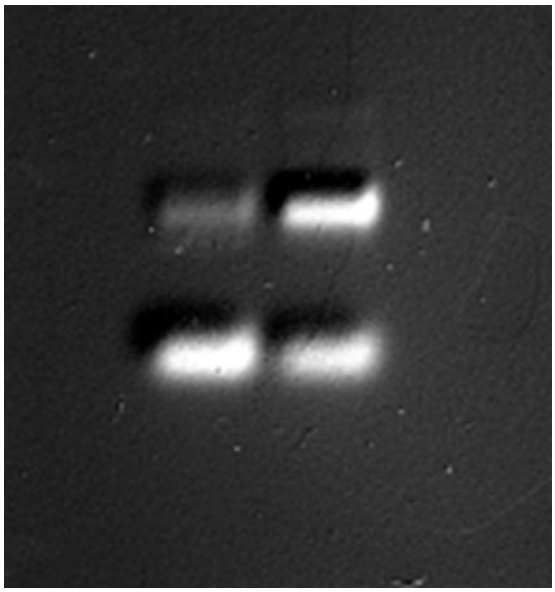

Figure 5. PCR amplified oligonucleotide fractions after 7th round of SELEX . Collected DNA library from 6th round was incubated with BRL26 *E.coli* cells. After incubation, cells were centrifuged to separate bound and un-bounds DNA strands. Un-bound DNA strands were removed by disregarding supernatant followed by two washes. Bound DNA strands were separated from targeted *E.coli* cells using heat at 94˚C for 15 minutes. The suspension was centrifuged, and the supernatant was collected. A SELEX negative selection was carried out in which collected DNA strands were incubated with BRL02 *E.coli* cells. Un-bounders were separated by centrifugation. Collected DNA strands were amplified by symmetric PCR amplification followed by asymmetric amplifications. Bands for 1PCR column represent amplified DNA strands after one round of amplification. Bands for 2PCR column represent amplified DNA strands after two rounds of amplification.

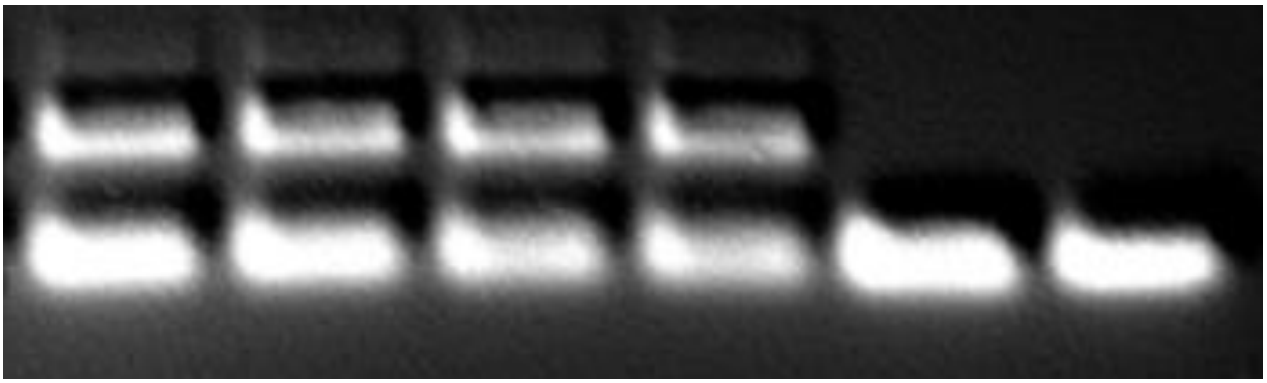

Figure 3. PCR amplified oligonucleotide fractions after 9^{th}, and 10^{th} round of SELEX . collected DNA from previus round was incubated with BRL02 and BRL26 *E.coli* cells. After incubation, cells were centrifuged to separate bound and un-bounds DNA strands. Un-bound DNA strands were removed by disregarding supernatant followed by two washes to remove DNA sequences that were non-specifically or weakly bound. Bound DNA strands were separated from targeted *E.coli* using heat at 94˚C for 15 minutes. The suspension was centrifuged, and the supernatant was collected. A SELEX negative selection was carried out in which collected DNA strands were incubated with the other *E.coli* cells. Un-bounders were separated by centrifugation. Collected DNA strands were amplified by symmetric PCR amplification followed by asymmetric amplifications. –ve control band on the gel represents PCR master mix without any DNA. Indetical procedure was applied for successive rounds.

Binding Affinity and Specificity of Aptamer pools

Aptamer pools were assessed for binding affinity and specificity to the target molecule using both fluorescent intensity and EC_{50} value. 200 nM fluorescently labelled aptamer pools were incubated with targeted positive and negative *E.coli*cells. After incubating for 15 min, DNA sampleswere washed once with PBS+$MgCl_2$ buffer. *E.coli* cells were suspened in 85µl PBS+$MgCl_2$ buffer and the florescent intensity of each pool was measured using plate reader. Fluorescent intensity of fluorescently labelled DNA library and labelled forward primer was assessed as well (controls). Both *E.coli* strains incubated in PBS+$MgCl_2$ were loaded on plate as well in order to determine background noises that might be accompanied by *E.coli* cells. For simplicity, DNA aptamers selected against BRL 02 and BRL 26 will be abbreviated to DNA-02 and DNA-26 respectively.

Binding Affinity and Specificity of DNA-02 against BRL02 (Figures 7 & 8).

Ten rounds of selection were performed in order to select aptamers for BRL02 *E.coli* cells. Binding affinity of performed rounds was assessed using fluorescent intensity of each round. Fluorescent intensity correlates with binding affinity of each DNA pool, as higher binding affinity, would lead to higher concentration of binders and hence higher fluorescent intensity will be observed.Binding affinity was performed in two sets of conditions, one incubated with around 250 nM masking DNA in PBS+$MgCl_2$ buffer and the other incubated with PBS+$MgCl_2$ buffer only, without masking DNA. In both cases, throughout all ten rounds the binding affinity between DNA-02 strands and BRL02 *E.coli* cells were higher than DNA-02 and BRL25/26 *E.coli* cells. This confirms the selectivity that DNA-02 possesses between BRL02 and BRL26/25.

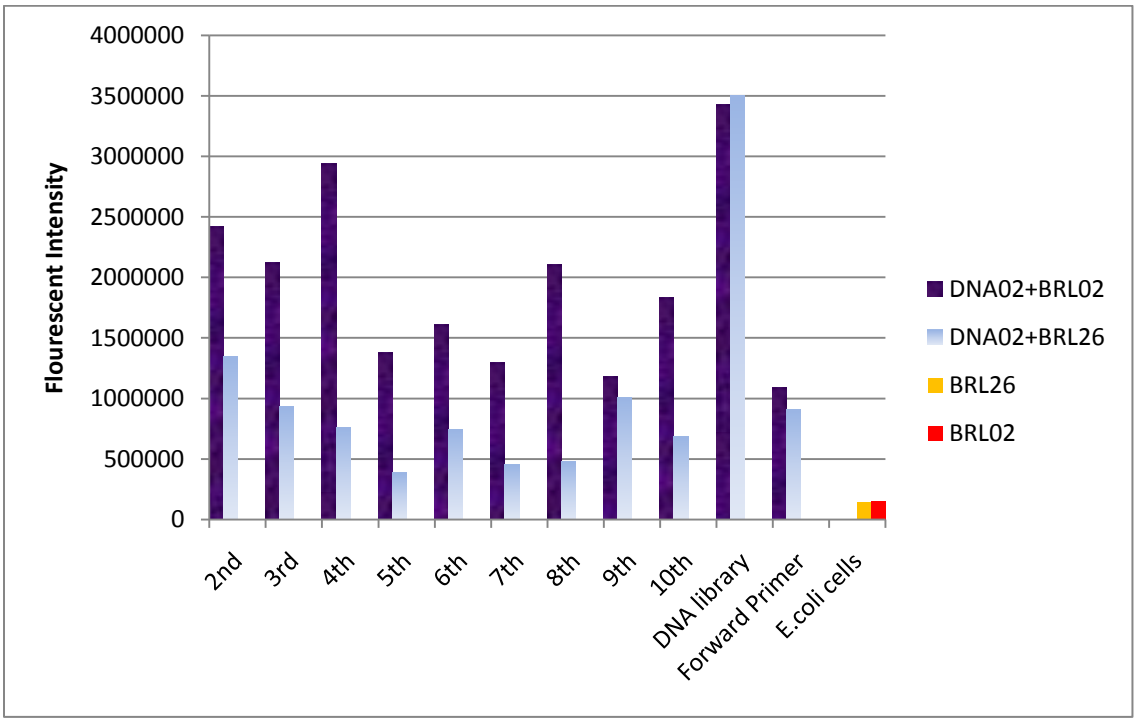

Figure7. Fluorescent intensity of collected DNA for live BRL 02. Aptamer pools obtained after each round of selection were fluorescently labelled at the 5' end with a fluorescent labelled primer. For each round 200nM of labelled aptamer pool and 10^7 E.coli cells were incubated (in the absence of making DNA) for 25 minutes. The suspension was washed once with PBS+$MgCl_2$. The intensity of each pool was measured using plate reader. Binding affinity of 2^{nd} up to 10^{th} round is shown.

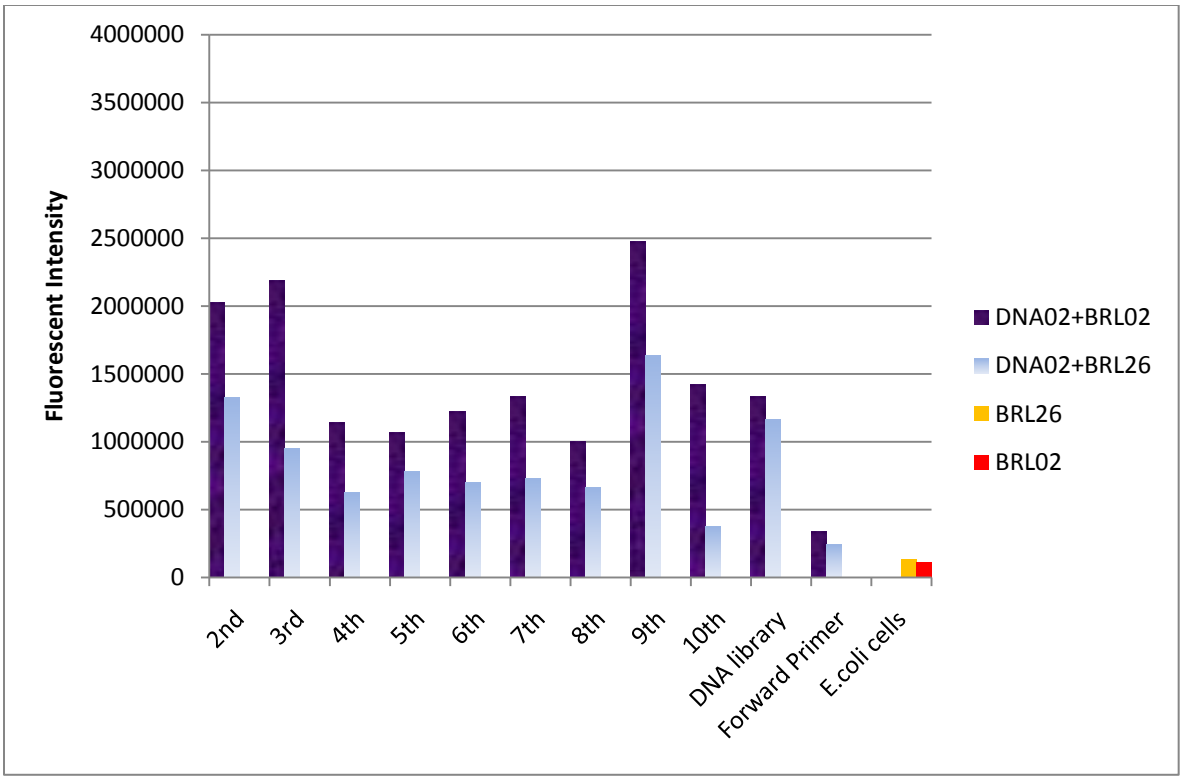

Figure 8. Fluorescent intensity of collected DNA for live BRL 02. Aptamer pools obtained after each round of selection were fluorescently labelled at the 5' end with a fluorescent labelled primer. For each round 200nM of labelled aptamer pool and 10^7 E.coli cells were incubated (in the presence of making DNA) for 25 minutes. The suspension was washed once with PBS+$MgCl_2$. The intensity of each pool was measured using plate reader. Binding affinity of 2^{nd} up to 10^{th} round is shown.

Binding affinity with masking DNA (figure 7). A dramatic decrease in binding affinity after 2nd round is observed. This is mainly due to removing huge number of non-binders and loosely binders from DNA pool. Non-binders can contribute to secondary interactions as well, which can contribute to false positive results. However, there is a gradual increase in binding affinity after 4th round that is evidently due to concentrating DNA pool with binders in each successive round of selection.

DNA strands from 9th round exhibits highest binding affinity between DNA-02 and BRL02 *E.coli* cells. Even though 9th round shows the highest binding affinity it does not possess the best selectivity amongst all ten rounds since the binding affinity between DNA-02 and BRL 26 (negative binders) is highest relative to other rounds. However, DNA-02 selected in 9th rounds is considered candidate aptamers because of their significantly high binding affinity to the target molecule. The pool from 9th round would be considered for more successive rounds of selection and even for cloning and sequencing.

There is a noticeable decrease in binding affinity in 10th round. This is something that one would not expect since typically the binding affinity should be increasing with each successive rounds of selection. This deselection may be due to a decrease in aptamer pool complexity or to inefficient separation of bound from unbound sequences during 10^{th} round selection process (1). Furthermore, the fluorescent intensity of 10th round's negative binders is noticeably low (lowest amongst all ten rounds) as well. This suggests that the overall fluorescent intensity of 10th round might be low due to usage of a smaller amount of DNA-02 during 10^{th} round of selection. This can be due to errors in calculations and/or calibrations in adjusting DNA's

concentrations. Additionally, DNA strands are highly subjected to mutations and modifications (especially during the PCR amplifications). These mutations might have introduced a sudden dramatic change in DNA-02 nucleotide sequence that has disrupted its binding to the target molecule. Similarly the same mutations might have disrupted even negative binders and hence very low binding affinities for negative binders is observed.

DNA library exhibits some bindings as well that is due binders and loose binders. DNA library exhibits almost same binding characteristics for both *E.coli* cells. Labelled forward primers have shown some bindings as well. However, by considering the fluorescent intensity of E.coli cells, the binding affinity of forward primers is so minimal that it is negligible.

Binding affinity without masking DNA (Figure 8). Masking DNA (salmon sperm) can block non-specific binders and competes with loose binders. DNA library showed highest binding affinityin absence of masking DNA since; it contains a lot of non-specific and loose binders. The overall trend did not change, however, DNA's from 9th round changed from highest to lowest binding affinity. This confirms the fact that most of binders in 9^{th} round are specific binders since with masking DNA the binding affinity dose not decrease. Without masking DNA 3rd round possessed best binding affinity and selectivity hence most of binders in 3^{rd} rounds are most likely non-specific binders. This confirms the fact that using masking DNA is necessary to block non-specific binders since there exist a lot of non-specific binders that can interfere with our results.

Binding Affinity and Specificity of DNA-26 against BRL26 (Figures 9 & 10)

Ten rounds of selection were performed in order to select aptamers for BRL 26 *E.coli* cells. Binding affinity of performed rounds was tested using plate reader. The fluorescent intensity of each round is an approximate representative of binding affinity.Binding affinity was performed in two sets of conditions, one incubated with around 250 nM masking DNA in PBS+$MgCl_2$ buffer and the other incubated with PBS+$MgCl_2$ buffer only, without masking DNA Two separate procedures were performed, one incubated with masking DNA in PBS buffer the other incubated with PBS buffer without masking DNA. In both cases, throughout all ten rounds the binding affinity between DNA-26 strands and BRL26 *E.coli* cells (positive binders) were higher than DNA-26 and BRL02 *E.coli*cells (negative binders). This confirms the selectivity that DNA-26 possesses between BRL26/25 and BRL02.

DNA-26 shows gradual and uniform increase in binding affinity which was expected since typically the binding affinity should be increasing with each successive rounds of selection. BRL 26 *E.coli* cell is lacking one of it's major componential sugars. This would give less chance to non-specific binders and loose binders to bind to BRL26 since there are fewer targets on the surface for them. Best binding affinity and selectivity is observed in 10th round of selection. Trend is same in both cases (with and without masking DNA) and in both cases 10th round exhibit best binding affinity amongst all rounds.

In general, masking DNA reduces the binding affinity since it blocks non-specific binders from binding to *E.coli* cells. Therefore, we can conclude that in most of DNA pools there were some non-specific binders that were reduced by masking DNA (Appendix 1).

Overall the fluorescent intensity was higher for DNA-02 pools comparing to DNA-26 pools (Appendix 2). BRL 26 has less targeted molecules on its surface for aptamerstherefore; selected aptamers would be less concentrated. On the other hand wild type *E.coli* cells possess a lot of target molecules on their surface for aptamers and therefore a more concentrated pool is generated. More rounds of selection should be done to eliminate this disparity. With performing more rounds of selection the selectivity of aptamer pools can be improved. Additionally DNA-26 is being selected for the absence of a structure and hence target molecule is unknown. Size and abundance of this unknown target molecule can effect DNA-26 concentrations. Small size molecules are usually single site binders because of small surface are for aptamers to interact with. On the other hand relatively large molecules (i.e, poly-N-acetyl glucosamine) are usually multi-site binders which means multiple aptamer can interact with target molecule simultaneously. This increases DNA concentrations and therefore more binding affinity can be obsereved. The abundance of target molecule also effects the concentration of DNA aptamers. More abundant target molecule, more DNA strands have the chance to bind to target and therefore less of binders get removed.

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