Aptamer selection for targeting AXL protein receptor expressed on cells and human IgG FC fragment

By

Angelique Masibag

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Supervisor: Dr. Maxim Berezovski

Department of Chemistry
University of Ottawa
Ottawa, Canada

ABSTRACT

Just like antibodies, aptamers have high sensitivity and specificity to its target. Aptamers are single stranded DNA that adapts to 3D structure. In this project two proteins were used for aptamer selection. The first project (Chapter 1) focused on aptamer selection against AXL tyrosine kinase using cell SELEX. HEK293 were transfected to express the AXL tyrosine kinase, which was used for aptamer selection. The selected aptamers were sequenced, and its structure were identified. The binding of the aptamers were evaluated on cells expressing and not expressing AXL using flow cytometry. The Apt-AXL-1615 displayed the best binding. The Apt-AXL-1615 was evaluated using whole blood leukocytes expressing AXL using flow cytometry and mass spectrometer. Overall, aptamers are used as probes for detection and isolation of the target cells. The second project (Chapter 2) focused on aptamer selection against human IgG FC fragment using SELEX. The rounds 5 to 7 aptamer pool binding to Fc fragment were evaluated using flow cytometry. However, the result displayed no binding at all. With an optimization on the recent protocol for aptamer selection, new selection was performed using agarose gel to verify the amplification of the selected pool. The results displayed a low quantity of amplified product. Overall, it requires more improvements on the method used for the aptamer selection against human IgG Fc fragment. In conclusion, aptamers against AXL were successfully selected and it was used for a successful isolation and sorting of the target cells; while, aptamers against human IgG Fc fragment requires further studies and improvement in SELEX method.

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LIST OF ABBREVIATIONS

Symbol	Description
nt	nucleotide
bp	base-pair
ssDNA	single stranded DNA
dsDNA	double stranded DNA
SELEX	Systematic Evolution of Ligands by Exponential enrichment
AptaBID	Aptamer-facilitated Biomarker Discovery
IgG	Immunoglobulin G
FC	Fragment of constant region
FAB	Fragment of antigen binding
R#	Aptamer pool round #
CD	Cluster of differentiation
ePCR	Emulsion PCR
hFc	Human IgG Fc region

STATEMENT OF CONTRIBUTION

Conception:

The idea of aptamer selection for the cancer cells expressing AXL receptors were originated by Dr. Maxim Berezovski and Shahrokh Ghobadloo (PhD Candidate). Dr. Maxim Berezovski and Dr. Ana Gargaun originated the idea of developing aptamers that binds to the human FC fragment for antibodies recruitment against cancer cells.

Experimental:

For the project, aptamer selection for cells expressing AXL receptors, transfection of the cells, aptamer selection, aptamer sequencing, phylogenetic tree analysis and aptamer binding affinity were performed by Shahrokh Ghobadloo (PhD Candidate). The purification of the biomarker using the whole blood, and the isolation of the proteins bound to Apt-AXL-1615 were performed by author along with the assistance of Shahrokh Ghobadloo (PhD Candidate) and Constance You (NSERC Student).

For the project, aptamer selection for human FC fragment, the first selection of aptamer pool from round 4 to 7 were performed by Ana Gargaun. The amplification, extraction, and purification were performed by the author with the assistance of Dr. Sabina Sperandino. The preparation of the samples and analyzing the flow cytometry results were performed by the author with the assistance of Dr. Sabina Sperandino, Shahrokh Ghobadloo (PhD Candidate) and Dr. Chris Clouthier. For the next selection of aptamer pool from round 1 to 3 were performed by the author with the exception of the initial selection, which was performed by Sabina Sperandino. The amplification, extraction, and purification for the new selections were accomplished by the author. The protocol used was optimized by Dr. Sabina Speradino.

Writing:

The final research report was written by the author, with the assistance of Shahrokh Ghobadloo (PhD Candidate). The thesis was edited by the author and Elishah Cornelio.

1. INTRODUCTION

1.1 APTAMERS: ITS IMPORTANCE AND DIFFERENCE WITH ANTIBODIES

Antibodies are glycoproteins released by the immune system for the detection and removal of foreign antigens in the human body [1,2]. Antibodies are produced by the B-lymphocytes or T-lymphocytes and are generated in the bone marrow [1,2]. The basic structure of antibodies are composed of four main chains - two identical H domains and two identical L domains [2,3]. Each domain is composed of approximately 110 amino acids and the L domain serves as the "antigen-binding site" [2,3]. Antibody has high affinity and high specificity[4] making it an ideal molecule to use for defence mechanism to protect the human body from any harmful molecules or viruses such as HIV [5]. With its great properties, it is used in biological applications such as probes, radioimmunotherapy, imaging and many other more [2].

Just like antibodies, chemically synthesized oligonucleotide sequences, which are known as aptamers, have high affinity and high specificity to their target [4,6]. Aptamers are nucleic acid probes [7] that are made out of short single stranded RNA or DNA [4]. Despite their nucleic acid properties, the aptamers do not follow the Watson and Crick base pairing for recognizing its target [4]. Instead, an aptamer has a unique tertiary structure that makes it very ideal for binding [4]. Hence, its name was derived from the Latin word *aptus* meaning "to fit" and from the Greek word *meros* meaning "region" [4]. The nucleotide bases of the aptamer interact to one another or to its target protein resulting for its unique aptameric structure [4]. A single stranded DNA or RNA can conform into a hairpin, loop, pseudoknots, kink-turn, zipper and many other motifs [8,9]. As a result, aptamers are a great candidate in bioanalytical applications such as protein detection, electrochemical sensor, small molecule sensors and many other more [10].

Despite the similarities of antibodies and aptamers, there are still differences between the two. First, aptamers are easy to chemically synthesize [4,6] and it can be performed in *in vitro*. Meanwhile, antibodies are synthesized *in vivo* making it very dependable in animals or cells [4]. Second, aptamers have high affinity and specificity more than antibodies due to the absence of immune response[4]. Third, antibodies do not have the capability to be attached to receptor molecules, while, aptamers have this ability to attach on receptor molecules such as biotin [4]. Fourth, aptamers are cheaper than antibodies [11]. Lastly, aptamers are better tool for diagnosis than antibodies because they do not have any immunogenic response[11].

With its great advantages, it comes with limitations. DNA aptamers are more stable than RNA aptamers [11,12]. The nuclease degrades RNA aptamers faster than DNA aptamers because of the presence of hydroxyl group in the 2' ribose sugar [11,12]. DNA aptamers requires longer random sequences to form better 3D structures while RNA aptamers are more diverse in the formation of 3D structures [12].

1.2 APTAMER SELECTION USING SELEX

Aptamers are chemically synthesized oligonucleotides *in vitro* due to their advantages [4,13,14]. The *in vitro* synthesis of 10^6 of RNA or DNA pools is able to generate random sequences and unique structures of the oligonucleotides [13]. Back in the 1990s, a research was performed involving the RNA sequences from a random sequence pool, which were isolated base on the their affinity on the target protein [13]. This resulted in the development of "systematic evolution of ligands by exponential amplification" or also known as SELEX [13,14]. The SELEX follows the general overview seen in Figure 1. Generally, the aptamer generation starts from a library containing 10^{14} to 10^{15} random sequences [6,14]. The ssDNA sequences contain a fixed region on their 5' and 3' ends where complemented primers, which are 18 to 21 nt long, are able to

bind [15]. In between these fixed sequences contains the 20 to 80 nt long of random sequences [11,15]. For the first selection, the bound ssDNA molecules are separated from the unbound ssDNA by incubating the library with the target protein [15]. The separation can be done in various ways such as affinity chromatography, magnetic beads and many more [15]. The washes, incubation time, and concentration increase as SELEX rounds increases, which drive the evolution of the population of the pool resulting to an increase in its binding affinity to its target [14,15]. The bound ssDNA is eluted and amplified using PCR followed by purification. This creates the new enriched ssDNA pool that is used for the following rounds of SELEX [15]. Ideally, the 6 to 20 SELEX rounds are performed to acquire the DNA sequence with the best binding affinity to its target protein [15,16]. Once the desired round was achieved, the last SELEX round stops in the amplification process. The amplified products are used for cloning, sequencing, alignment analysis, and secondary structure analysis [15]. Overall, the SELEX is composed of basic steps which are binding, partition, elution, amplification, and condition [15].

1.3 MODIFIED SELEX: CELL-SELEX

Modified SELEX is performed for a more specific target. One of many kinds of modified SELEX is known as cell-SELEX [15,17]. Generally, cell SELEX is used to develop aptamers that target cells [15,17,18] , since aptamers are ideal for diagnosis and drug effective for pathogenic organisms [6]. The ssDNA is incubated with whole living cells compared to a standard SELEX, where it is incubated with proteins [18]. The great thing about aptamers is it can bind to the target protein on the cell surface without any great knowledge about the target's characteristic [18]. It typically follows the same procedure as the standard SELEX. However, a counter selection is usually performed in which the non-specific aptamers are removed from specific aptamers [17]. This is done by incubating the ssDNA to negative cells to select ssDNA binding only to

pathogenic cells and not to the non-pathogenic cells [17]. This process is known as the negative selection step, which the ssDNA is exposed to cell with non-target protein [6].

Studies used cell-SELEX for development of aptamers against bacteria cells with great affinity and specificity [19–21]. Dwivedi and colleagues successfully developed DNA aptamer that was used to sort Sa*lmonella* Typhimurium[19]. Interestingly, it used biotinylated DNA aptamer instead of dye-DNA aptamer for the recognition of the cells to avoid the destruction of the 3D structure[19]. Bitaraf and colleagues revealed in their studies that using cell-SELEX for aptamer selection amplified the binding affinity and signal strength compared to the normal SELEX [20].

Cell-SELEX is ideally used for development of probes using aptamers, specifically for detecting cancer cells [6,17,18,22,23]. A research was performed by Prabodhika et al, which aptamers were developed using cell-SELEX as probes for the target cells [24,25]. In this project, they identified protein expression in the leukemia cells in which they successfully used aptamer to recognize Burkitt's lymphoma membrane proteins [24,25].

Moreover, cell-SELEX is modified to detect transmembrane proteins [6]. Aptamers were developed through cell-SELEX for the detection and inhibition of the RET receptor tyrosine kinase, which is a transmembrane protein [6,26]. Interestingly the aptamer selection against transmembrane protein required a negative selection step because of the difficulty of in obtaining a purified transmembrane protein [6]. The general overview of the modified cell-SELEX is seen in figure 1.2. Using this technique, aptamers are used as probes for medical and pharmaceutical applications.

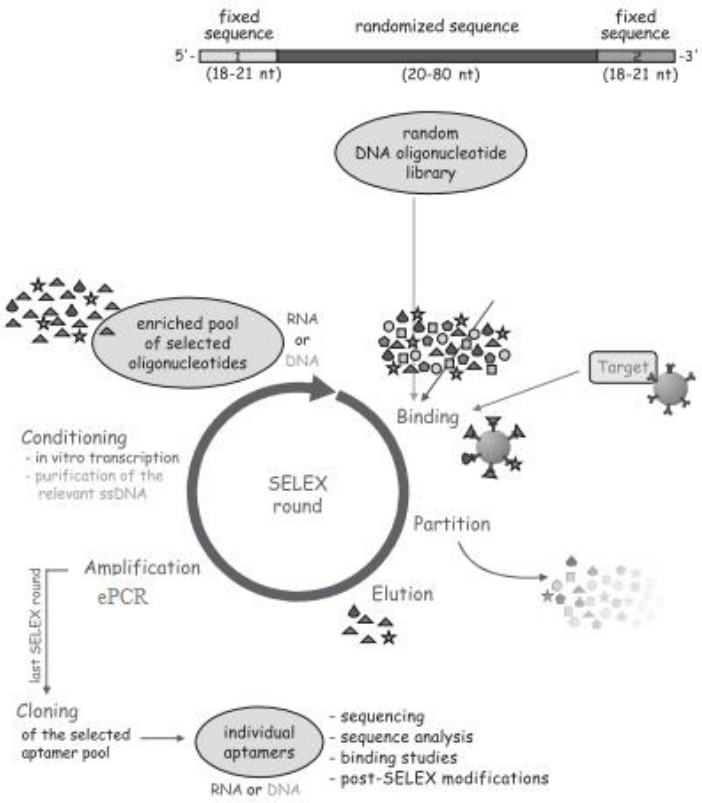

Figure 1.1 Aptamer selection using SELEX protocol. This image was modified from [15]. Displays the general SELEX protocol used to develop aptamers against the target proteins for this project.

1.4 APTABID USED FOR VERIFICATION OF TARGET PROTEIN

Aptamers are great candidates to use as probes for biomarkers because of their high sensitivity and specificity to their targets [18]. Biomarkers are used to diagnose and to get information on the development of the disease [27,28] . Moreover, biomarkers can be used to determine the susceptibility of one to a disease and to identify the pathological process[27]. In the past few years, biomarkers were developed through various techniques such as Western blotting and 2D gel electrophoresis accompanied with mass spectrometry [28]. These techniques

increases the chances of false positive and false negative results, which makes these huge barrier for biomarker development [28].

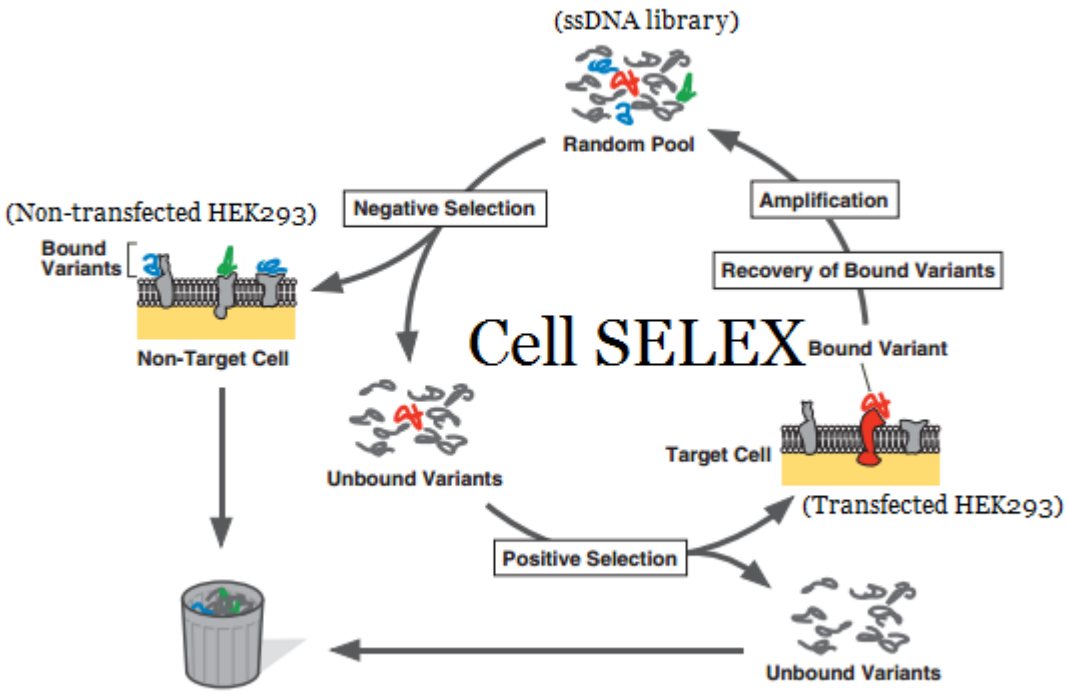

Figure 1.2 Modified Cell-SELEX schematic overview. The cell-SELEX is the technique used to perform aptamer selection on the whole living cells. Aptamers were selected against transmembrane protein. To remove non-specific binding to other proteins, negative selection is performed. This image was modified from [6].

The discovery of AptaBID, aptamer-facilitated biomarker discovery, overcame this limitation. The general overview of AptaBid is displayed in figure 1.3. First, aptamer selection is performed using cell-SELEX [28]. To develop aptamers for the biomarkers, the selected aptamers are incubated with the target cells [28]. The bound aptamers are then extracted from the target cells followed by their analysis using mass spectrometry [28]. Using this technique, it lessens the false negative and false positive results [28]. With its great advantage, aptaBID is greatly used for the isolation and verification of the target proteins.

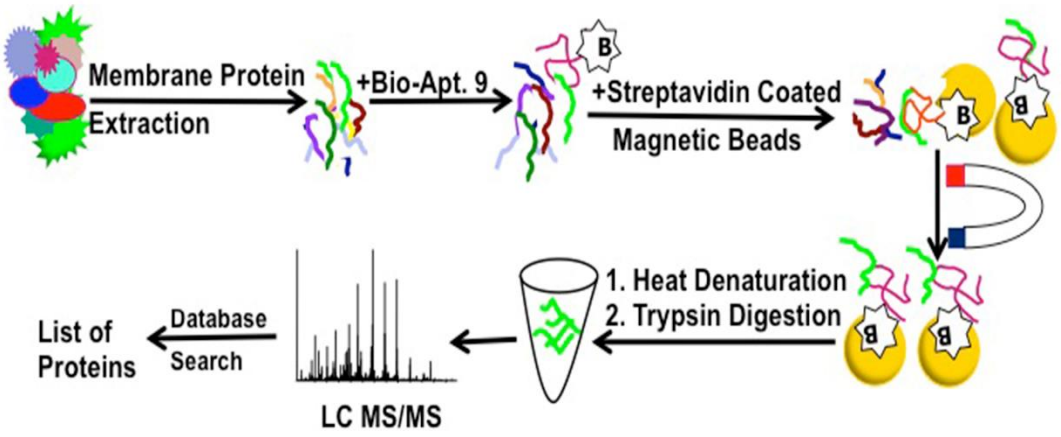

Figure 1.3 AptaBID scheme used for verification of target proteins. The targeted protein was incubated with the selected aptamer conjugated with biotin (Bio-Apt. 9). The bound aptamer is extracted using the streptavidin coated magnetic beads. Afterwards, the aptamer is eluted by exposing the solution in high temperature. (This figure is used with the permission of Shahrokh Ghobadloo's thesis).

1.5 AXL: A TYROSINE KINASE RECEPTOR

AXL is a gene encoding for tyrosine kinase receptor found on the cell surface of the cells [29,30]. It is a transmembrane protein composed of an extracellular N-terminal domain and an intracellular C-terminal domain with a molecular weight between 100 to 140 kDA [31]. Tyrosine kinase receptors are responsible for activating signalling pathway inside the cells when hormone binds to the receptor [29]. Basically, it activates cell proliferation, differentiation, migration, survival, and adhesion [29,32] as seen in figure 1.4. The AXL gene is found in chromosome 19q13.2 and encodes 20 exons. Its name was derived from the Greek word *anexelekto,* which means uncontrolled [29,32].

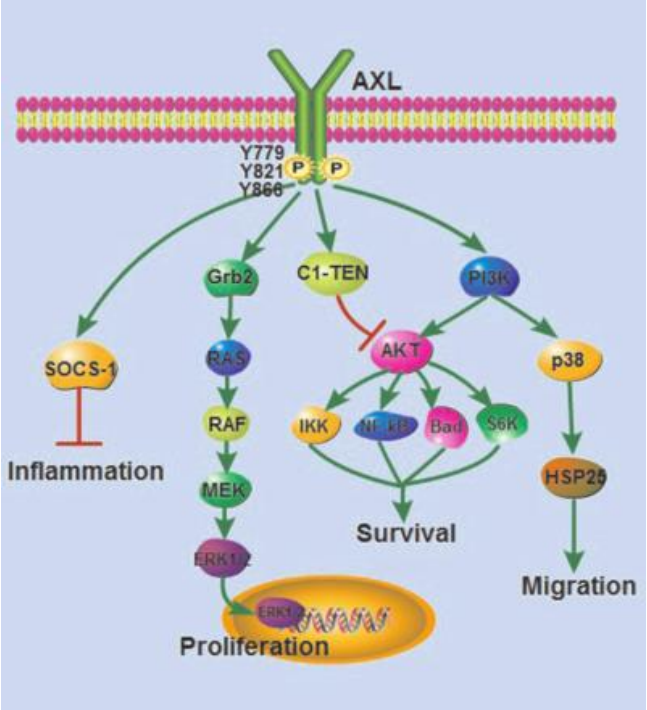

Figure 1.4 Activation of signalling pathway via AXL. Once the protein binds to the AXL receptor, the AXL dimerizes and it becomes phosphorylated. This activate the signalling pathway inside the cell. It is responsible for the inflammation, proliferation, survival and migration. (This figure is taken from [32].

Since AXL is from a tyrosine kinase family, it plays an important role in cancer development [30]. Hence, AXL was discovered from myeloid leukemia cells [30,32]. Many researches were performed in the understanding of AXL expression in cancer cell lines. The overexpression of AXL in prostate cancer and pancreatic adenocarcinoma promotes cell migration and higher metastasis frequency [32,33]. Moreover, it is highly expressed in lung cancer, which it promotes cell migration [32]. AXL expression is increased in breast cancer, thyroid cancer, ovarian cancer, lung cancer and other types of cancer [22,32,34]. Hence, AXL could be a great cancer marker.

AXL receptors are not only present in cancer cells but also in chronic immune disorders, [31] because AXL is important for the inflammatory response of T helper cells [35]. AXL is used

for diagnosis of cardiovascular diseases [31] due to its role in the survival of T lymphocytes [36]. Overall, the presence of AXL in cancer, chronic immune disorders, and cardiovascular diseases makes it a great candidate as the target protein for developing probes using aptamers.

1.6 IGG FC REGION AS THE TARGET PROTEIN

Just like transmembrane proteins, antibodies can be used as the target proteins for aptamer development. Since antibodies play a crucial role in the immune system, many studies have been done for aptamer development. One of the most common types of antibodies in human serum is called immunoglobulin G (IgG) [37]. IgG plays an important role in neutralizing non-self-molecules such as viruses and toxins [37]. Every antibody consists of the Fab, which is the area on the antibody that antigen binds to, and Fc, which is the area that activates the effector functions [38]. Fc fragment is the constant region in the antibodies. Since this is constant, a great interest is performed for the aptamer selection against the FC fragment of IgG. So far, only RNA aptamers were developed against the IgG FC regions. The research performed by Yoshida and colleagues developed RNA aptamer pool against rabbit IgG using a different amplification steps[39]. Another study performed by Miyakawa and colleagues explored the development of RNA aptamers against the human IgG Fc region with high affinity and specificity [40]. Ma and colleagues successfully developed DNA aptamers against the mouse IgG FC region using the target replacement SELEX within 8 rounds of selection[41]. Target replacement SELEX was used in order to develop DNA aptamers that universally recognizes the FC fragment of all types of IgG [41].

1.7 RESEARCH OBJECTIVE

The objective of this project is to first, develop aptamer probe that recognizes cells expressing AXL receptor. To eventually evaluate the binding of selected DNA aptamers to cells expressing AXL receptor in the whole blood leukocytes using mass spectrometry and flow cytometry. Thus, single stranded DNA aptamers function like antibodies and it is able to recognize its target protein. Second is to develop aptamer pools with best binding to the human FC fragment. To determine the aptamer pool with the best product yield and binding using agarose gel electrophoresis and flow cytometry. Therefore, the higher rounds of aptamer pool SELEX will result to aptamers with higher binding affinity.

2. Chapter 1.

Aptamer selection for cells expressing AXL receptors

2.1 MATERIALS AND METHODS

2.1.1 PREPARATION OF THE CELL LINES

In this project, the cell line that was used was HEK 293, which is short for human embryonic kidney. The cells were acquired from Clonetech Laboratories and using Dulbecco's modified Eagle medium with the addition of 10% fetal bovine serum, the cells were cultured. Doxycycline (Clontech; Cat# 631311) with a concentration of 500 ng/mL was added to the media in order to culture transfected cells with the expression of AXL receptor.

2.1.2 LIST OF ANTIBODIES USED

Multiple antibodies were used to detect the presence of the target protein, AXL protein. These antibodies were primary antibodies for the AXL receptor (R&D Systems, Cat#MAB6965), PE conjugated antibody for the AXL receptor (R&D Systems, FAB154P), and antibody for rabbit IgG with conjugate Dylight 488 (DI-1488) (Vector Laboratories). For the flow cytometric analysis, CD markers were used, which were antibodies against CD3 conjugated with FITC (Cat #60011FI.1) that were obtained from Stemcell Technologies. Moreover, antibody against CD4 conjugated with PCy7 (Cat#344611) were acquired from Biolegend.

2.1.3 AXL CDNA INCORPORATION TO PLVX-TRE3G VECTOR

The open reading frame of AXL gene was obtained from the bacteria with a plasmid (pDONR223-AXL) acquired from the addgene (Plasmid# 23945) [42]. The bacteria were cultured and their vectors were removed. Using the CloneAmp HiFi PCR Premix (Clonetech Laboratories, INC., Cat# 639298), cDNA was created from the AXL vector. Specific forward and reverse primers for the AXL gene were used with the sequence of 5' ATG GCGTGGCGGTGCCCCA 3' and 5' TCAGGCACCATC CTCCTGCCCT 3' respectively. Along with these sequences, the primers had 15 basepair (bp) extension with a similar sequence to the vector ends of pLVC-TRE3G. The forward primer extension had a sequence of GCCCCCGGGACGCGT, while the reverse primer extension's sequence was CTACCCGGTAGAATTC. The pLVC-TRE3G was linearized by digesting the pLVC-TRE3G with the restriction enzymes MluI (New England Biolabs; Cat# R0198S) and EcoRI (New England Biolabs, Cat# R0101S). The digestion was performed for 3 hours at 37°C. To purify the linearized vector, it was ran in 1% agarose gel followed by an extraction with GeneJet Extraction Kit (Thermo Scientific, Cat# K0692). Afterwards, the PCR products were

cloned into pLVC-TRE3G using Fusion HD Cloning kits (Clontech Laboratories, Inc., Cat. # 639648).

2.1.4 CELL TRANSFORMATION

The 2.5 ng pLVX-TRE3G mixture was added to 50 µL of competent cells. The mixture of the pLVX-TRE3G with cells was incubated for 30 minutes on ice. It was followed by heat shocked at 42°C for 45 seconds. The solution was immediately placed on ice for another 1 to 2 minutes. Before the solution was cultured, Super Optimal Broth with Catabolite repression medium (SOC) was mixed to the solution-to create a total volume of 500 µL. Agar medium with 100 ampicillin µg/ml was used to culture the pLVX-TRE3G with cells solution.

2.1.5 PRODUCTION AND TRANSDUCTION OF LENTIVIRUS

To construct the lentivirus, the lenti-XTM Tet-On 3G Inducible Expression System was used (Clontech, Cat# 631187). By using the In-Fusion HD (Clontech, Cat# 638909), the AXL was incorporated to the pLVX-TRE3G vector. The 293T cells and pLVX-TRE3G- Axl were transfected using Lenti-X HTX Packaging System (Clontech, Cat# 631187) to create lentiviruses. For 48 hours, the transfection of the cells took place and afterwards, the virus particles were obtained. Using PLVX-Tet3G, the HEK293 cells were infected. The infected HEK293 cells were cultured and selected with 1 mg/mL of G418 (Clontech, Cat# 631307). The selected cells were collected and they were once again infected with pLVX-TRE3G-AXL viruses. The cells were then cultured in a 10cm plate with 25 µg/mL of puromycin (Clontech; Cat# 631305) for a new selection. In order to induce the AXL expression, 500 ng/mL of doxycycline (Clontech; Cat# 631311) was added.

2.1.6 CELL STAINING USING IMMUNOFLUORESCENCE

In order to stain the cells, 4% of paraformaldehyde was used to fix the cells and the cells were permeabilized with 0.25% Triton X-100 (Sigma, St. Louis, MO). It was by the blocking of the cells with 10% fetal bovine serum (Gibco, CA). Primary antibody against AXL (R&D Systems, Cat# MAB6965) was added to the cells and they were incubated for 1 hour at room temperature. Dylight 488-conjugated secondary antibodies (Vector Laboratories) were placed on the cells and it was incubated for another 1 hour at room temperature. This was followed by multiple washes to remove unbound antibodies. The 4′, 6′-diamidino-2-phenylindole (DAPI; Sigma) were used to counterstain the cells. The cells were viewed with the fluorescence microscope and confocal microscope (Nikon).

2.1.7 FLOW CYTOMETRIC INVESTIGATION FOR THE EXPRESSION OF AXL RECEPTOR

The cells that were transfected, non-transfected, and induced with doxycycline were suspended in a single-cell suspension. The PE-conjugated antibody against Axl receptor (R&D Systems, FAB154P) was added to the cells and it was incubated for 1 hour. The expression of the AXL on the cells was analyzed by Beckman Coulter FC500 flow cytometry.

2.1.8 CELL-SELEX FOR APTAMER SELECTION AGAINST AXL RECEPTOR

Single stranded (ss) DNA library, which contained 60 nucleotides (nt) random sequences and a 5’ and 3’ fixed ends, was used for the aptamer selection. The library sequence was 5’ – CTCCTCTGACTGTAACCACG – N60 – GGCTTCTGGACTACCTATGH – 3’ (IDT DNA Technologies, USA). The ssDNA library was subjected to the PBS with Ca^{2+}/Mg^{2} and it was denatured at 95°C. After 10 minutes of denaturation, the solution was immediately placed on ice. To select aptamers against AXL receptor, the ssDNA library was added to cells

that expressed AXL receptors. The ssDNA library was incubated with the cells at 37°C for 1 hour. The unbound ssDNA was removed by washing the cells first with PBS with Ca^{2+}/Mg^{2+} followed by centrifugation at 200g. The collection of the bound ssDNA was performed by heating the solution for 5 minutes at 95°C. For a specific binding of the ssDNA, negative selection was performed in order to remove the non-specific binding in which the non-transfected HEK293T was incubated with the collected ssDNA for 1 hour at 37°C. The non-specific ssDNA was bound to the HEK293T, in which the supernatant was collected since it contained the specific ssDNA. For every 10 rounds of positive selection, one round of negative selection was performed. Once the desired ssDNAs were collected, they were amplified with 25 cycles of PCR using Thermo Scientific Phire Hot Start II DNA Polymerase (Thermo Scientific). The master mix used contained 1x Phire Reaction Buffer, 3% Dimethyl Sulfoxide (DMSO), 1 µL of the Phire Hot Start II DNA Polymerase (Thermo Scientific), 200 µM dNTPs, 0.5 µM of 5'-Cy5-conjugated forward primer, and 0.5 µM 5´-phosphorylated reverse primer. To complete the PCR mixture, the ssDNA was added and it was transferred to PCR tubes. The amplification was performed with the thermal cycler with a set program. It first starts on melting step for 30 seconds at 95 °C, followed by annealing step for 15 seconds at 58°C, and it was finished by extending step for 10 seconds at 72°C. Double stranded (ds) DNA molecules were acquired after the amplification and they were digested at their 5' phosphorylated reverse strand by Lambda Exonuclease (New England Biolabs, Cat#M0262S) by following the manufacturer's protocol, in order to obtain ssDNA.

2.1.9 SSDNA SEQUENCING AND ANALYSIS OF PHYLOGENETIC TREE

Once again, PCR was used to amplify only the pools with the best binding to the target receptors. The barcode primers were used this time. Afterwards, the amplified pool was ran in

a 4% agarose gel (Invitrogen) and purified using the GeneJET Gel Extraction Kit (Thermo Scientific, Canada, Cat# K0691). The 200 ng of purified products were obtained and they were sequenced using Erofins MWG Operon LLC (Eurofins Genomics company). The Illumina MiSeq paired end was used to read the sequences twice for 150 bases. Afterwards, the Illumina sequencing gave 20 million reads, which were the total number of reads for both forward and reverse read. The Galaxy project platform (https://usegalaxy.org) was used to upload the obtained fastq files. The fastq files were then transformed to FASTA [43], which led to the creation of the FASTA format, which made it easier for data organization using their barcodes. The data was organized into groups using their common motifs, and MEME (http://meme.nbcr.net/meme/) was used to analyse the most prevalent sequences present in each group. This was followed by studying the phylogenetic tree through the use of Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Finally, for each clusters of the analyzed sequences, which was acquired from the organized group, was synthesized by IDT (DNA Technologies, USA).

2.1.10 BINDING AFFINITY OF THE OBTAINED APTAMER

Assay was performed using flow cytometry, Beckman Coulter Gallios, to analyze the binding affinity of the aptamers obtained. First, different concentrations (0, 50, 100, 200 and 400 nM) of the aptamer, Cy5 labelled Apt-AXL-1615, were added to the HEK293 cells in order to identify 50% of the aptamers bound (EC_{50}) to HEK293 cells. After incubation, the cells were washed and were ran in flow cytometry; then the obtained data was analyzed using Kaluza analysis software. The EC_{50} was measured using the mean fluorescence intensity acquired.

2.1.11 PURIFICATION OF THE BIOMARKER

The hypotonic buffer was prepared using 50 mM Tris-HCl with a pH of 7.5 and it was mixed with Halt Protease Inhibitor (Thermo Scientific, Cat#78430). The hypotonic buffer was used to lyse the whole blood leukocyte cells and it was incubated for 30 minutes at 4 °C. Afterwards, the hypotonic buffer was used to wash the cells three times, followed by incubation of the cells on ice with 1 mL of PBS with Ca^{2+}/Mg^{2+} containing 1% Triton X-100 for 30 minutes. The cells were lysed using 25G needle. After lysing, the supernatant was collected by centrifugation at 17 000g in 4 °C for 5 minutes. The yeast RNA was added to the supernatant with a concentration of 1 mg/mL. After the incubation with the yeast, 150 pmol of 5' biotin conjugated Apt-AXL-1615 was added to the sample. The control sample was prepared by adding the 5'biotin conjugated ssDNA library to sample and it was incubated for 30 minutes on ice. In order to isolate bound ssDNA from unbound ssDNA, 2 mg of streptavidin-coated magnetic beads were added the sample. The samples with the beads were incubated for 15 minutes on ice. After incubation, the bound ssDNA was isolated using the magnetic holder by washing four times using 1 mL of PBS containing with Ca^{2+}/Mg^{2}. The 20 mM of EDTA was added to the water creating a total volume of 30 µL and it was added to the ssDNA bound to the magnetic beads for the elution of the ssDNA. The supernatant with the desired ssDNA was isolated from the magnetic beads using the magnet holder.

2.1.12 IDENTIFICATION OF PROTEIN

The proteins were first isolated with 12% of Sodium Dodecyl Sulfate-Polyacrylamide gel (SDS-PAGE) followed by gel staining with QC Colloidal Coomassie Stain (161-0803). The desired bands were Apt-AXL-1615 bound to the protein. The bands were excised and digested with Pierce Trypsin Protease, MS Grade (Thermo Scientific, Cat#90057) *in situ.* The digestion of the bands was performed according to the manufacturer's protocol. The pipet tip

packed with C18 (ThermoScientific, Cat# 87782) was used to purify the obtained peptides followed by the addition of 10 µL of 50% methanol/1% formic acid for further peptide analysis. The peptides were analyzed using Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer.

2.2 RESULTS

2.2.1 TRANSFECTION OF AXL RECEPTOR

The target of this project was to find an aptamer that binds to the AXL receptor. To perform aptamer selection, Cell-SELEX was used [44] in which it required transfected human embryo kidney (HEK293) with the lentiviral gene containing the AXL receptor gene. For the confirmation of the successful cell transformation, immunofluorescence and flow cytometry were performed by Shahrokh Ghobadloo. The transfected cells were exposed to 500ng/mL of doxycycline. As seen in figure 1a, expression of the AXL receptor increased in the presence of doxycycline. In addition, figure 1b displays the flow cytometric comparison of the expression of AXL receptor in transfected cells induced by doxycycline. The induced cells (purple curve) have higher intensity than the non-induced cells (black curve).

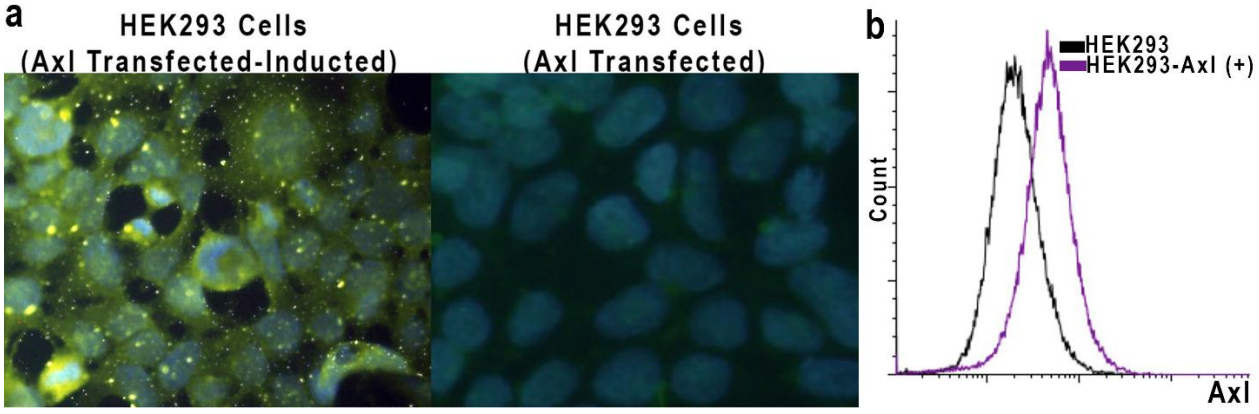

Figure 2.1 Verification of AXL receptor expression in transfected HEK293 using immunofluorescence and flow cytometry. (a) The transfected HEK293 cells induced with

doxycycline (left) or without doxycycline (right) for the expression of AXL was checked using immunofluorescence. The cells were first incubated with primary antibody that is against the AXL. The cells were washed twice before it was incubated with dylight 488 conjugated secondary antibody. The cell was stained by DAPI. (b) The expression of AXL was checked using flow cytometry in the presence and absence of doxycycline. The purple peak represents the doxycycline induced cells, while the black peak represents the non-induced cells. (This image is used with the permission of Shahrokh Ghobadloo's Thesis).

2.2.2 APTAMER SELECTION AGAINST AXL RECEPTOR

Once target cells with and without AXL receptor were ready, they were used in the cell-SELEX for the aptamer selection. Ten rounds of cell-SELEX were performed in which Cy5-conjugated single stranded (ss) DNA library was used as the starting pool for the first round of selection. For each round, it generated Cy5-conjugated ssDNA pool that was used in the subsequent rounds of selection. The obtained ssDNA pools were analyzed using flow cytometry. The pools generated from higher cycles, which were the 9^{th} and 10^{th} round pools displayed better binding to the target cell compared to the initial ssDNA library, as seen in Figure 2.2. The increase in binding was indicated by the right shift of the peaks in Figure 2.2. The pool with the best binding was amplified using the barcode primers and then was purified and sequences afterwards (NGS). The sequences were read twice by Illumina MiSeq paired end for 150 bases. FASTA format of the data was created from the fastq files, which were organized and categorized based on the common motifs using Galaxy. As a result, four different aptamer sequences were obtained (Table 2.1), which were the most abundant sequences from the generated clusters. Once the sequences were obtained, the secondary structures of each sequence was determined using the RNAstructure web server, which is displayed in Figure 2.3.

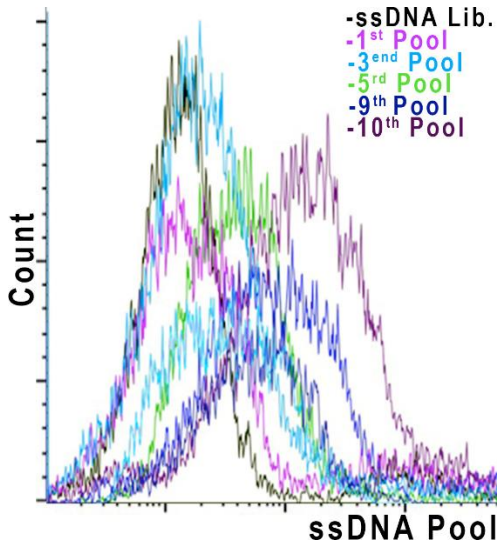

Figure 2.2 Different aptamer pool selection binding to AXL receptor of transfected HEK 293 cells. Each ssDNA, selected by Cell SELEX, was conjugated of Cy5. The histogram obtained from flow cytometry displayed the binding of the ssDNA pool to the AXL receptor of the transfected HEK 293 cells. (This image is used with the permission of Shahrokh Ghobadloo's Thesis).

Table 2.1. Obtained aptamer sequence. The ssDNA pools were sequenced using Eurofins MWG Operon LLC. The sequences were read twice by Illumina MiSeq paired-end for 150 bases. The FASTA format sequences were clustered and categorized based on its common motifs. Afterwards, the most abundant sequences in each cluster were sequenced and arranged in this table. (This is used with the permission of Shahrokh Ghobadloo's Thesis).

Aptamer	Sequence
Apt-Axl-1615	CTCCTCTGACTGTAACCACGGCGTACGTGACCACATACGCACTCTAGCCA CATACGTTCGCCCACGCATGTAGCGCGCGGGCATAGGTAGTCCAGAAGCC
Apt-Axl-25926	CTCCTCTGACTGTAACCACGACACATGCAGTGGTGTTTGTTCATGCGTAC ATGTCTACGTGTGCGAGTTTGATGCGCGTCGCATAGGTAGTCCAGAAGCC
Apt-Axl-22481	CTCCTCTGACTGTAACCACGACACATGCAGTGGAGTTTGTGTCATGCGTA CATGTCTACGTGTGCGAGTTTGATGCGCGTGCATAGGTAGTCCAGAAGCC
Apt-Axl-25504	CTCCTCTGACTGTAACCACGACACATGTAGTGGTGTTTGTGTCATGCGTA CATGTCTACGTGTGCGAGTTTGATGCGCGTGCATAGGTAGTCCAGAAGCC

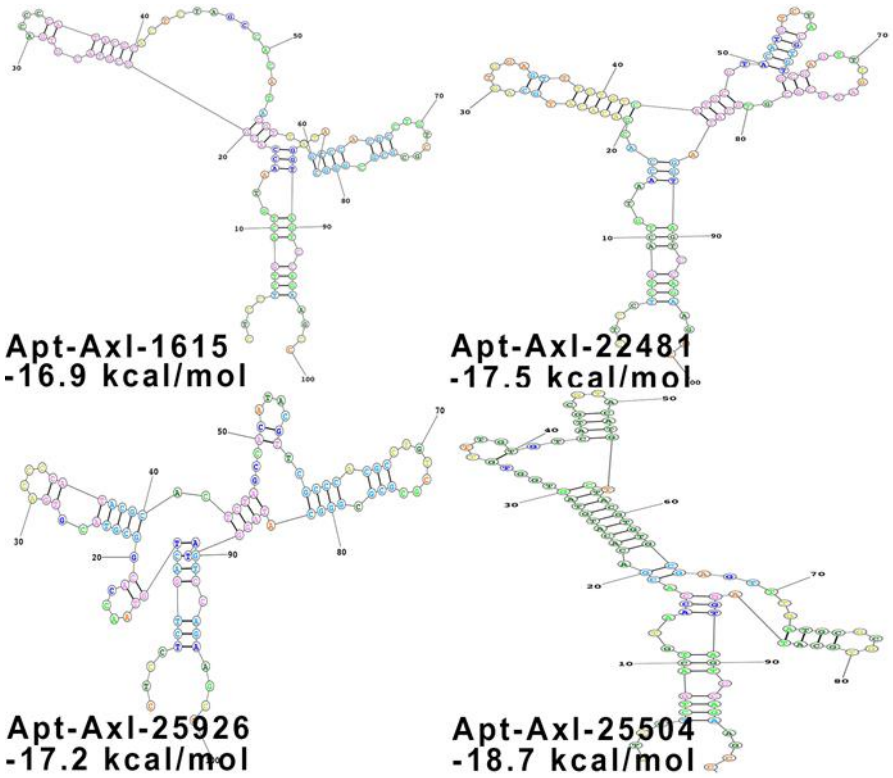

Figure 2.3 The selected aptamer's structure. The sequences of the selected aptamer obtained from table 1 were used to determine its secondary structures. Along with this, the minimum free energy were also assessed for each of the sequences. In order to obtain this, RNAstructure web software was used [45]. (This is used with the permission of Shahrokh Ghobadloo's Thesis).

2.2.3 SPECIFIC BINDING OF SELECTED APTAMERS

The four selected aptamers were labelled as Apt- AXL-1615, Apt-AXL-22481, Apt-AXL-25926, and Apt-AXL-25504. Flow cytometric analysis was ran with these 4 selected aptamers to compare their binding with Cy5 conjugated ssDNA library to the target cell. The Cy5 conjugated ssDNA library was incubated with the transfected HEK293 induced with doxycycline, which the black peak (Figure 2.4) represents the intensity of its binding to the target cell. The selected Cy-5 conjugated aptamers were incubated for both cells expressing AXL (purple peak) and not expressing AXL (yellow peak) cells. Figure 2.4 displayed an increase in intensity in the selected aptamers in the cells with positive expression of AXL

compared to the cells with negative expression of AXL and to the ssDNA library. Moreover, Apt-AXL-1615 had the lowest overlap between the yellow and the purple peaks. The rest of the selected aptamer displayed big overlap between the yellow and the purple peaks.

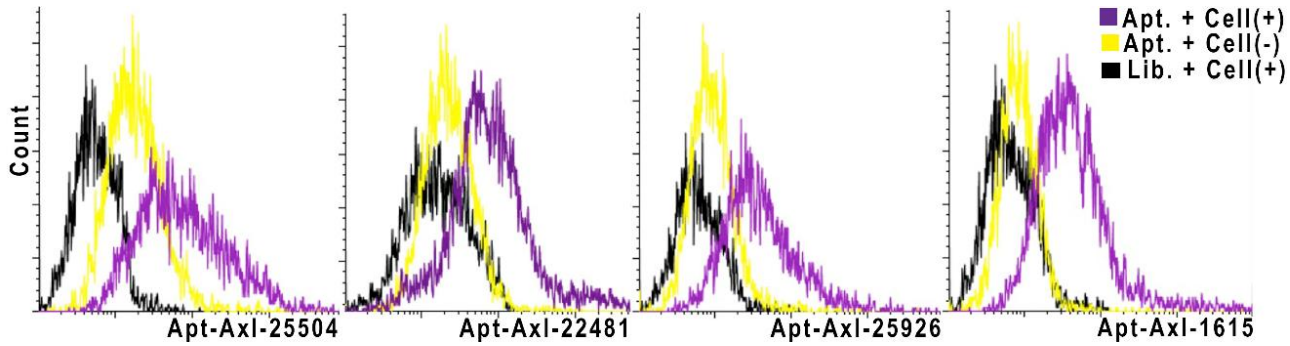

Figure 2.4 Different kinds of aptamers with specific binding to transfected HEK 293 cells. The binding of the aptamer conjugated with Cy5 was evaluated with flow cytometry. Its binding was compared with the ssDNA library against the transfected and non-transfected HEK 293 cells. The black peak represents the ssDNA library binding to the cells with positive expression of AXL. The yellow peak represents the selected aptamer to the cells with negative expression of AXL. The purple peak displays the aptamer binding to the cells with positive expression of AXL. (This image is used with the permission of Shahrokh Ghobadloo's Thesis).

2.2.4 VERIFICATION OF APT-AXL-1615 BINDING

Further studies were done using Apt-AXL-1615 in which fluorescence microscopy was used. This experiment was performed to verify the binding of Apt-AXL-1615 to the AXL receptor. First, the transfected AXL receptor HEK 293, without the Apt-AXL-1615 was subjected to a primary antibody against the AXL receptor. Followed by incubation and washes, the secondary antibody conjugated with dylight 488 was added and incubated with the cells. After the antibody staining, the cells were viewed under the microscope. Then, Apt-AXL-1615 was added to the medium and images were taken. The images with and without Apt-AXL-1615 were merged together as seen in Figure 2.5 to verify the binding of the Apt-AXL-1615. The overlapping green and red generated a brighter yellow colour, which indeed verified the binding of the Apt-AXL-1615 to the AXL receptors.

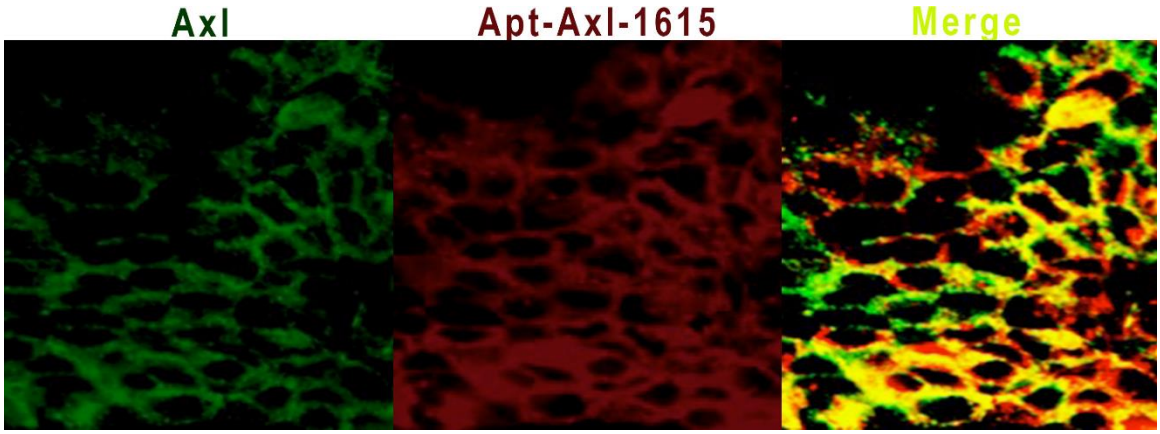

Figure 2.5 Verification of aptamer binding to AXL receptor. Using microscopic studies, the transfected AXL receptor HEK 293 without the aptamer was compared to the transfected AXL receptor HEK293 with the aptamer. A primary antibody against the AXL receptor was used. In order to view the image, a secondary antibody conjugated with dylight 488 was used, which was against the primary antibody. (This image is used with the permission of Shahrokh Ghobadloo's Thesis.)

2.2.5 SORTING OF WHOLE BLOOD CELLS BOUND TO APT-AXL-1615

Using whole blood cells, the binding of the Apt-AXL-1615 in the heterogeneous tissues can be validated. First, the leukocytes from the whole blood cells were collected and it was incubated with the Cy5- conjugated Apt-AXL-1615 in PBS with Ca^{2+}/Mg^{2+}. Using FACS, 11.3% of leukocytes were bound to the Apt-AXL-1615 and these were isolated and gated. The sorted leukocytes were stained with PE conjugated antibody against the AXL receptor, followed by flow cytometry analysis. Figure 2.6 displays the flow cytometry analysis in which the 98.6% of leukocytes were positively bound to Apt-AXL-1615 while 1.4% of the leukocytes were negatively bound to Apt-AXL-1615. Further analyses were done to the leukocytes with positive binding to Apt-AXL-1615, in which antibody against the AXL receptor was added. When both antibodies against AXL receptor and Cy5 conjugated Apt-AXL-1615 were present, these were categorized as double positives, which were displayed by the 93.8% of the sorted leukocytes. On the other hand, when only the antibody against the

AXL receptor was present, it was categorized as negative of Apt-AXL-1615, which was represented by 3.9% of the sorted leukocyte population. Similarly, the 2.2% of the Apt-AXL-1615 was not bound to the leukocyte cells, which were categorized as negative of leukocytes.

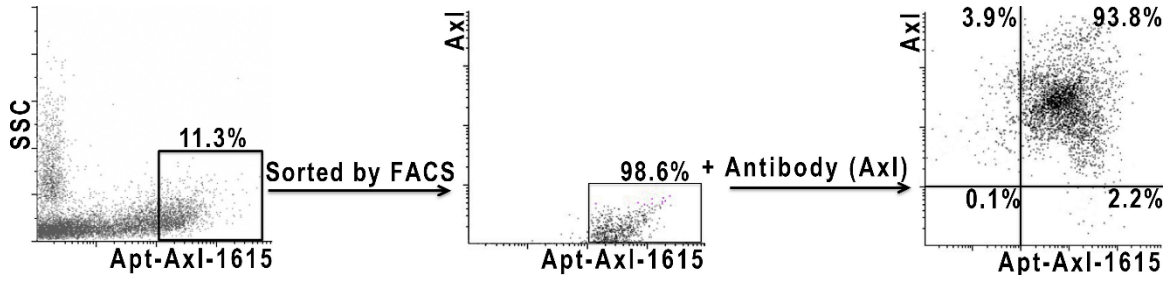

Figure 2.6. Binding of Apt-AXL-1615 to whole blood cell. The Cy5 conjugated aptamer binds to the leukocyte of the whole blood cell. The Apt-AXL-1615 bound to the leukocytes were isolated by FACS. The isolated leukocytes were stained with PE conjugated antibody that is against the AXL receptor of the cell. The cells were gated with the side scatter (ssc) on the y-axis against the Apt-AXL-165 on the x-axis. (This image is used with the permission of Shahrokh Ghobadloo's Thesis).

2.2.6 WHOLE BLOOD ANALYSIS

The human whole blood leukocytes were incubated with PE conjugated antibody against the AXL receptor and they was analyzed using flow cytometry. In Figure 2.7, only 12% of the leukocytes had AXL receptor present. Further analyses were performed, which the 12% of leukocytes were incubated with multicolor antibodies against CD3 and CD4. The flow cytometry dot plot displayed 78% of the sorted leukocytes as double positive in CD3 and CD4. On the other hand, 2% were CD4 positive but CD3 negative, 16% were CD3 positive but CD4 negative, and 4% were CD4 and CD3 negative. The remaining whole blood leukocytes (88%) were also further analyzed using flow cytometry and they were incubated with multicolour antibodies against CD3 and CD4. About 44% of the leukocytes were CD4 and CD3 positive, 9% were CD4 positive CD3 negative, 14% CD4 negative and CD3 positive, and 33% were both CD4 and CD3 negative.

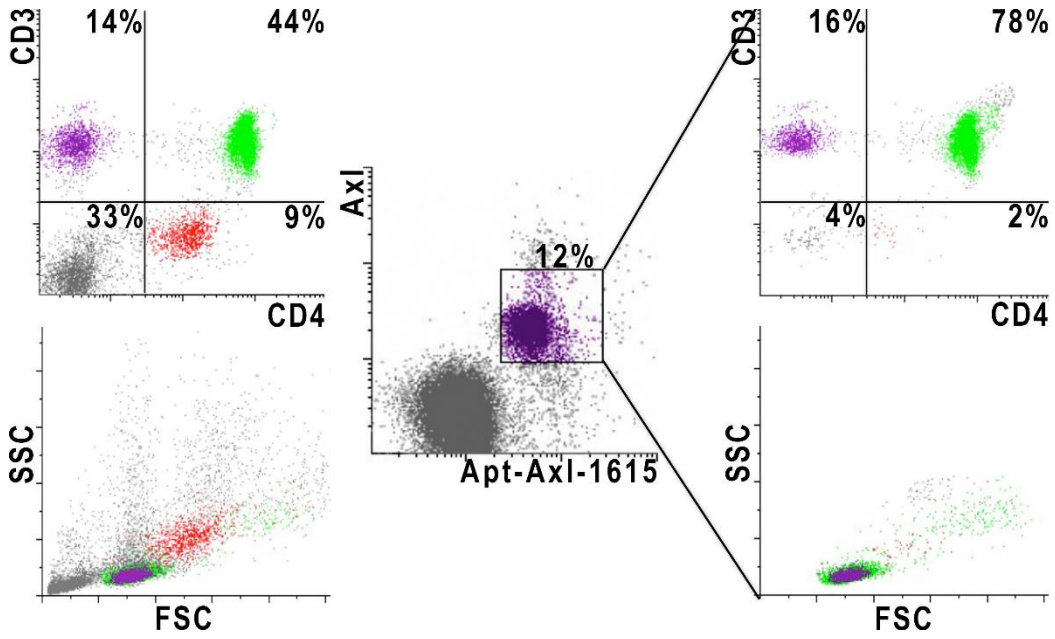

Figure 2.7 Identification of whole blood leukocytes. Using flow cytometry, the whole blood leukocytes were gated using antibody against AXL tyrosine kinase, CD3 and CD4. After gating, the cells with positive binding between the Apt-AXL-1615 and AXL tyrosine kinase were sorted. The side scatter (SSC) and forward scatter (FSC) dot plot displayed the group of the leukocytes with the negative and positive binding. (This image is used with the permission of Shahrokh Ghobadloo's Thesis).

2.2.7 BINDING ASSAY OF APT-AXL-1615

Different concentrations of Apt-AXL-1615 ranging from 0 to 400 µM were added to the transfected HEK293. The cells were incubated at room temperature in PBS with Ca^{2+}/Mg^{2}. After incubation, the cells were washed and ran in the flow cytometry. The flow cytometry generated the graph displayed in Figure 2.8 in which the x axis, Apt-AXL-1615, displays the intensity of the aptamer bound to the cell. This experiment was performed in order to identify the 50% of the aptamers bound to the transfected HEK293 cells. As seen in Figure 2.8, as there is an increase in the Apt-AXL-1615 concentrations, the more the peaks shifted to the right, which corresponds to the increase in intensity.

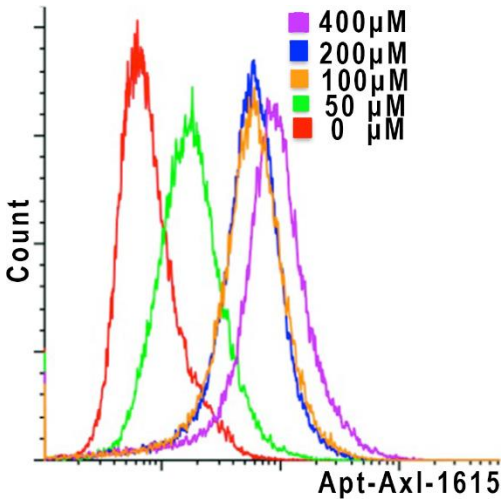

Figure 2.8 Different concentration of Apt-AXL-1615 in transfected AXL receptor of HEK 293 cells. A titration of the aptamer was performed in which different concentrations ranging from 0 to 400 µM of the Apt-Axl-1615 was incubated with the transfected AXL receptor of HEK 293 cells. The cells were incubated at room temperature in PBS with Ca^{2+}/Mg^{2}. The incubation time was 30 minutes. After the incubation, the cells were washed. This is followed by the flow cytometry analysis, where the titration graph was obtained. The x axis displayed the cell count and the y axis is the intensity of the Cy5 conjugated Apt-AXL-1615. (This image is used with the permission of Shahrokh Ghobadloo's Thesis).

2.2.8 ISOLATION OF APT-AXL-1615 WITH ITS TARGET PROTEIN

The whole blood leukocytes were lysed and their membrane proteins were extracted in PBS with Ca^{2+}/Mg^{2}. Afterwards, the extracted membrane proteins were incubated with 5'-biotinylated aptamer 9 that was developed by Sharkokoh Ghobadloo. This created the biotinylated Apt-AXL-1615 for an easier isolation of the membrane protein bound to Apt-AXL-1615, which was pulled by the streptavidin coated magnetic beads. The magnetic beads were isolated from the membrane protein bound to Apt-AXL-1615 by heating the solution for 5 minutes at 95 ˚C. The bound Apt-AXL-1615 in leukocytes were isolated using 12% SDS-PAGE. The target band was excised and prepared for the mass spectrometry analysis using nanoLC-Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer. The mass spectrometer identified two proteins which where the AXL and fibronectin type III with Ig-like.

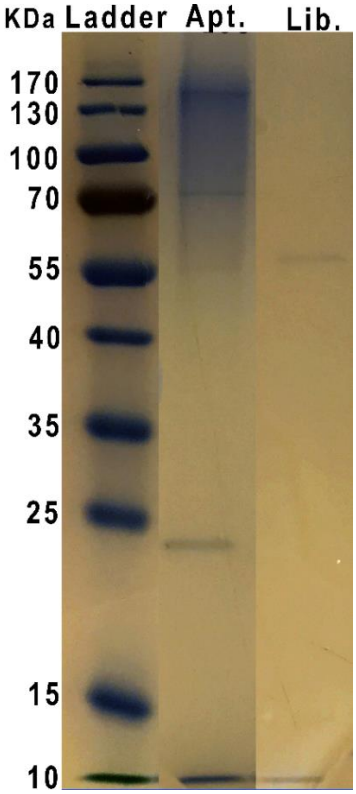

Figure 2.9 Isolation of proteins bound to Apt-AXL-1615. The biotinylated Apt-AXL-1615 bound to the proteins were isolated using the streptavidin coated magnetic beads. The proteins from the leukocytes were isolated using SDS-PAGE. .The protein marker was ran in lane 1 ranging in MW between 10 to 170 KDa. The biotinylated Apt-AXL-165 bound to the proteins were ran in the second lane. While on the third lane, the ssDNA library bound to the proteins were ran. (This image is used with the permission of Shahrokh Ghobadloo's Thesis).

2.3 DISCUSSION

Aptamers are great nucleic acid probes[7], which are great candidates to use for detection of transmembrane proteins[6]. In the first part of this project, aptamers were used to evaluate cells expressing AXL receptors. For selection of aptamers, cell SELEX was performed in order to specifically target cells that express the transmembrane protein [6,26] , AXL tyrosine kinase. Using the successful transfection of HEK293 as seen in Figure 2.1, it was used for the aptamer selection against AXL tyrosine kinase. Recently, RNA aptamers were successfully developed *in vitro* against AXL receptor with high binding affinity [46]. Similarly, this project explores the development of DNA aptamers against cells positively expressing AXL. The selection of

ssDNA against AXL was first performed. According to *Nature protocol*, enrichment pool can be selected base on the fluorescence intensity that compares the initial ssDNA library pool to the subsequent rounds of enrichment pools [47]. The selection started of choosing the aptamer pool with the highest fluorescence intensity. Remember that the principle of SELEX is that, as the more rounds of aptamer selection is performed, the more the enrichment of the ssDNA with the highest binding affinity to the target are amplified. Looking at Figure 2.2, the 10th pool has the highest fluorescence intensity, which is the selected pool with the highest binding affinity to AXL. Likewise, in many research studies, cell-SELEX was used for a successful development of DNA aptamers against bacteria[19–21], which are used for identification, detection, and isolation. In the research performed by Bitaraf and colleagues, they used the SELEX round that displayed a huge amount of binding of the ssDNA to the target cell [20]. Similar results were used for DNA aptamer pool selection against cancer cells [18,22,23]. These supports that 10th round of SELEX does contain the most ssDNA pool bound to the target cells.

Using the 10th pool, the pool was amplified and cloned [47], in which four different aptamers were obtained, and sequenced (Table 1). From this, the structure and minimum free energy were determined[45]. The four selected aptamers displayed stable structure as indicated by the low minimum free energy (Figure 2.3). However, out of the four selected aptamers, Apt-AXl-25504 displayed the lowest minimum free energy (Figure 2.3), indicating that it has the highest binding affinity. Despite Apt-AXl-25504 having the lowest minimum free energy, it still displayed high amount of non-specific binding (Figure 2.4). The non-specific binding is represented by the aptamer binding on cells without the expression of AXL (Figure 2.4). Looking at Figure 2.4, the huge overlap between the peaks of Apt-AXl-25504 binding on cells

expressing AXL and not expressing AXL, which indicates that there is a high amount of non-specific binding. Similar pattern are seen in Apt-AXL-22481 and Apt-AXL-252926, in which, non-specific binding is highly seen (Figure 2.4). Out of the four aptamers, Apt-AXL-1615 displayed the highest minimum free energy (Figure 2.3) but with less non-specific binding (Figure 2.4). Apt-AXL-1615 is used for further studies due to its high specificity to the target AXL tyrosine kinase receptor. Regardless of the effort of removal of the non-specific binding by performing 1 round of negative selection within 10 rounds of positive selection, the non-specific binding is still present. It is hard to remove all of the non-specific binding. As the rounds of SELEX increased, the non-specific binding increased. By performing more negative selection within ten rounds of positive selection in the future, it can decrease the non-specific binding and increase the specific binding of aptamers [6,47].

Many researches were done in the development of aptamer against cancer cells. Xu and colleagues developed DNA aptamers that recognizes and binds to liver cancer cells with high affinity[22]. Likewise, Shangguan and colleagues selected aptamers that recognizes T cells of leukemia cells[23]. DNA aptamers does display a great potential to use as probes for diagnosis and therapeutics[22,23]. With great similarities, the purpose of this study was to develop DNA aptamer probe that specifically binds to AXL receptor. The binding of the Apt-AXL-1615 to the target protein was verified using microscopic studies. Indeed, Apt-AXL-1615 specifically binds to the target protein as seen in figure 2.5. Further testing was performed to validate the binding of Apt-AXL-1615 to AXL in heterogeneous tissues using whole blood leukocytes. About 11.3% displayed a positive binding between the AXL and Apt-AXL-1615, which indicates that AXL receptor tyrosine kinase is present in some population of the whole blood leukocytes and within this population about 98.6% has positive binding as seen in Figure 2.6.

The missing 1.4% was lost during the sorting. Within this population, 93.8% displayed antibody AXL positive and Apt-AXL-1615 positive, which indicates that aptamers bind to the AXL receptor. However, 3.9% of antibody AXL displayed positive but negative pm Apt-AXL-1615, which indicates that some AXL either lost their binding with the aptamer during washes or some were not able to bind to the aptamer. This results to the presence of 2.2% of negative antibody AXL and positive Apt-AXL-1615. The 2.2% can also represent aptamer with non –specific binding.

To identify which population of the whole blood leukocytes specifically binds to the aptamer, a flow cytometry was performed. CD3 and CD4 are antigens present in the leukocytes and they can be used to know which population of leukocytes have positive binding to the aptamer. CD3 is related with T cell receptor, which is seen in T cells[48], while CD4 is seen in T helper cells and monocytes[48]. Since tyrosine kinase has a crucial role in the immune system, especially in the regulation of T cells[49], it is expected that Apt-AXL-1615 will bind to T cells. Looking at Figure 2.7, about 16% of the population was CD3+/CD4- and about 78% of the population was CD3+/CD4+. As expected, Apt-AXL-1615 binds to the lymphocytes of whole blood leukocytes. However, looking at the sorted SSC vs FSC dot plot of Figure 2.7, some binding of monocytes are noticeable.

Just to confirm that the target proteins were AXL, the protocol laid out by the aptaBID was performed. The biotinylated aptamer was used to efficiently isolate the bound proteins from unbound proteins using magnetic beads. The use of biotinylated aptamers do not destroy the 3D structure of the selected aptamer[19]. Since AXL has a molecular weight between 100 to 140 kDA[31], the bands on 70 kDa and 150 kDa were cut to verify what proteins were bound. The mass spectrometry did indicate the presence of AXL in the sample. Indeed, the Apt-AXL-

1615 specifically binds to AXL transmembrane protein. In conclusion, the project successfully developed an aptamer probe recognizing cells positively expressing AXL tyrosine kinase receptor.

Further studies can be performed using Apt-AXL-1615 to determine its regulation and effect upon binding to the AXL tyrosine kinase. Recently, it was reported that RNA aptamer against AXL tyrosine kinase was able to inhibit its signalling pathway[26,46]. *In vivo* studies should be another step since aptamers do not have an immune response and they can quickly penetrate tissues compared to antibodies[4]. Since Apt-AXL-1615 is able to recognize AXL tyrosine kinase, which is highly present in cancer cells, immunogenic disease, and cardiovascular disease, it can explore its binding to these cells and its effectivity for diagnosis and therapeutic.

In summary, this study explored the development of aptamer probe against whole cell using cell-SELEX. The 10^{th} round of aptamer pool was selected (Figure 2.2) because of the highest fluorescence intensity. From this, selected aptamers were sequenced (Table 2.1) and cloned. Their structure and minimum free energy were also identified (Figure 2.3). Apt-AXL-1615 had the least non-specific binding (Figure 2.4) compared to the other three selected aptamers. It was used to identify AXL receptor in whole blood leukocytes. The whole blood leukocytes were gated and sorted (Figure 2.6) in order to evaluate the binding of the aptamer to the whole blood. For identification of specific population of whole blood leukocytes, the CD3 and CD4 antibodies were used. As expected, the lymphocytes (T cells and T helper cells) bind to Apt-AXL-1615 (Figure 2.7). The AXL protein was verified using mass spectrometry (Figure 2.9), which confirmed that the protein the Apt-AXL-1615 was bound to was indeed

AXL tyrosine kinase receptor. Overall, DNA aptamer was an effective probe for detection and isolation of cells expressing AXL tyrosine kinase receptor.

3. CHAPTER 2

Aptamer selection against human IgG Fc fragment

3.1 MATERIALS & METHODS

3.1.1 REAGENTS USED FOR THE PREPARATION OF REQUIRED SOLUTIONS

Mineral Oil (SIGMA, product# M5904), Triton x-100 (SIGMA, product# T8787), Tween 80 (SIGMA-ALDRICH, product# P1754), Span 80 (SIGMA, product# S26760), and 1X Phosphate Buffered Saline (PBS) buffer with Ca^{2+} and Mg^{2+} (ThermoFisher, cat# 14040133) were used to prepared the required solutions for each steps.

3.1.2 COUPLING OF HUMAN IGG FC FRAGMENT AND BOVINE SERUM ALBUMIN WITH MAGNETIC BEADS

The magnetic beads used were from 1µM Carboxy flexibind magnetic beads (PureProteome, cat# LSKMAG1CBX02). The magnetic beads were coupled with the natural human IgG Fc fragment (hFc) (Abcam, cat# ab90285) using the protocol by the manufacturer (EMD Millipore protocol via EDC). Prior to the use of the magnetic beads, it was washed 3 times using the coupling buffer, which contained 50mM MES, pH 6.0 and 0.01% of Triton X-100. The carboxyl groups on the beads were activated using EDC, in which the magnetic beads

were washed with the coupling buffer with EDC. Once activated, the beads were ready to be coupled with hFc. After the coupling, the beads-hFc complex was re-suspended in Carboxy beads SELEX buffer (CBSB) which was composed of 1X Phosphate Buffered Saline (PBS) buffer with Ca^{2+} and Mg^{2+} mixed with 0.005% Triton X-1000. The beads-hFC complex solution was then stored in -20 ˚C.

Similarly, the Bovine Serum Albumin (BSA) (abcam, cat# ab186531) was coupled with 1µm Carboxy flexibind magnetic beads. Just like the coupling for hFc, the BSA was coupled following the protocol EMD Millipore protocol via EDC. Afterwards, the CBSB was used to re-suspend the beads-BSA complex. The coupled beads-BSA beads were used for pre-clearing before the selection of the DNA sequences.

3.1.3 APTAMER SELECTION USING SELEX

There were a total of 3 rounds of aptamer selections done. The first selection started from the 1.1 µM of 100 nucleotide (nt) Harvard Structure Library (H-Lib) with 20 bp fixed sequence on both ends and 80 random sequences in between. The H-Lib was diluted to 1.1 µM with 1X PBS buffer with Ca^{2+} and Mg^{2+}, followed by 5 minute heating at 95 ˚C and 10 minutes incubation on ice. This was performed in the preparation for the pre-clearing, which was the removal of the non-specific hFc binding of the H-Lib. The H-Lib was coupled with the beads-BSA complex. It was incubated for 1 hour in 37 ˚C with shaking at 1 400 rpm using the Thermomixer (Eppendorf). The unbound H-Lib and bound H-Lib were separated using the PureProteome magnetic stand. The H-lib bound to beads-BSA complex were discarded. While, the supernatant contained the desired DNA sequences and it was used for selection. The selection were performed after each round of amplification. The first selection was done using the supernatant in order to get the R1 elution. For each rounds, the solution was

denatured at 95 ˚C for 5 minutes and it was immediately placed on ice for another 10 minutes. The solution was coupled with the beads-hFc complex and it was incubated at 37 ˚C for 1 hour accompanied with shaking of Thermomixer at 1 400 rpm. Note that prior to the use of the beads-hFc complex, the beads-hFc complex was first washed twice using CBSB in order to remove the unbound proteins. After the coupling with the DNA sequences, the beads was washed with pre-warmed CBSB at 37 ˚C. For the first round of selection, the beads were washed 5 times. However, for the second round of selection, the beads were washed 4 times. Later on, as the rounds increased, the number of washes increased by 1. The desired DNA sequences were on the beads-hFc complex and they were collected. The solution with DNA bound-beads-hFc complex were heated at 95 ˚C for 5 minutes in order to break the complex. The PureProteome magnetic stand was used to separate the DNA from the beads-hFC complex, by immediately removing the solution after heating it.

3.1.4 EMULSION PCR AMPLIFICATION OF THE DNA SEQUENCES

Two separate experiments were amplified using the ePCR. The first experiment used the aptamer pools from round 1 to round 7 that were already selected by Ana Gargaun. These pools were amplified and the samples were ran in 4% agarose gel in order to select the best aptamer pool. On the second experiment, the ePCR was used to amplify the newly selected aptamer pools. The selection was followed by the amplification of the DNA sequences using emulsion PCR (ePCR). The first step was the denaturation of DNA at 98 ˚C for thirty seconds. It was followed by 40 cycles of the denaturation of the DNA at 98 ˚C for 10 seconds, the binding of the primer at 58 ˚C for 15 seconds, and the primer extension at 72 ˚C for 5 seconds. After the cycles, the DNA was exposed at 72 ˚C for 20 seconds followed by fully annealing of the double stranded DNA (dsDNA) at 4 ˚C for 2 minutes. In this process, 5nM of DNA

template was added to the master mix which contained ddH_2O, 7% DMSO, 1X Phire Buffer with $MgCl_2$ (Thermofisher, cat# F527L), 0.2 mM dNTP, 0.4 µM F4 forward primer, 0.4 µM R4 reverse primer, and U/25 µL rxn of Phire II poly. The general sequence of the F4 forward primer was 5' – CTC CTC TGA CTG TAA CCA CG – 3'. While, the R4 reverse primer had the sequences 5' – GGC TTC TGG ACT ACC TAT GC – 3'. For the F4 forward primer, it had the Cy-5 fluorophore conjugated at the 5' end, which made the DNA sequence fluorescent. In the process of ePCR, it required two immiscible solutions which were the master mix with the DNA template and the oil mixture. The oil mixture contained mineral oil (Sigma), 10% Span 80 (Sigma), 10% Triton X (Sigma) and 10% Tween (Sigma) with a total volume of 200 µL. Small amount, ideally 10 µL, of the master mix with DNA template was added to the oil mixture. After the addition of the master mix, the solution was vortexed for 10 seconds. Once the whole master mix was used, the solution was vortexed for another 5 minutes. Afterwards, the solution was run in the thermocycler (Eppendorf). And then, the samples were recollected and they were spun at 9 000 g for 5 minutes using the centrifuge (Eppendorf). This was followed by the removal of the top oil layer and the addition of ether, in which the ether was added for the extraction of the dsDNA. The solution was vortexed in order to fully extract the DNA and remove the excess oil. However, since the desired products were only the dsDNA, the ether was removed and the ether left on the sample was dissipated by heating the sample at 50 ˚C for 30 minutes using the thermomixer. Since the single stranded DNA sequences of aptamer pools were desired, the amplified samples were incubated with the 10X exonuclease buffer and lambda exonuclease for 2 hours in 37 ˚C.

3.1.5 VERIFICATION, PURIFICATION AND PRECIPITATION OF THE AMPLIFIED PRODUCTS

The samples were loaded on 4% agarose gel to test if the ePCR or exonuclease worked. Once the samples were ran, they were viewed under FluorChemQ (AlphaInnotech). The amplified aptamer pools were evaluated to verify if the ePCR and exonuclease worked. Usually for the verification, ssHLib and dsHLib were loaded as the controls. With this process, the amplified aptamer pools were also assessed for presence of high background or by-products. The amplified aptamer pools were ran in the 4% preparative agarose gel for purification. Using the FluorChemQ, the desired band on the gel was cut and it was further cut into smaller pieces. The small pieces of gel were incubated overnight with ddH_2O to elute the ssDNA to the water. This was followed by the extraction of the ssDNA and its purification through the Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore), following the laid out protocol by the company.

After the purification, the amplified aptamer pools were precipitated using the 300mM of NaAcetate with pH of 5.2 and 100% EtOH. The whole solution was vortexed and incubated at -20 ˚C for 1 hour. After the incubation, the solution was centrifuged at 1 400 rpm for 15 minutes. The pellets were collected and 75% EtOH was added. The solution was centrifuged at 1 400 rpm for another 5 minutes. The supernatant was removed and the pellets were air-dried until all EtOH were gone. The pellets were re-suspended with ddH_2O and they were stored at -20 ˚C.

3.1.6 APTAMER POOLS BINDING TO THE HFC USING FLOW CYTOMETRY

Once all the aptamer pools were collected, the binding to hFc was verified using Beckman Coulter Gallios Flow Cytometer. Each pool was prepared by amplifying the aptamer pools four to six times using ePCR, in order to get the total concentration of 80 to 200 nM. Afterwards, the amplified aptamer pools were changed from dsDNA to ssDNA using lambda

exonuclease and the concentrations were checked using the 96 well Biotek multimode plate reader. The ssDNA pools were incubated with hFC-magnetic bead complexes and were washed. The magnetic beads were eluted with PBS with Ca^{2+}/Mg^{2}.

3.2 RESULTS

3.2.1 VERIFICATION OF THE AMPLIFIED ROUNDS 4 TO 7 POOLS

The target of this project was to develop 10 ssDNA enrichment pools using SELEX against the human FC fragment. The initial selection of rounds 4 to 7 aptamer pools were initially selected by Ana Gargaun. These pools were amplified using ePCR following the protocol optimized by the lab. The amplified samples were ran in 4% agarose gel for better separation of the 100 nt Harvard library. Along with the amplified pools, MassRuler low range ladder from Thermofisher and the dsDNA and ssDNA Harvard library were ran. In Figure 3.1, round 4 amplified (R4A) contained one pronounced band slightly above 80bp. On the other hand, the round 5 amplified (R5A) started to develop a second band at around 100 bp. While, round 6 amplified (R6A) and round 7 amplified (R7A), displayed 2 bands, first bands were both around 80 bp while the second bands were below the 80 bp.

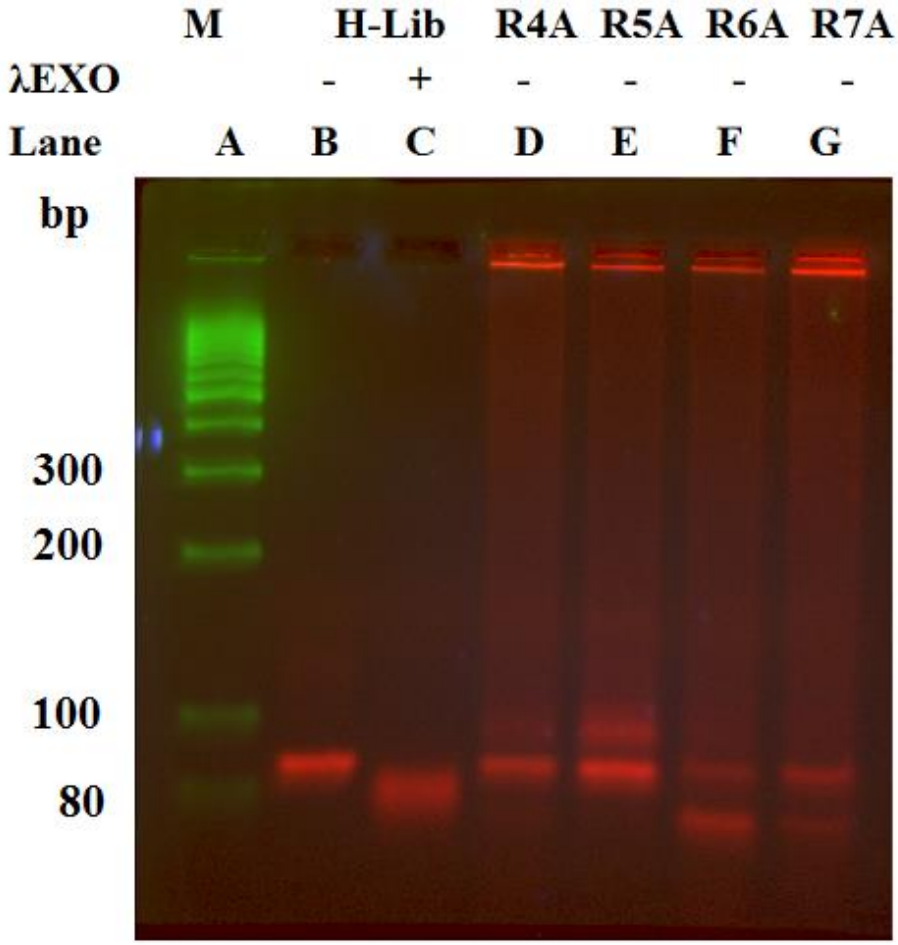

Figure 3.1 Amplification of ssDNA pool from 4 to 7 cycles of SELEX. Small amount of Cy5-conjugated ssDNA pools from 4 to 7 enrichment from SELEX were taken from and mixed with prepared master mix. Each pools were amplified using ePCR. After amplification, the samples were extracted using ether. The each amplified products were ran in 4% agarose gel along with the mass ladder and the Cy5-conjugated double stranded and single stranded Harvard library. The gel was viewed under FluoroChemQ system.

3.2.2 APTAMER BINDING TO FC FRAGMENT USING FLOW CYTOMETRY ANALYSIS

Following the amplification, the R5A, R6A, and R7A were digested with λ exonuclease. In order to use the desired Cy5 – conjugated ssDNA, it was purified. The ssDNA was incubated for 5 minutes in 95 ˚C and it was placed on ice. The ssDNA pools were incubated

with the carboxyl-magnetic beads bound to the human FC fragment at room temperature followed by washes to remove unbound Cy5 – conjugated ssDNA. Each pool was prepared for the flow cytometry with a concentration of 80 nM. The 80 nM of Harvard library was ran along with the samples as the control. In figure 3.2, the histogram for R5, R6 and R7 displayed similar characteristic as the Harvard library. However, there were no peaks visible on the higher intensity. All peaks for R5, R6 and R7 were below to 10^0 of FL6.

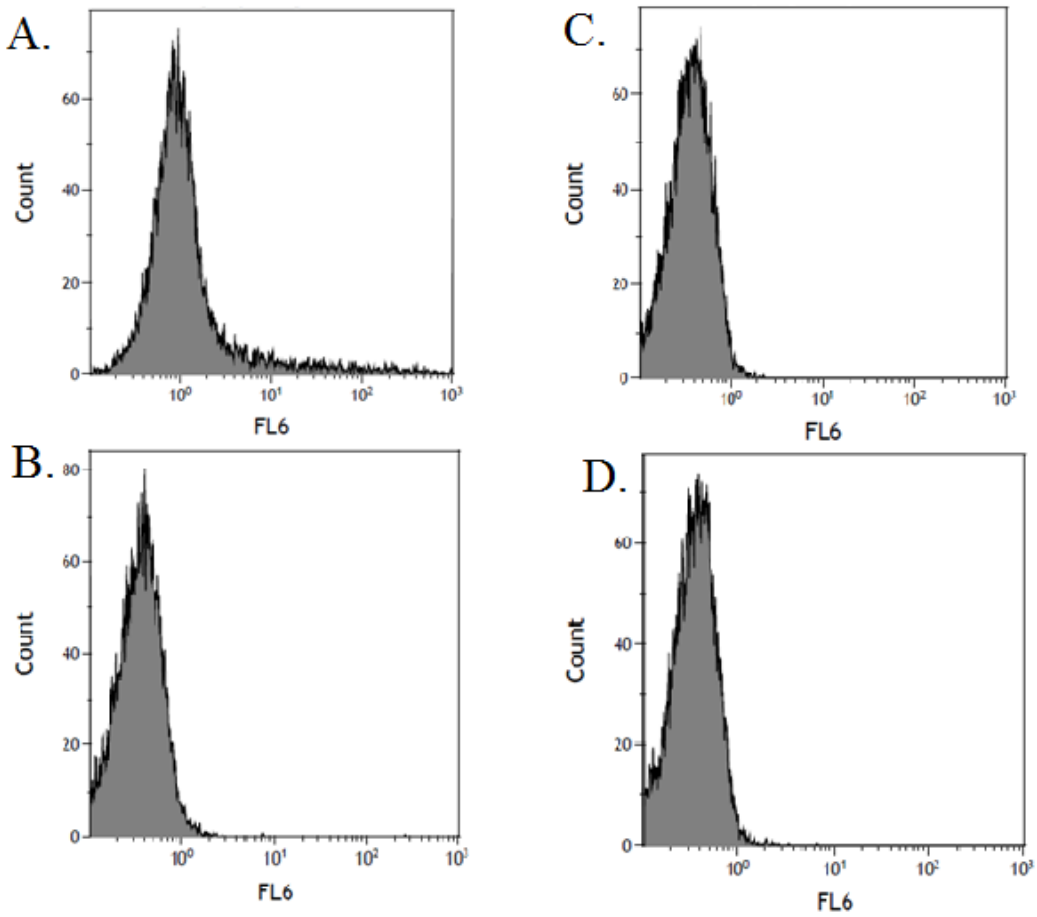

Figure 3.2 Flow cytometric analysis of ssDNA binding to Fc fragment. The (A) H-lib (B) R5, (C) R6 and (D) R7 pools were amplified and 80nM of each samples were ran in flow cytometry. The fluorescence intensity were compared for the verification of the binding of the enriched pools to the target protein.

3.2.3 NEW APTAMER SELECTION: ROUND 1 AMPLIFICATION

A new selection was done using 100 nM of Cy5 – conjugated ssDNA Harvard library as the initial pool for the first round of selection (R1). The carboxyl magnetic bead bound to BSA (bovine serum albumin) were incubated with the ssDNA Harvard library to remove non-specific binding. The supernatant contained the unbound ssDNA, in which it was incubated with the carboxyl magnetic beads bound to human FC fragment. After multiple washes, the bound ssDNA on the magnetic beads was eluted by placing the solution in 95 ˚C for 5 minutes by using the magnetic stand. The eluted ssDNA was amplified by ePCR and it was ran in 4% agarose gel. In Figure 3.3, two amplified R1A were created, in which both bands have approximately 80 bp. Both bands of R1A were slightly above the Harvard library.

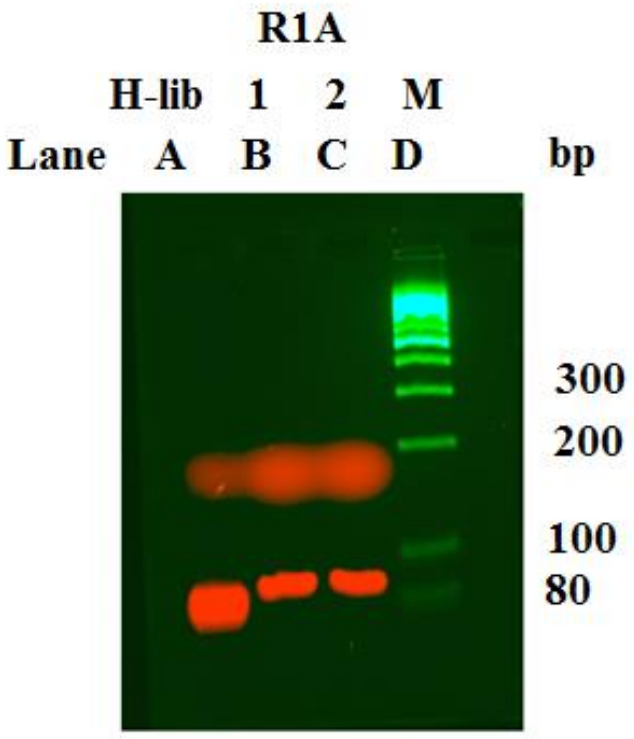

Figure 3.3 Amplified ssDNA pool from the first pool selection. The Cy5- conjugated Harvard library was used for the first round of SELEX. The ssDNA Harvard library was first incubated with the magnetic beads bound to BSA and the supernatant contained the unbound

ssDNA, which was incubated with the magnetic beads bound to human FC fragment. The ssDNA were eluted from the magnetic beads and it was amplified using ePCR. Two samples of R1A were amplified and it was compared along with the ssDNA Harvard library (H-lib). M corresponds to the ladded used. These were ran in a 4% agarose gel and viewed under FluoroChemQ system.

3.2.4 VERIFICATION OF AMPLIFICATION FOR ROUND 2 SELECTION

After the first round of selection, the generated first round ssDNA pool was used as the starting pool for the second round of selection. The 100 nM of ssDNA was incubated at 95 ˚C for 5 minutes and it was immediately placed on ice. The ssDNA was incubated with the magnetic beads bound to the human FC fragment for 1 hour in 37 ˚C. Afterwards, it was washed 4 times. The bound ssDNA were eluted in 95 ˚C and it was amplified using ePCR. The amplified solution were exposed to λ exonuclease for 2 hours in 37 ˚C. Both of the round 2 amplified (R2A) incubated with and without the λ exonuclease were compared with the dsDNA and ssDNA Harvard library. As seen in Figure 3.4, the round 2 amplified for both samples (R2A1 and R2A2) without the exonuclease, were seen on the same row as the 100 bp. Hence, the samples were on the same location as the dsDNA of the Harvard library. Once the samples were digested by the λ exonuclease, both bands (lane b and d) were in 80 nt. Thus, it has on the same location as the ssDNA of the Harvard library.

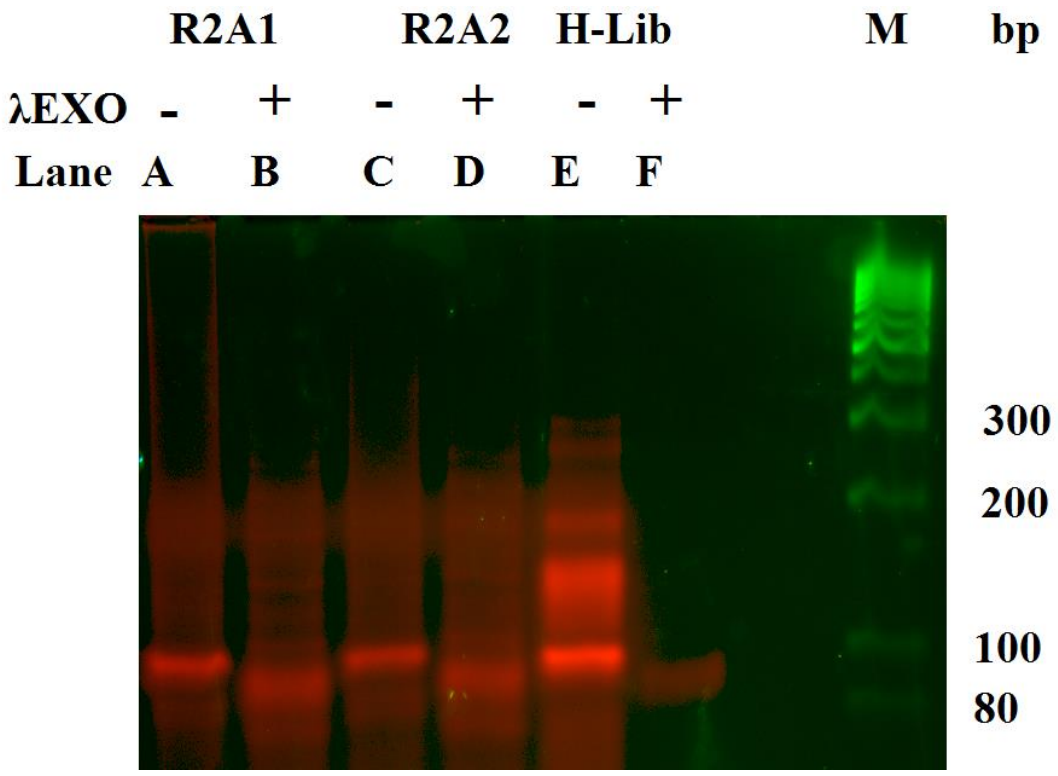

Figure 3.4 Second round of aptamer pool selection. A second enrichment was performed using SELEX. One hundred nanomolars of first ssDNA pool was incubated with the carboxyl magnetic beads bound to the human FC fragment. After 4 washes, the bound ssDNA was eluted by heating the sample at 95 ˚C and isolated the magnetic beads from the ssDNA using the magnetic stand. After the completion of the SELEX, the eluted ssDNA was the round 2 ssDNA pool. Two samples of the pools were amplified and it was ran in 4% agarose gel and the gel was viewed under the FluorChemQ system. R2A corresponds to round 2 amplified; H-lib corresponds to Harvard library; M corresponds to the DNA ladder; bp corresponds to base pair.

3.2.5 VERIFICATION OF AMPLIFICATION FOR ROUND 3 SELECTION

Another SELEX was ran, which corresponded to the third round of selection. Similar steps from round 2 were taken, where the purified Cy5- conjugated ssDNA pool from the second round of enrichment were used as the initial pool. The ssDNA were incubated with the carboxyl magnetic beads bound to the human FC fragment for 1 hour in 37 ˚C with continuous shaking. After incubation, the beads were washed 5 times, and the bound ssDNA were eluted in 95 ˚C using the magnetic stand to separate the beads from the desired ssDNA. The ssDNA

was amplified using ePCR. The amplified round 3 pool (R3A) were ran in 4% agarose gel to verify for a successful ePCR. The bands were viewed under the FluorChemQ system, which developed the image on figure 3. In this figure, the R3A1 and R3A2 both displayed a band at around 100 bp. However, the R3A1 had a higher band intensity than R3A2. The R3A2 had a higher primer band intensity than the desired dsDNA.

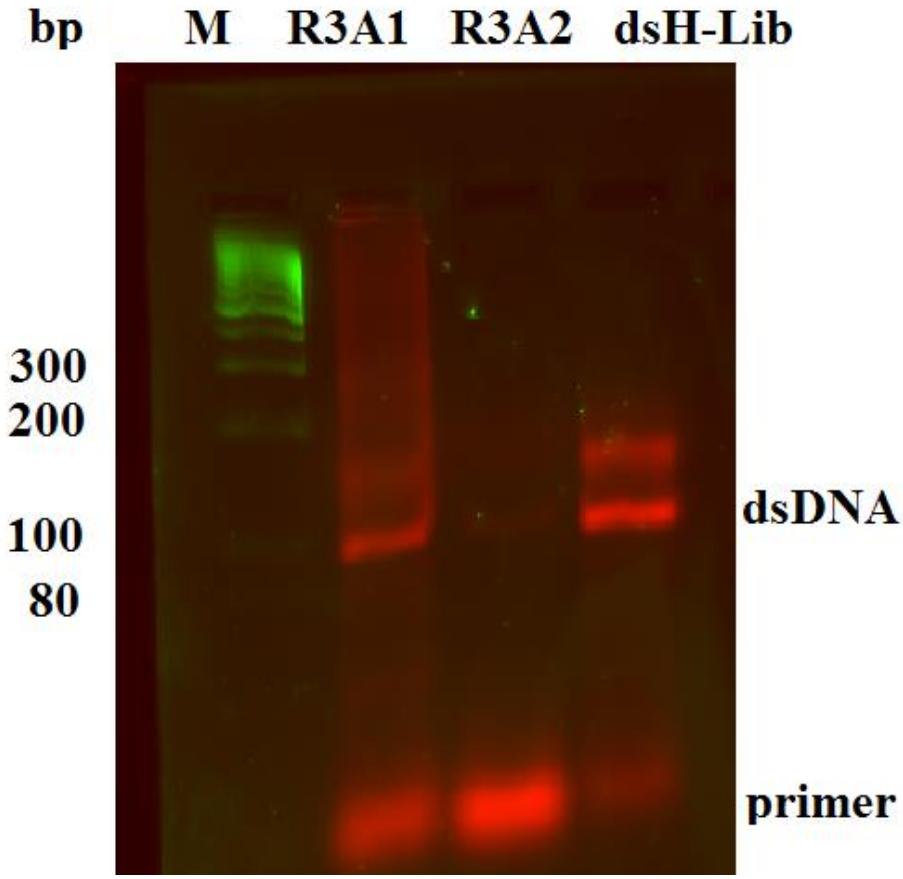

Figure 3.5 Amplified of the third SELEX selection. The 100 nM purified R2A ssDNA pool was used as the initial pool for the SELEX selection. The ssDNA was first heated in 95 ˚C for 5 minutes and it was placed on ice. The ssDNA were incubated with the carboxyl magnetic bound human FC fragment for 1 hour in 37 ˚C followed by 5 washes. The solution was placed in 95 ˚C for 5 minutes, to elute the bound ssDNA from the magnetic beads. The eluted ssDNA were amplified using ePCR, where the amplified ssDNA pool was called round 3 amplified pool (R3A). Using 4% agarose gel, the sample were ran along with the double stranded Harvard library (dsH-lib) and DNA ladder (M).

3.3 DISCUSSION

Not many studies have been done in aptamers against IgG specifically the Fc. The purpose of this study was to explore the development of DNA aptamer targeting the human IgG Fc Fragment using SELEX. Recently, RNA aptamer was successfully developed targeting rabbit IgG antibody [39]. The RNA aptamer started to become enriched in 4th round and the enrichment of the high binding affinity of ssRNA drastically increased by 7th round[39]. It is expected in this project, by 7th round, a high enrichment of ssDNA pool will be seen with high fluorescence intensity. However, in Figure 3.2, it is seen that the 7th round (Figure 3.2 D) were exactly the same as the control, which was the ssDNA library (Figure 3.2 A). It seems that at 7th round, the ssDNA with high fluorescence intensity were lost compared to the ssDNA library, which small peaks in high fluorescence intensity are present. High fluorescence intensity indicates ssDNA with high binding affinity to the target protein. Similar results are seen in 5th and 6th rounds, which displays no peaks in high fluorescence intensity meaning that the ssDNA with high binding affinity to target was lost from prior rounds.

The start of the formation of by-product can be the reason for losing the desired ssDNA. Looking at Figure 3.1, starting from 4th round, formation of 100 bp by-products started to form. Likewise, the 5th round had 100 bp by-product formation and it had increased in band intensity, which means that there is increase in by-product formation. Similar results were seen in the research conducted by Tolle and colleagues, it displayed that dsDNA by-product started to form in the 4th round of SELEX and its formation increased in the subsequent rounds[50]. By-product formation can be caused by the binding of two or more on the forward or reverse primers on the random region of the DNA and there are repeated primers on the binding site[50]. Moreover, product-product hybridization is a probable cause of the formation of the by-

product due to the heterogeneity[51] of the H-lib. The by-production formation in early rounds were expected to be avoided because of the use of ePCR. According to Shao and colleagues, the by-product formation are expected to be seen after 25 rounds[52]. However, this was not the case seen in the results obtained. It was expected that ePCR creates emulsion or droplets that only contains one DNA template[52]. However, the by-product formation on the 4^{th} and 5^{th} rounds indicates that each droplets have contained more than one DNA template. The amplification performed in this project requires improvement. The increase in by-product formation is caused by the high amount of DNA template concentration added to the master mix. It is suggested to use lower concentration of DNA template because the higher the concentration of the DNA template. The higher the by-product formation occur[52].

In the case of 6^{th} and 7^{th} rounds (Figure 3.1), by-products are seen below the 80 bp band. This by-product was unexpected because the amplified products were not exposed to λ exonuclease. This type of result is only expected on the degradation of the DNA in the presence of exonuclease. This by-product can be the cause of non-uniform amplification. It is probable that at this point the DNA template contained high amounts of GC rich regions creating stable structure such as loops [51,53]. The GC rich regions tends to cause the polymerase to jump from one sequence to another creating a shorter product [51]. Since the sequence amplified contained approximately 10^{15} different sequences, it is still unknown if the amplified products contained huge amounts of GC region. It requires further experiments, such as sequencing, to support this assumption.

New selection was performed to further understand the loss of desired products seen in Figure 3.2. The new selection used the H-lib as the initial ssDNA pool for the SELEX. Base on Figure 3.3, there was no by-product present at all, which indicates that the

amplification worked. It shows that the droplet formed during ePCR contained only one DNA template, which explains why product-product hybridization is not present.

With this success, 2nd round of selection was carried. In the process of 2nd round of selection, only 4 times of washing were performed, due to the loss of products in higher washes. Washes are very important for the aptamer selection. Ideally, washing is performed to remove non-specific binding or to remove ssDNA with less binding affinity to the target[54]. However, when 6 washes were performed for the selection, the samples were lost. By doing 4 washes instead, samples were seen and a successful amplification was performed (Figure 3.4). The amplified dsDNA products were exposed to λ exonuclease in order to create ssDNA. The intensity of the band for ssDNA was weaker than the dsDNA, which is expected since there is less Cy5 – conjugated ssDNA. The R2A was purified to remove unwanted products (Figure S5.1), because contamination and primer dimer were present. Purification step is a very important step to ensure that during amplification step, the amplified products are the desired templates and not any of the by-products. Amplification of by-products will contaminate the solution and they will affect the selection for the following rounds.

Continuing the SELEX process, 3rd round of SELEX was performed. The amplification of R3A displays low band intensity due to low yield (Figure 3.5). The R3A2 in Figure 3.5 displayed higher band intensity for the primer than desired dsDNA. This indicates that the primers did not successfully bind to the ssDNA. The high primer concentration and faint bands of dsDNA indicate that the polymerase did not work properly. Similar problem is seen for R2A amplified products (Figure S5.1). The amplified products displayed low yield with high concentration of primers present. It seems that the primers were not binding to the DNA template instead they bind to one another creating by-products, called primer-dimers. It

seems that the high concentration of primers have more non-specific binding, which leads to the decrease of dsDNA. However, according to Shao and colleagues, the primer concentration does not affect the yield of the desired product [52]. In this project the primer concentration used was 0.4 μM, which was the similar concentration used by Shao and colleagues[52]. This means that the probable cause of the decrease in dsDNA yield is due to the polymerase. The polymerase could have been degraded since that 40 cycles of PCR were performed. By increasing the concentration of the polymerase, it is possible that there will be an increase in the yield of dsDNA. The increase in polymerase concentration indeed increased the PCR product in Shao and colleagues study[52].

The addition of the DMSO to the master mix was expected to decrease the amount of by-product[55]. However, even in the presence of DMSO, the R4, R5, R6, and R7 (Figure 3.1) still displayed by-product formation. Moreover, primer-dimer started to form in the R2 amplification (Figure S5.1). It also displayed low yield of PCR product. According to Kang and colleagues, the addition of DMSO and betaine did increase the amplification product of the DNA library[55]. To increase the yield of amplified product in future experiments, it is suggested to use not only DMSO but also betaine.

The purpose of this study was to develop an aptamer pool with the best binding affinity to the FC fragment. Most aptamer development targeted FAB region because it is still a challenge to develop aptamer against FC fragment [41]. Ma and colleagues successfully developed ssDNA aptamer against the mouse IgG Fc region[41]. It took 8 rounds of SELEX to develop aptamer pool with high affinity to the Fc region [41]. In this project, high enrichment of ssDNA with high binding affinity is expected as rounds are increased; since SELEX is designed to enrich the pools of ssDNA with high affinity and specificity to its target protein[15].

Thus, it is expected that around 6th rounds[15] there should be increase of ssDNA with high affinity and specificity to the target protein. Ma and colleagues used target replacement SELEX that developed DNA aptamers with high affinity to the target protein [41]. In future studies, trying a new SELEX method to develop ssDNA pools with high binding affinity to the target protein at lower rounds of SELEX.

In conclusion, aptamers bind with high specificity and affinity to the target protein. However, this project was not able to develop aptamer pools with great affinity and specificity to the human IgG Fc region. The amplified R4, R5, R6, and R7 aptamer pool lost the ssDNA with high affinity and specificity to target protein because as the rounds increased, by-product formation increased. Moreover, new selection was performed and it displayed low yield of PCR products. It requires further improvement on the amplification process for a better yield. Overall, higher SELEX rounds are required in order to develop aptamer pool with high affinity and specificity to the human IgG FC fragment.

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5. Appendix

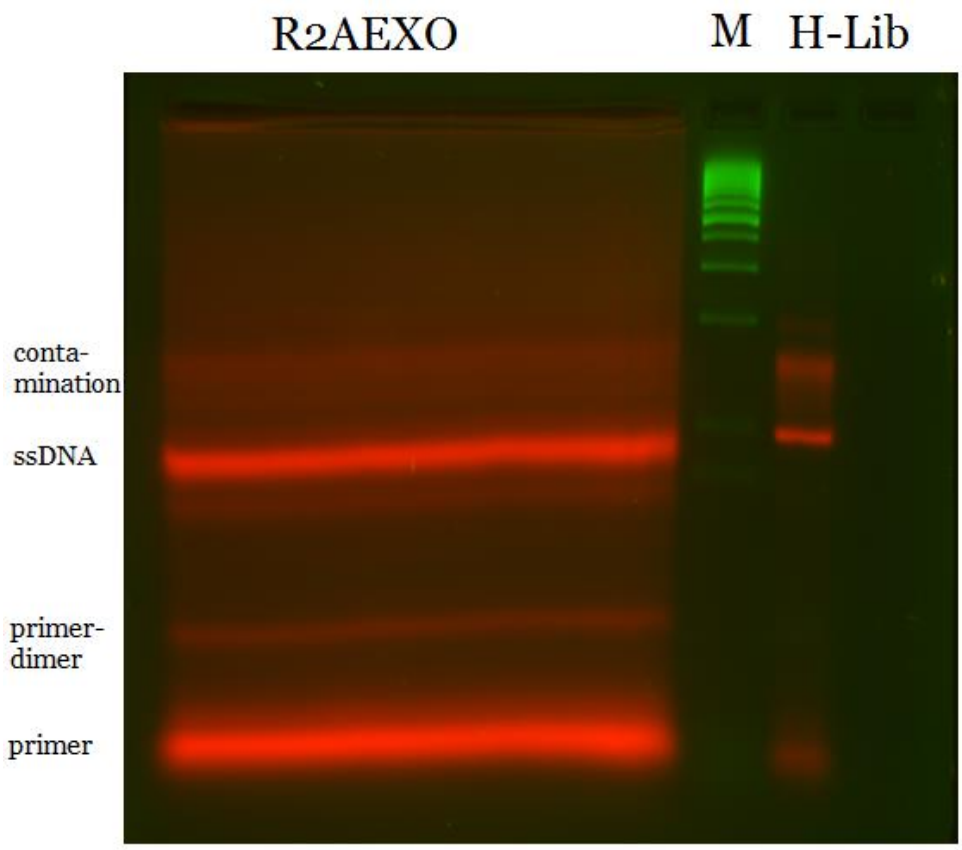

Figure S5.1 Preparative gel performed on R2A. The R2A were exposed to λ exonuclease in 37 C for 2 hours. The ssDNA were ran in 4% agarose gel for the isolation of the target ssDNA from any by-products or contamination.