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Development Of A DNA Aptamer That Specifically Inhibits Human Carbonic Anhydrase II

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DEVELOPMENT OF A DNA APTAMER THAT SPECIFICALLY INHIBITS HUMAN CARBONIC ANHYDRASE II

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To my

FAMILY with love

DEVELOPMENT OF A DNA APTAMER THAT SPECIFICALLY INHIBITS HUMAN CARBONIC ANHYDRASE II

by

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THESIS

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ABSTRACT

Carbonic anhydrases (CA) are a family of enzymes that catalyze the rapid interconversion of carbon dioxide and water to bicarbonate. Carbonic anhydrase II is the most abundant protein in the cytosol. CA II inhibitors are emergent therapeutic targets for the development of antiglaucoma agents. Glaucoma is a disease affecting large number of people and characterized by an elevated intraocular pressure due to excessive secretion of aqueous humor. The enzyme CA II is responsible for the secretion of aqueous humor. Isoforms of CA II such as CA IV and CA I are present in many tissues and organs other than the eye. The commercially available CA inhibitors such as Brinzolamide tend to inhibit CA II and other isozymes such as CA IV and CA I. This leads to undesired side effects such as numbness and tingling of extremities, metallic taste, depression, fatigue, etc. In order to avoid these side effects an isozyme specific inhibitor is required. Here, we sought a novel DNA-based agent to inhibit specifically carbonic anhydrase II. Toward this goal, an 86 base pair, single-stranded DNA aptamer library containing a 40 base pair variable region was screened for CA II binding candidates, using ten rounds of positive and negative selection. Positive selection rounds has been used to evolve aptamers of extremely high binding affinity toward the target CA II whereas negative selection cycles were performed to determine the specificity of the aptamers toward CA II. Next generation sequencing (NGS) revealed sequences of the aptamer library. The most active anti-CAII aptamer had a random central sequence 5'-CGT AGC TAT GAT ATT GATT GTT TTT AAA TCC ACC CG TGCA -3' and was designated as CA II inhibitory aptamer (CAII-A8). This aptamer was found to be isozyme specific inhibitor determined by the enzymatic assay of carbonic anhydrase for Wilbur Anderson Units (EC 4.2.1.1). CAII-A8 is the first CA II isozyme specific inhibitory aptamer to be described as an antiglaucoma agent.

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CHAPTER 1 : INTRODUCTION

1.1 Glaucoma

Glaucoma is an eye disease that damages the optic nerve due to high intraocular pressure and leads to irreversible blindness. Usually Glaucoma has no symptoms at early stages which makes the disease more dangerous by the time it is noticeable, the disease has already progressed to the level that irreversible blindness has already occurred and the treatment becomes more difficult. The most common type of glaucoma known as primary open angle glaucoma estimated to affect 60 million people worldwide and more than 2.2 million people in the United States [1]. The risk factors of glaucoma are elevated intraocular pressure, a family history of glaucoma and high blood pressure. Eye pressure greater than 21 mmHg is considered as high intraocular pressure. A fluid called aqueous humor is continuously produced from the ciliary body and flows from posterior to anterior chamber and leaves the eye through trabecular meshwork. Continuous secretion and regulation of outflow of aqueous humor is very important to maintain the intraocular pressure in the normal range. When this mechanism is interrupted, the fluid inside the eye builds up and results in increased eye pressure, therefore causes glaucoma [2]. Routine examinations are very important for protecting the eye. An early treatment can help to prevent irreversible blindness due to glaucoma.

1.1.1 Types of Glaucoma

Glaucoma is categorized on the basis of the angle formed between the iris and the cornea. This angle formation is important for the drainage of aqueous humor from the eye. Glaucoma is mainly divided into two forms, open-angle glaucoma (OAG) and closed-angle glaucoma (CAG). OAG is one of the most common form of glaucoma which is estimated to affect 75% of glaucoma

cases in the population. CAG and OAG is further divided into primary and secondary forms. In the case of Primary closed-angle glaucoma (PCAG), the drainage canal of aqueous humor is blocked by the iris where the iris is in contact with the trabecular meshwork. In secondary closed-angle glaucoma (SCAG), the drainage angle becomes narrow or closed which prevents the outflow of aqueous humor. In Primary open-angle glaucoma (POAG) there is no significant abnormality in the trabecular meshwork but there are some problems with the cells of the trabecular meshwork that prevents the trabecular meshwork from carrying out its normal functions. The result is that the outflow of aqueous humor is slowed down. The fluid inside the eye builds up and causes the pressure inside the eye to increase. Secondary open-angle glaucoma also occurs for other reasons such as Pseudoexfoliation syndrome or use of steroids for long period of time [3]. Finally, glaucoma can be further classified as congenital and juvenile glaucoma.

1.1.2 Aqueous Humor pathway

The regulation of secretion and drainage of aqueous humor are physiologically important to maintain intraocular pressure (IOP). IOP is important to fill the eye and retain its shape as well as optical properties of the eye. Flow of aqueous humor against resistance produces an average IOP of 15 mmHg [4]. Aqueous humor secretion involves three important processes such as passive diffusion and ultrafiltration and active secretion [4]. Passive diffusion takes place when lipid soluble substances are transferred through the membrane which occurs between the capillaries and the chamber. On the other hand, ultrafiltration involves passive flow of water and water-soluble substances through the ciliary epithelial membrane. Active secretion is one of the most important process in aqueous humor formation as this is considered to contribute 80% to 90% of total aqueous humor secretion. Nonpigmented epithelial cell is the main site for active secretion [5].

The enzyme carbonic anhydrase which is present in nonpigmented and pigmented ciliary epithelial body, facilitates the transport of bicarbonate ion through ciliary epithelium. Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide to form bicarbonate ions and protons as shown by the following reaction [6]:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

Formation of bicarbonate affects aqueous humor transport by affecting Na+, feasibly by regulating the pH for optimal active ion transport [7]. Proper IOP is dependent on the regulation of inflow and outflow of aqueous humor. When the drainage canal gets blocked, the fluid in the anterior chamber builds up and elevates the intraocular pressure. High IOP damages blood vessels and optic nerves.

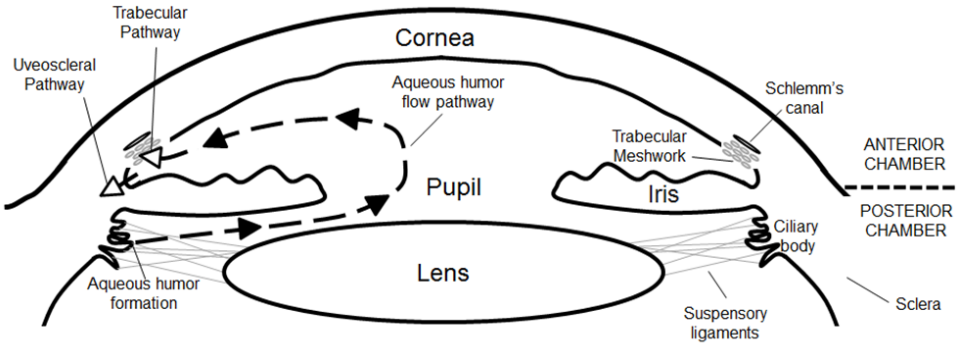

Figure 1.1: Aqueoushumor pathway [82].

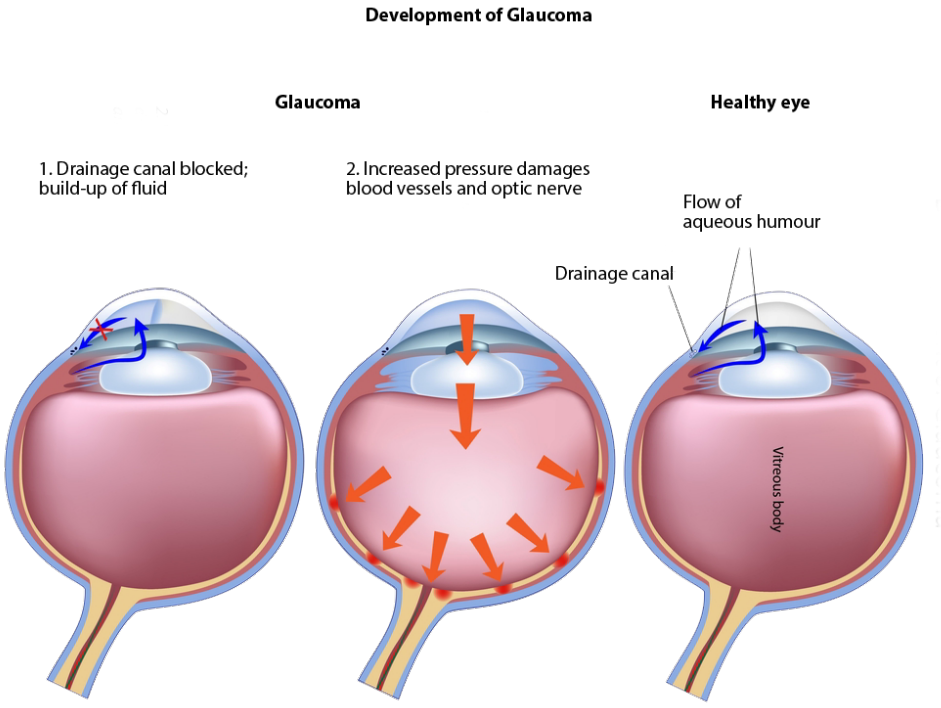

Figure 1.2: Aqueous humor flow in glaucoma patient [83].

1.1.3 Risk factors of Glaucoma

Several factors are associated with increase in risk for developing glaucoma such as age - people over age 60 are at greater risk for glaucoma. Glaucoma can even develop in younger people but the risk increases as we get older. Glaucoma can be inherited. So a family history of glaucoma increases the risk of developing glaucoma. Race is another risk factor for developing glaucoma. African-Americans are more likely to be at higher risk for developing glaucoma than Caucasians. Asians and Alaskans at higher risk of angle-closure glaucoma. Japanese people have greater tendency to develop low-tension glaucoma. People who have diseases such as diabetes, myopia, long term steroid or cortisone use and severe eye injury have tendency to develop glaucoma as well [8].

1.1.4 Signs and Symptoms of Glaucoma

Usually open-angle glaucoma is painless and it doesn't have any clear symptoms. Due to the lack of significant symptoms, glaucoma is not detected at early stage. That's why regular eye-checkup is very important. The only signs are gradual loss of vision and changes in optic nerve. In case of closed-angle glaucoma, the symptoms are characterized by severe eye pain, seeing halos around lights, red eye, high IOP, nausea, vomiting, decrease in vision and dilated pupil. Acute angle closure glaucoma patients need immediate attention [9].

1.1.5 Antiglaucoma agent

High intraocular pressure can be lowered with medications. Different classes of medications are available to treat glaucoma. Various topical and systemic medications are available as anti-glaucoma agent. Prostaglandin analogs increases uveoscleral outflow of aqueous humor and thereby decreasing the accumulation of aqueous humor in the anterior chamber. Prostaglandin drugs includes latanoprost, travoprost and bimatoprost increases the trabecular drainage [10]. Beta-adrenergic receptor antagonist decreases IOP by decreasing the secretion of aqueous humor from ciliary body epithelium. This class of drugs include timolol, levobunolol and betaxolol. Alpha2-adrenergic agonist works by both decreasing aqueous humor formation and increasing the outflow. Brimonidine and apraclonidine are examples of alpha2- adrenergic agonist. Alpha agonists such as epinephrine decreases the secretion of aqueous humor by constricting the blood vessels of ciliary body. Another class of anti-glaucoma agent known as miotic agents or parasympathomimetics such as pilocarpine increases the drainage of aqueous humor by constricting the ciliary muscle and the opening of the trabecular meshwork. Carbonic anhydrase inhibitors decrease high IOP by decreasing aqueous humor formation. Aqueous humor production

is decreased by inhibiting the enzyme carbonic anhydrase II in nonpigmented ciliary body epithelium [11].

1.2 CARBONIC ANHYDRASE

1.2.1 Introduction

Carbonic anhydrase (CA) is a family of enzymes that catalyzes the reversible hydration of carbon dioxide to form bicarbonate ions and protons and they are catalytically one of the fastest of all enzymes. The reaction catalyzed by carbonic anhydrase is [12]:

$$CO_2 \rightleftharpoons + H_2O \rightleftharpoons H_2CO_3\ HCO_3^- + H^+$$

They are ubiquitous metalloenzymes, they have zinc ion in their active site. There are five different CA families such as α, β, γ, δ and ζ and they are found in a wide variety of organisms such as vertebrates, algae, bacteria and plants. The most essential CAs are α-CAs as they are found in humans and they play vital roles in our body [13,14]. Mammalian CA isozymes are divided into four categories such as the CAs in the cytosol (CA I, CA II, CA III, CA VII, CA XIII), the CAs in mitochondria (CA VA and CA VB), secreted CAs (CA VI) and CAs bound to membrane (CA IV, CA IX, CA XII, CA XIV and CA XV) [15]. Among mammalian α-class CAs, CA II is the one of the crucial enzyme and it was the most studied of the CA isoforms. It was first isolated and characterized in 1993, CAII was first reported by Meldrum and Roughton when they discovered an enzyme in an erythrocyte that catalyzed the interconversion between carbon dioxide and bicarbonate ion. They play a vital role in many of the physiological processes such as respiration and transport of CO_2/bicarbonate between lungs and metabolizing tissues, pH and CO_2 homeostasis, secretion of ions in different organs and tissues and biosynthetic reactions such as

lipogenesis and gluconeogenesis, bone resorption, calcification, tumorigenicity, and many physiologic processes [16,17].

1.2.2 Catalytic and inhibition mechanism of carbonic anhydrases

The zinc ion present in the active site of CA II is very important for its activity. The data of X-ray structure revealed that the Zn^{2+} ion is located at the bottom of a 15 A° deep active site cleft and in coordination with three histidine residues (His 94, His 96, His 119) and a water molecule [11,18,19]. The hydroxyl moiety of Thr 199 forms a hydrogen bond with the zinc bound water molecule which in turn forms a bridge to the carboxylate moiety of Glu 106. These interactions help to increase the nucleophilicity of the zinc bound water molecule and adapt the substrate CO_2 in such a way that it favors the nucleophilic attack. The strong nucleophile (hydroxide bound to Zn^{2+}) attacks the CO_2 molecule bound in a hydrophobic pocket which leads to the formation of bicarbonate ion in coordination with Zn^{2+}. Later on, a water molecule displaces the bicarbonate ion and releases the bicarbonate ion into the solution. As a result, the enzyme becomes catalytically inactive. In order to make the enzyme active, a proton transfer reaction from the active site to the environment takes place which may be aided by active site residues or by the buffers present in the medium [20].

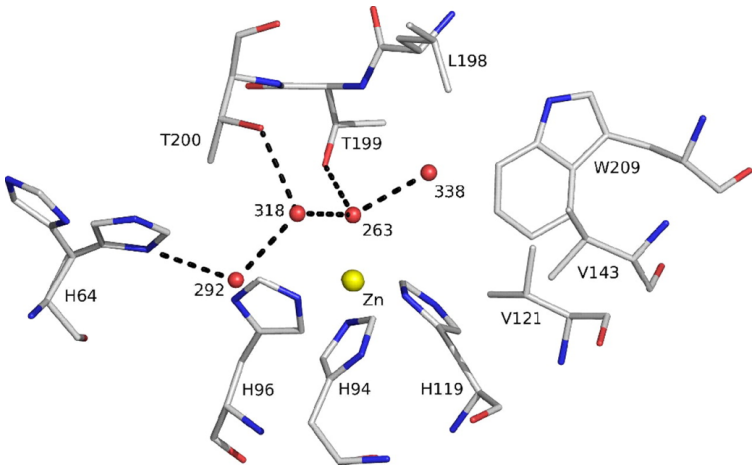

Figure 1.3: Active site of human Carbonic Anhydrase II [84].

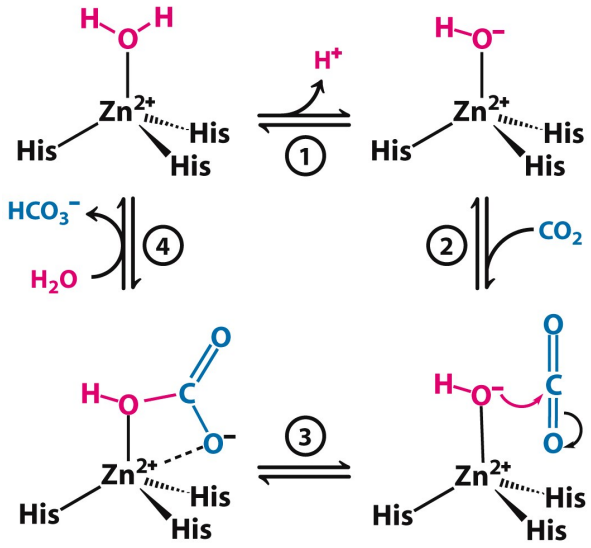

Figure 1.4: Schematic representation of the catalytic mechanism of carbonic anhydrases [85].

There are two main classes of carbonic anhydrase inhibitors which includes metal complexing anions and the unsubstituted sulfonamides. Both the inhibitors exhibit similar inhibition mechanism. They work by binding to the Zn^{2+} ion in the active site of CAs by either replacing the non-protein zinc ligand or adding to the metal ion and producing trigonal-bipyramidal species. The most important carbonic anhydrase inhibitors, sulfonamides in deprotonated state binds to the Zn^{2+} ion in a tetrahedral geometry. Metal complexing anions such as thiocyanate act by binding to the metal ion either in tetrahedral geometry or as trigonal bipyramidal adducts [21,22].

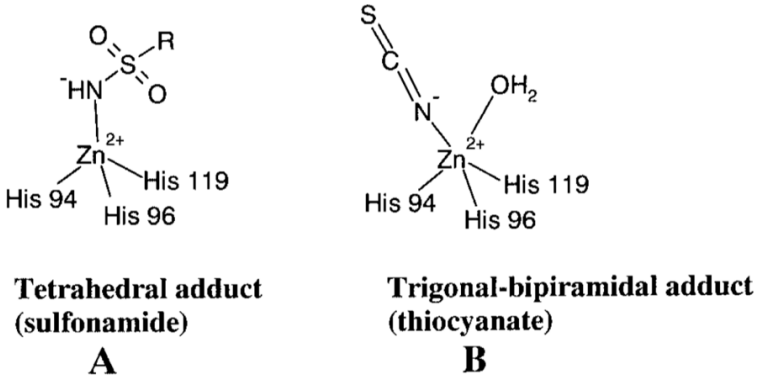

Figure 1.5: Mechanism of carbonic anhydrase inhibitors such as sulfonamide and thiocyanate [84].

1.2.3 Role of Carbonic Anhydrase in aqueous humor secretion

The enzyme carbonic anhydrase plays a vital role in the secretion of aqueous humor and inhibition of this enzyme will lead to reduction of aqueous humor secretion and pressure in glaucoma patients. Carbonic anhydrase is found in nonpigmented ciliary body epithelium. We all have a HCO_3^- storing system in the aqueous humor, based on the hydration reaction of carbon dioxide $CO_2 + OH^- \rightarrow HCO_3$ in the ciliary body epithelium. Carbonic anhydrase catalyzes this reaction but the reaction without the enzyme proceeds at measurable rate both chemically and physiologically. The secretory cells deliver OH^- ion to the luminal surface, and at the same time CO_2 is delivered from blood and cells [23,24]. 40% of Na^+ transported is linked to $HCO3^-$. Nascent fluid is isotonic with respect to sodium, but $HCO3^-$ ion is much higher whereas Cl^- is in lower concentration than in plasma. Inhibition of carbonic anhydrase removes about 70% of $HCO3^-$ concentration and 30% of Na^+ concentration. Chloride concentration remains unchanged. The decrease in Na^+ uptake reduces the secretion of aqueous humor. Thus it is clear that the enzyme is present in great amount of physiological needs and over 99% inhibition is necessary to produce a pharmacological effect.

1.2.4 Carbonic Anhydrase inhibitors as antiglaucoma agents

Carbonic anhydrase inhibition by sulfanilamide was discovered by Mann AND Keilin. [25]. Sulfonamides with CA inhibitory properties are mainly used as antiglaucoma agents, antithyroid drugs and some anticancer agents [26]. Two types of sulfonamides are developed as antiglaucoma agents: systemic and topical sulfonamides. Four systemic sulfonamides have been used clinically for a long time: acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide. Systemic sulfonamides are efficient in reducing high intraocular pressure by inhibiting the enzyme carbonic anhydrase II

and leads to 25 – 30% decrease of IOP, but the concern is that sulfonamide is susceptible to isozyme CA II and CA IV. They indiscriminately inhibit CA isozymes present in tissues other than that of the eye whereas the target should be only CA II for antiglaucoma effect. Inhibition of various CA isozymes leads to entire range of side effects such as numbness, tingling of extremities, metallic taste, depression, malaise, metabolic acidosis, etc. [13, 26]. The Fig 1.6 below displays the inhibition data for sulfonamides against several CA isozymes abundant in organs such as the eye, kidney, blood, lungs, CNS, etc.

Isozyme	*Catalytic activity (CO_2 hydration)*	*Affinity for sulfonamides*	*Sub-cellular localization*
CA I	Low (10% of that of CA II)	Medium	Cytosol
CA II	High	Very high	Cytosol
CA III	Very low (0.3% of that of CA II)	Very low	Cytosol
CA IV	High	High	Membrane-bound
CA V	Moderate-high[a]	High	Mitochondria
CA VI	Moderate	Medium-low	Secreted into saliva
CA VII	High	Very high	Cytosol
CARP VIII	Acatalytic	*	Probably cytosolic
CA IX	High	High	Membrane-bound
CARP X	Acatalytic	*	Unknown
CARP XI	Acatalytic	*	Unknown
CA XII	Active (no quantitative data)	Unknown	Membrane-bound
CA XIII[b]	Probably high	Unknown	Unknown
CA XIV	Low	Unknown	Membrane-bound

Figure 1.6: CA isozymes, Their relative CO_2 Hydrase activity, affinity for sulfonamide [81].

As systemic sulfonamides are not isozyme specific, it was a major concern to make it isozyme specific in order to reduce side effects. To minimize the side effects the route of administration was changed from systemic to topical. Thus topical sulfonamides were formulated which is administered directly into the eye. The first topically acting sulfonamide is Dorzolamide entered in clinics in 1995 [27], and the second drug Brinzolamide which is structurally related to Dorzolamide entered in clinics in 1999 [29]. But none of the clinically used inhibitors proved to be effective when administered topically in reducing elevated IOP. These two new drugs which show much less side effects as compared to the systemic sulfonamides but they also inhibit physiologically relevant CA isozymes (CA I and CA IV) present in ciliary epithelium of the eye. Thus the goal of this research project is to develop an isozyme specific inhibitor which will only inhibit CA II but not CA I and CA IV and reduces the high IOP by decreasing the secretion of aqueous humor.

1.3 APTAMERS

Aptamers are short (usually 20 to 60 nucleotides) single stranded RNA or DNA oligonucleotides with a specific and complex three-dimensional structure that are able to bind to target molecules with high specificity and affinity. Binding of aptamers to the target molecule results from structure compatibility which includes stacking of aromatic rings, van der Waals and electrostatic interactions [30, 31].

1.3.1 Theory and History

In 1990 Professor Larry Gold and his graduate student Craig Tuerk developed the process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) during an experiment at the University of Colorado. The experiment's results led to the concept that nucleic acids can bind to a wide variety of targets ranging from simple inorganic molecules to large protein complexes and entire cells [32]. Ellington & Szostak named the RNA molecule as "aptamer" referring to the latin word aptus – meaning "to fit", and the Greek word meros – meaning "region" [34]. Aptamers are ligands with high affinity and specificity that are able to bind to functionally important parts of their targets, hence acting as inhibitors [33]. SELEX is an efficient method for producing a protein binding drug. This method relies on the ability of a DNA-binding protein to select high affinity DNA ligands from a randomized pool of DNA sequences. SELEX is performed over several rounds with each round resulting in increased enrichment of DNAs capable of binding to the protein. This method enables to separate bound DNAs from unbound DNAs and the bound DNAs are amplified to generate a new pool. This process is repeated until aptamers with desired affinities are identified [35].

1.3.2 Technology

1.3.2.1 Random DNA oligonucleotide library

The starting point of SELEX is usually a chemically synthesized random oligonucleotide library. The oligonucleotide libraries contains two fixed primer binding sites and a region of randomized bases maximal 20 – 60 nucleotide in length. The primer binding sequences are required for PCR amplification at every selection cycle. In order to obtain strongest binding aptamers, the random pool must cover as much sequence space as possible [36]. The RNA or ssDNA libraries are used in SELEX procedures. In principal, the specificity and the affinity of ssDNA and RNA ligands is not different. The benefit of RNA aptamer is that they can be expressed inside cells which may have significant importance in vivo experiments. On the other hand, DNA aptamers are much more stable than RNA aptamers. Another potential advantage of DNA aptamers over RNA aptamers is that the selection process is much simpler and faster. The RNA aptamer selection needs reverse transcription and in vitro transcription. It also requires initial transcription for producing the RNA library from the DNA library. In case of DNA aptamer selections, these steps are not necessary . In addition, the cost of DNA aptamer production is lower than that for RNA aptamers. That is the reason DNA aptamers have become more prevalent recently [37].

The random region of ssDNA library is synthesized by adding the mixture of all four deoxyribonucleic acid derivatives to the reaction mixture allowing the random incorporation of a nucleotide into the growing molecule. The promoter sequence for the RNA polymerase of bacteriophage T7 is introduced into the 5' terminal region of the ssDNA library to generate an RNA library, dsDNA is obtained by a polymerase chain reaction (PCR), and then an in vitro transcription is carried out. The synthesis of random sequences are relatively cheap [38].

The size (n) of random domain determines the complexity of the library, which is calculated as 4^n (n is the number of positions in the random sequence. For example, the complexity of a library with thirty-five fully randomized nucleotides is 4^{35} or 10^{21}. Enough randomized nucleotides should be used to get a high affinity ligand and most probably a stable structure. So far the successful aptamers selected in the SELEX experiments represents 1 in 10^9 to 10^{13} of the molecules in the starting library [39].

Aptamers are chemically modified to achieve particular purposes such as to expand the potential varieties of oligonucleotides, to introduce new features e.g. addition of functional groups in order to provide new possibilities for the interaction with the target molecules, to improve the stability of the aptamers and to increase their resistance to nucleases [40]. Typical modifications include the 2'-position of the sugar in RNA libraries. The ribose 2'-OH group of pyrimidine class nucleotide is substituted with a 2'-NH_2 or F group, this shields the RNA from nuclease degradation [41]. There is another technique applied to overcome the instability of aptamers is the creation of spiegelmers. The idea of spiegelmers is that the aptamers composed of natural D-oligonucleotides can be selected against mirror image targets, such as D-amino acid peptides rather than natural L-amino acid peptides. Once the aptamers are isolated, they can be chemically synthesized as L-oligonucleotide and they are called Spiegelmer. They will bind to the natural L-amino acid peptide targets [42]. As Spiegelmers are composed of unnatural monomers, they are insensitive to nuclease degradation and have shown to be stable over 60 hours in biological fluids [42, 43].

1.3.2.2 Standard Selection Process (SELEX)

The standard SELEX procedure starts with subjecting the oligonucleotide library to any target molecule. The oligonucleotide sequences in the library which binds to the target are considered as aptamers. Non-binding aptamers are then separated from binding aptamers. Those

that binds to the target molecule are amplified by polymerase chain reaction (PCR) to enrich the pool and then they are selected through several additional selection cycles. The amplification is performed by PCR in the case of DNA, and by RT-PCR followed by *in-vitro* transcription in the case of RNA. The number of high affinity binding molecules are reduced to a small number through several rounds of selection and then the individual aptamers are isolated, sequenced and analyzed.

The partitioning of the aptamer-target complex from non-specific molecules can be achieved by various techniques. The most commonly used method for protein targets partitioning is filtration through nitrocellulose filters [44]. The selection processes using nitrocellulose membranes usually require up to 12-15 selection cycles. Alternatively, the use of functionalized magnetic absorbent particles with a magnetic separation system has also been considered to be a useful tool for the separation of protein and nucleic acids [45]. Also, using affinity tags like glutathione S-transferase and streptavidin - derivative surfaces [46, 47] or column matrices like sepharose can be used to reduce the number of selection cycles [48].

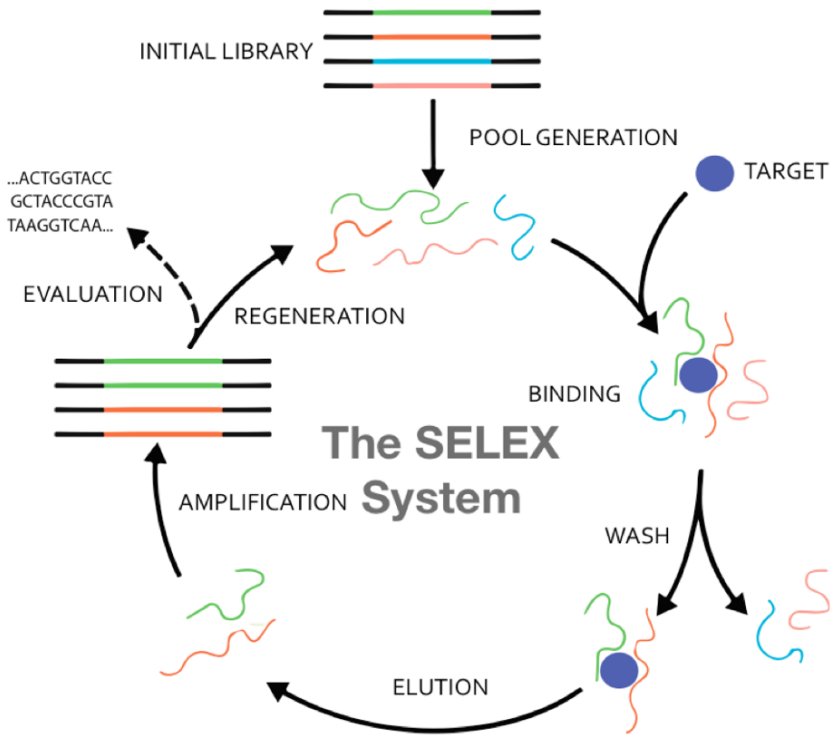

Figure 1.7: General scheme of SELEX system [86].

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Electrophoresis (CE), Flow Cytometry (FC) [50], Electrophoretic Mobility Shift Assay [51], Surface Plasmon Resonance (SPR) [52].

1.3.2.3 RNA and DNA SELEX

The basic process of *in vitro* selection is same for both RNA and DNA SELEX. In order to select RNA aptamers, before starting the first round of SELEX, the random DNA oligonucleotide must be converted into an RNA library. A particular forward primer flanked at the 5'-end comprising of T7 promoter sequence and reverse primers are required to transform the ssDNA library into a double stranded DNA (dsDNA) library by amplification. An *in vitro* transcription of dsDNA was carried out by the T7 RNA polymerase and producing a randomized RNA library. In every cycle of SELEX a reverse transcription of RNA aptamers should be done and afterwards the aptamer library should be amplified by RT-PCR. For every cycle, the RNA library is produced by *in vitro* transcription.

The procedure of DNA SELEX is much simpler since it involves only one PCR amplification. The library can be used straight in the first cycle of the SELEX in case of DNA aptamer selection. Forward and reverse primer resulting from the specific sequences at the 5'- and 3'- end enable amplification of the selected oligonucleotides in each SELEX round. After PCR amplification, ssDNA preparation must be performed to generate a ssDNA pool for the next round. Several methods are commonly applied for the generation of ssDNA. Double stranded DNA is separated into single stranded DNA by streptavidin coated magnetic beads. A biotin residue is introduced into one of the primers used for amplification, and the DNA strands are denatured with streptavidin [53].

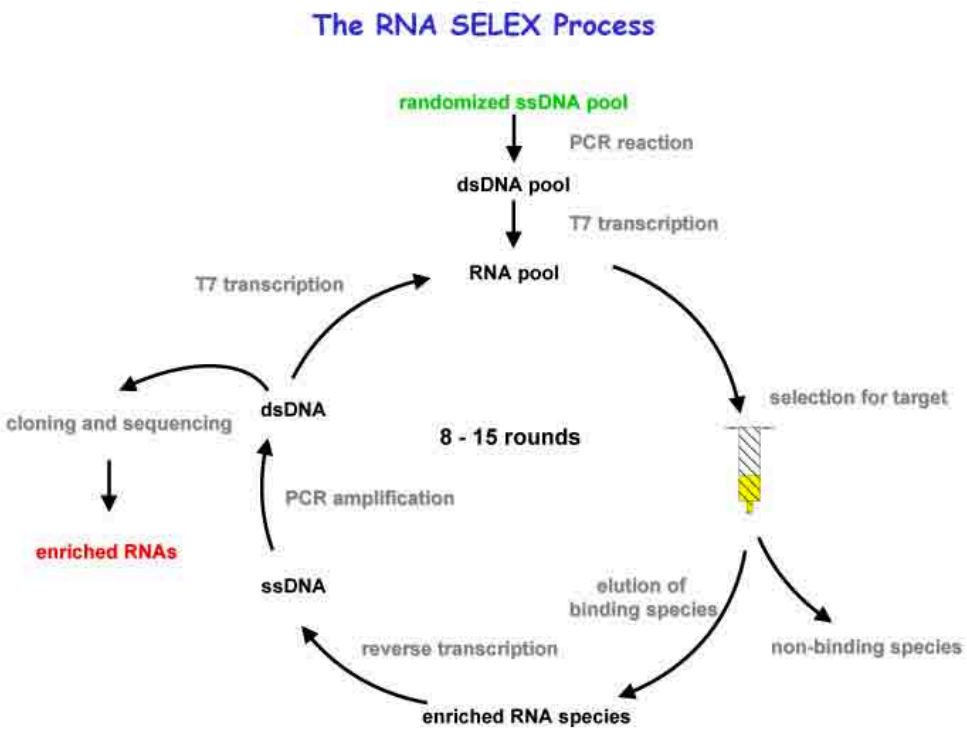

Figure 1.8: Scheme of RNA SELEX [87].

Asymmetric PCR amplification is another method to generate ssDNA. In this process, different ratios of primers are used and the major product is the single stranded DNA. As asymmetric PCR progresses, the lower concentration reverse primer is producing double-stranded DNA and the forward primer which is in excess amount continues to produce the target strand. [31]. An enzyme digestion method is also applied to produce ssDNA. A phosphate group is introduced into the 5' – end of one primer and the dsDNA obtained by amplification is treated with the lambda exonuclease that cleaves the phosphorylated strand of DNA [54]. Another method to generate ssDNA can be done by adding hexaethyleneglycol (HEGL) spacer and an elongation of some adenine nucleotides (polyA) at the 5'- end of the reverse primer. The HEGL turns as a terminator for the Taq polymerase. The extension of the target strand stops, whereas the non-target strand continues to grow. The two strands can be separated by electrophoresis under denaturing

conditions [55]. Until now, a number of both DNA aptamers and RNA aptamers have been selected against various targets. Investigation shows that there is no difference between RNA and DNA aptamers in terms of affinity and specificity [49]. Therefore, DNA aptamers have become more prevalent during recent years [56].

1.3.3 Aptamers and antibodies

Antibodies has been used most extensively for molecular identification for more than three decades. Aptamers are commonly known as a replacement of antibodies as they overcome the limitations of antibodies. There are some advantages of aptamers over antibodies such as the thermal stability of aptamers. Oligonucleotides are more stable at higher temperature than antibodies and are able to retain their structure during multiple denaturation at 95°C in each selection rounds. However, proteins are irreversibly denatured at elevated temperature. That's why aptamers can be used under an extensive range of assay conditions. The production of antibodies are time consuming and very costly because it includes screening of large quantity of colonies [57]. Furthermore, immunoassays are needed to check the activity of the antibodies in each set, because the functioning of the same antibody has the tendency to differ from batch to batch. Conversely, aptamers can be synthesized with high precision and reproducibility. Selection of aptamers are more cost effective than the production of antibodies. Besides, modifications of aptamers are more easier and can be done to enhance their stability and resistance toward nucleases. Moreover, signal moieties such as fluorophores and quenchers can be used to produce biosensors. Aptamers are generally low-immunogenic, since our immune system cannot recognize oligonucleotides as external substances. Nevertheless, antibodies are known to be immunogenic, which prevents repeated dosing [58]. The FDA approved aptamer "Macugen" showed very little immunogenicity when administered to monkeys in 1000-fold higher doses (this data was

demonstrated by the Eyetech Study Group). Also, aptamers show a broad range of applications toward some ions or small molecules which cannot be detected by antibodies [59].

1.3.4 Aptamers in diagnostics

1.3.4.1 Aptasensors

Aptasensors are biosensor elements and their structural component is the aptamer [60]. Aptamers can be chemically modified without influencing their affinity and also they can easily be labelled to be used in diagnostics [61, 62]. Aptasensors can be classified into two main types, optical and electrochemical aptasensors, (1) Optical aptasensors include aptamers labelled with fluorescence, luminophore, enzyme, nanoparticles or aptamer with label-free detection systems (e.g., SPR, Surface Plasmon Resonance) [63]. E.g. an RNA aptamer was selected and used as a detector ligand in a sandwich assay to recognize vesicular endothelial growth factor (VEGF) [64]. (2) Electrochemical aptasensors depend on the immobilization of the aptamer on an electrode surface. Then the binding conditions with their targets can be monitored by the electrochemical current variations [65]. E.g. a novel electrochemical sensor system based on two different aptamers recognizing different epitopes of thrombin was developed. The first aptamer was thiol-modified and immobilized on a gold electrode for capturing thrombin while the second indicator aptamer was labelled with a pyrroloquinoline quinone glucose dehydrogenase [66]. Several aptasensors have been developed to detect microorganisms and viral proteins e.g. RNA aptamer chip was successfully manufactured for detecting HCV core antigen [67] and also RNA aptamer developed for the detection of the HIV-1 Tat protein [68].

1.3.4.2 Flow cytometry

The binding of aptamers to their target proteins presented on either cell surfaces or microspheres can be detected by flow cytometry. E.g. a fluorescently labelled DNA aptamer with high affinity to human neutrophil elastase (HNE) was used to stain HNE-coated beads for flow cytometry [69, 70] and also a fluorescently labelled RNA aptamer with a high binding affinity to mouse CD30 proteins had been evaluated for human CD30 protein recognition on intact cells by both, flow cytometry and fluorescence microscopy [71].

1.3.5 Aptamers in therapeutics

Aptamers have been proven to be a promising class of novel drug as they are characterized by small size, low or no immunogenicity, high stability, high specificity and high affinity to their targets. In addition, the synthesis and selection of aptamers is relatively easy and inexpensive. Aptamers have been validated as therapeutics in the areas of anti-infective, anticoagulation, anti-inflammation, anti-angiogenesis, anti-proliferation, and immune therapy [72]. Macugen, the first FDA approved aptamer in 2004 that was used against macular degeneration disease [73]. A peptide hormone ghrelin which is related to appetite and weight gain was used as a target molecule for the development of spiegelmers.

CHAPTER 2: OBJECTIVE

The goal of this research project is to select and characterize ssDNA aptamers specifically binding to carbonic anhydrase II (CAII) and to evaluate a potential *in vitro* inhibition of CAII activity that catalyze the rapid interconversion of carbon dioxide and water to bicarbonate ion. Inhibition of CAII has therapeutic application in the treatment of glaucoma, a disease affecting a large number of people characterized by an elevated intraocular pressure (IOP). As isoforms CA II/IV/I are present in many other tissues/organs, generally, systemic CA inhibitors possess undesired side effects such as numbness and tingling of extremities, metallic taste, depression, fatigue, etc. There's no CA inhibitor reported yet which can specifically bind to CA II but not CA IV and CA I.

In order to avoid above mentioned side effects, there should be an inhibitor which is isozyme specific and has high affinity toward CA II. Toward this goal a ssDNA (N_{40}) library will be screened by using SELEX method which includes ten rounds of positive selection against CA II and negative selection against CA I, CA IV and Brinzolamide. Next-generation sequencing will be used to sequence the aptamer candidates. Each sequence will be tested for inhibition of carbonic anhydrase II activity by Wilbur-Anderson method and Surface Plasmon Resonance will be used to analyze the binding affinity of an inhibitory aptamer. This could be the first step in the development of CA II isozyme specific DNA aptamers.

CHAPTER 3: METHODS & MATERIALS

3.1 CLONING AND EXPRESSION OF HUMAN CARBONIC ANHYDRASE II

The hCAII _pcDNA3.1+/C-(K) DYK construct (Genscript), was PCR amplified using Q5 high-fidelity DNA polymerase (NEB) with restriction enzymes specific primers. Restriction digestion using Nde I (NEB) and Sac I (NEB) enzymes was performed for the PCR amplified product and ligated to the corresponding sites in pET28a vector using 2X Instant sticky ligase Mix (NEB). The ligation product was transformed in the DH5α bacterial cells using electroporation method. Further, the colonies were inoculated and checked for the positive clones by performing restriction digestion using Nde I and Sac I.

The hCAII_pET28a construct was transformed into BL21De3 cells by electroporation method of transformation. The transformed cells were plated onto LB-Agar-Kanamycin (50 µg/ml) plate and incubated at 37°C overnight to form colony forming units. A single colony was inoculated into 10 ml of Luria Bertani (LB) media along with 50 µg/ml of kanamycin, 0.5 mM $ZnSO_4$ at 37°C for overnight at 200 rpm. Overnight grown primary 10 ml culture was added along with 50 µg/ml kanamycin and 0.5 mM $ZnSO_4$ into 1 liter secondary culture. Cells were grown at 37°C until 0.6-0.8 at $O.D._{600}$ and further induced with 0.1 mM IPTG at 18°C for 16 hours. Cells were harvested for 20 minutes at 6000 xg. and the cell pellet was resuspended into phosphate buffer and centrifuged again at 3700 xg for 15 minutes. The final pellet was thawed and resuspended in 30 ml of lysis buffer (20 mM MOPS at pH 7.5, 10% glycerol, 1 mM PMSF) and sonicated for 5 minutes with 10 seconds on pulse followed by 10 seconds off pulse. DNAse (1 µg/ml) was added to the sonicated cell lysate and gently stirred using magnetic stir bar on ice for 20 minutes. The cells were centrifuged at 38,759 xg for 45 minutes at 4°C and the supernatant was loaded onto the 1 ml crude FF-His column (GE healthcare) pre-equilibrated with loading buffer (50 mM MOPs at

pH 7.5) at 0.5 ml/min flow rate. Further, 15 column volume of loading buffer wash performed, followed by the elution step using elution buffer (50 mM MOPs at pH 7.5, 500 mM Imidazole). Elution was performed using 50 mM, 100 mM and 250 mM imidazole concentration. hCAII protein was eluted in all the three concentrations of imidazole, with the purest in the 250 mM imidazole fraction. The hCAII fractions were pooled and concentrated using 10 kDa cut-off concentrator (Millipore Sigma). Buffer exchange step was performed using 50 mM MOPs pH 7.5 buffer to get rid of imidazole. hCAII protein was analyzed for its size as well as purity by performing gel electrophoresis using 15% SDS PAGE gel. The protein concentration was estimated by absorbance value at 280 nm using spectrophotometer (Eppendorf). The purified protein was aliquoted, flash frozen in liquid nitrogen and stored at -80°C.

3.2 DNA TECHNOLOGY

3.2.1 Polymerase Chain reaction (PCR)

The PCR was first developed by Kary Mullis in 1983 [74]. PCR is a method to amplify a segment of DNA to generate millions of copies of a DNA sequence. In molecular biology PCR is one of the most widely used technique. It is an easy, cheap and most reliable way to replicate DNA. A PCR reaction requires Taq polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are incorporated in a PCR tube and are put through repeated PCR cycles of heating and cooling to allow the synthesis of DNA [75]. The basic steps of PCR includes denaturation at 96° C. This step denatures the DNA strands and provides single-stranded template for the next step. After denaturation annealing (55-65°C) is the next step where the reaction is cooled to allow the primers to bind to their complementary sequences on the single stranded DNA

template. Finally, extension at 72°C in which the temperature of the reaction is elevated so that the enzyme Taq polymerase can extend the primers, synthesizing new strands of DNA.

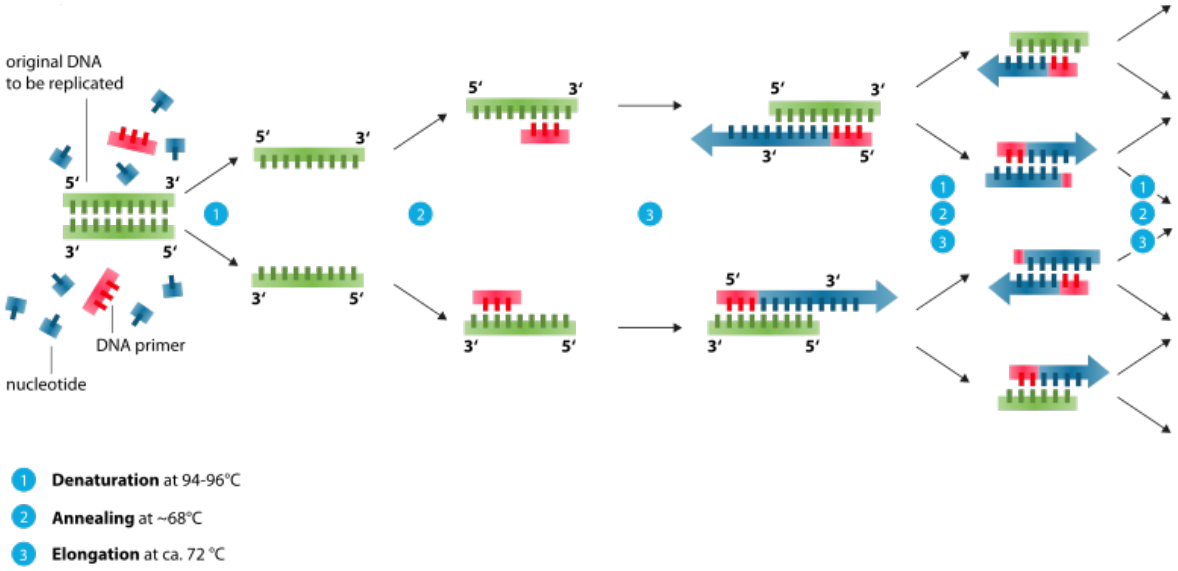

Figure 3.1: Steps of Polymerase Chain Reaction [88]

3.2.2 PCR during aptamer selection

3.2.2.1 Symmetric PCR

It is same as the conventional PCR. Symmetric PCR was carried out in a 50 μl reaction volume consisting of 50ng of ssDNA library, 1X Hot start PCR master mix, and equal ratios of biotinylated forward and reverse primer. Symmetric PCR cycle was run for total of 25 cycles (Table 3.1) and the temperature for denaturation was set at 94°C, annealing temperature was at 58°C and extension at 72°C.

Table 3.1 Symmetric PCR conditions

Step	Temperature	Time
Initial denaturation	94°C	2 minutes
Denature	94°C	30 seconds
Anneal	58°C	30 seconds
Extend	72°C	40 seconds
Hold	4°C	Indefinitely
Total PCR cycles	25 cycles	

3.2.2.2 Asymmetric PCR

Generation of ssDNA play key roles in ssDNA aptamer selection. Every new cycle must be initiated with ssDNA library. An asymmetric PCR is done in order to achieve this goal. An asymmetric PCR reaction involves amplification of one DNA strand in a double stranded DNA template. Asymmetric PCR is similar to symmetric PCR, except that the amount of primer for the targeted strand is much more than that of the non-targeted strand. As the asymmetric PCR progresses, the lower concentration limiting primer is quantitatively incorporated into newly synthesized double stranded DNA and used up. As a result, linear synthesis of the targeted single DNA strand from the excess primer are formed after depletion of the limiting primer [76].

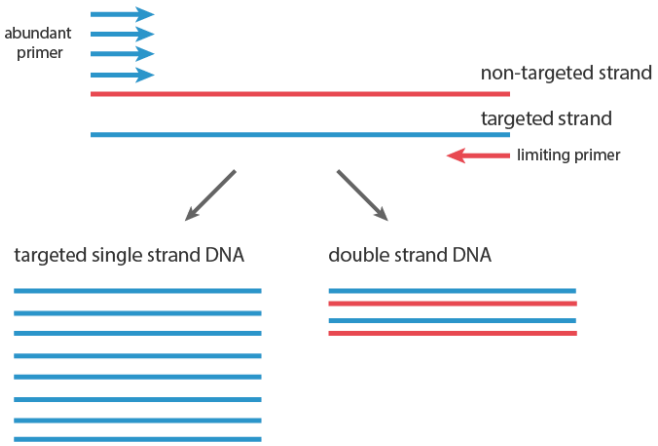

Figure 3.2: Steps of Asymmetric PCR [89].

Asymmetric PCR was performed in a 25 μl reaction volume, which consisted 1 μl of symmetric PCR product as an initial template, 20 pmol of nonbiotinylated forward primer and 1 pmol of biotinylated reverse primer (primer ratio was 20:1) and 12.5 μl of hot start PCR master mix. Asymmetric PCR protocol was as set for 25 cycles in total and the temperature for denaturation was at 94°C, annealing remparature was at 62°C which is little bit higher than symmetric PCR and finally the extension temperature was at 72°C (Table 3.2).

Table 3.2: Asymmetric PCR conditions

Step	Temperature	Time
Initial denaturation	94°C	2 minutes
Denature	94°C	30 seconds
Anneal	62°C	30 seconds
Extend	72°C	40 seconds
Hold	4°C	Indefinitely
Total PCR cycles	25 cycles	

3.2.3 Streptavidin Magnetic Bead Isolation: purification of ssDNA

Magnetic beads coated with streptavidin are used to capture the biotin-tagged PCR product. An asymmetric PCR generates mixtures of dsDNA along with ssDNA. Purification with streptavidin coated magnetic bead is applied to eliminate dsDNA and other by-products from asymmetric PCR product. At first, symmetric PCR using biotinylated primers was carried out to provide enough templates for asymmetric PCR. Secondly, asymmetric PCR was carried out with an abundant nonbiotinylated forward primer and a limited biotinylated reverse primer to generate ssDNA. Thus, both dsDNA and the by-products produced during the PCR cycles carried biotin. Finally, dsDNA and by-products were eliminated via streptavidin magnetic beads [77].

Streptavidin-coated magnetic beads of 20 μl were washed three times with 50 μl of normal saline and were captured using a magnetic bead after each wash. All asymmetric products were transferred into the tube that contains the washed streptavidin-coated magnetic beads, and the mixture was incubated at room temperature for 20 minutes with gentle mixing. Streptavidin-coated magnetic beads were captured using a magnetic stand, and supernatants were carefully collected without disturbing the streptavidin-coated magnetic beads.

3.2.4 Gel Electrophoresis

The symmetric, asymmetric and magnetic bead isolated products were separated on a 4% agarose gel. Each sample (10 μl) with 5 μl of 6X loading dye was loaded onto a 4% agarose gel, run in 1X TBE buffer at 80 volts for 70 minutes, and detected by SYBR gold nucleic acid gel stain. The gels were visualized under UV light.

3.2.5 Ethanol precipitation of DNA

The extracted or eluted ssDNA from selection cycles were concentrated by a widely used technique called ethanol precipitation. This method is carried out by adding salt (monovalent cations such as sodium ions) and ethanol to the solution containing ssDNA. Ethanol efficiently precipitates ssDNA in presence of salt. The precipitant of ssDNA is collected by centrifugation followed by the removal of the supernatant [78].

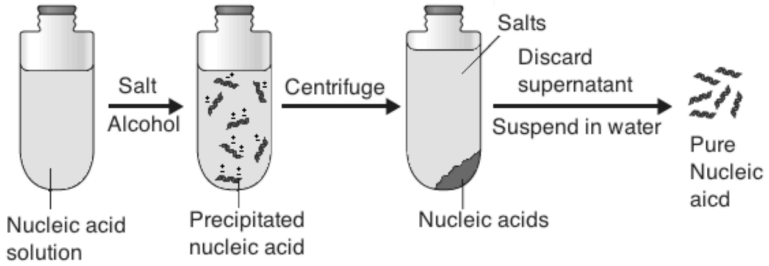

Figure 3.3: Steps of ethanol precipitation of DNA [90].

Ethanol precipitation of DNA protocol was started by adding 1/10 volume of 3M sodium acetate to the solution containing ssDNA followed by the addition of 3 μL of glycogen. Equal volume of isopropanol was added and incubated at -20°C overnight. The solution was centrifuged at 14000 rpm for 15 minutes and then the supernatant was discarded. The ssDNA pellet was rinsed with 70% ethanol. The solution was centrifuged again for 15 minutes. The supernatant was discarded and the pellet was dissolved in water.

3.2.6 Determination of nucleic acid concentration

The concentration of dsDNA and ssDNA was determined by spectrophotometer at wavelength 260nm (A_{260}). The function describing the concentration to absorbance relation is the Lambert-Beer law: OD = e* c* d

The optical density (OD) is the product of the substance specific extinction coefficient (e), the concentration of the absorbing sample (c), and the optical path length in cm (d). The commonly accepted absorbance to concentration conversion for nucleic acid is listed below:

dsDNA: 1 OD_{260} = 50 ug/ml

ssDNA: 1 OD_{260} = 33 ug/ml

The concentration of protein is determined at wavelength 280 nm (A_{280}).

3.3 IN VITRO SELECTION OF APTAMERS

3.3.1 Nitrocellulose filter partition method

Nitrocellulose membrane based SELEX was used for the selection of aptamers in order to isolate unbound ssDNA aptamers from DNA-protein complexes. The principle of this method is that most of the proteins nonspecifically binds to nitrocellulose membrane. Once the protein forms a complex with a nucleic acid, the complex also binds to the nitrocellulose filter. As a result, the unbound sequences are separated from the bound sequences.

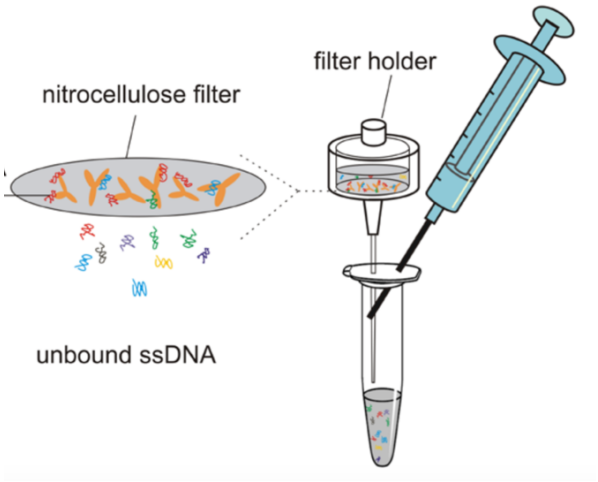

Figure 3.4: Nitrocellulose filtration partition method [91].

3.3.1.1 Selection procedure

SELEX method was used for the selection of aptamers which involved both positive and negative selections. The positive target of *in vitro* selection was CA II and the negative target was CA I, CA IV and Brinzolamide. Total ten rounds of consecutive positive and negative selection cycles were done. Generally, there is a competition between specifically and nonspecifically binding nucleic acid sequences. Sequences with low affinity can be removed by increasing the stringency of selection. A variety of parameters were used throughout the different rounds of

cycles to increase the stringency, for example using different ratios of ssDNA: protein, decreasing incubation time, and increasing number of washings with binding buffer.

Table 3.3: Conditions of stringency during SELEX rounds

Cycle	DNA (pmol)	CA II (pmol)	Incubation time (mins)	No. of washings
1	1000	400	30	2
2	800	300	20	4
3	800	200	15	6
4	800	100	15	8
5	800	50	10	10
6	800	30	10	12

3.3.1.2 Pre-selection of aptamers

In order to refold aptamers into 3 dimensional structures, the ssDNA library was dissolved in 150 μL of binding buffer and it was heated to 95°C for 15 minutes. After 15 minutes the ssDNA library was cooled at room temperature for 10 minutes. The ssDNA library was filtrated through nitrocellulose membrane filter in absence of positive and negative target to eliminate nitrocellulose membrane binders.

3.3.1.3 Positive selection of aptamers

The pre-selected aptamers were incubated with the positive target CAII at 37°C for 30 minutes. The CA II-aptamer complex. The nitrocellulose membrane filter was soaked into the reaction mixture. The mixture was allowed to incubate at 37°C for 30 minutes to let the complex

to bind to the filter. The concentration of the unbound sequences left in the solution was measured by spectrophotometry. The filter was removed from the reaction mixture and placed in a clean tube. The filter was washed several times with 1 ml of binding buffer in order to get rid of loosely bound and unbound sequences. Finally, the bound sequences were extracted from protein by adding an elution buffer. In order to elute the sequences, the filter was cut into pieces and 200 μL of elution buffer was added. The mixture was heated at 95°C for 5 minutes followed by vortexing. Elution was done several times to make sure that all the tighter binding sequences are recovered. At the end of elution step, the bound sequences were concentrated and precipitated by the method called ethanol precipitation. The precipitated aptamers were resuspended in water and amplified by symmetric PCR using biotinylated forward and reverse primer. To generate ssDNA asymmetric PCR was done after symmetric PCR. The symmetric PCR product was used as a template for asymmetric PCR where the reverse primer was biotinylated and the forward primer was nonbiotinylated. The asymmetric PCR products contains mixtures of ssDNA and dsDNA. To eliminate dsDNA and other by-products, asymmetric PCR product was purified by streptavidin magnetic bead isolation.

3.3.1.4 Negative selection with CA I and CA IV

A negative selection with other CA isozymes (CA I and CA IV) was done to make sure that the aptamers specifically binds to CA II but no other isozymes. In order to get isozyme specific aptamers, positively selected aptamers were incubated with the negative target CA I and CA IV at 37°C for an hour. After incubation, the mixture was treated with a nitrocellulose filter. The bound aptamers were discarded and the unbound aptamers were collected. At this stage the unbound

aptamers are CA II binders only. This negative selection was done twice to make sure that all the CA I and CA IV binders were eliminated.

3.3.1.5 Negative selection with Brinzolamide

Brinzolamide is a sulfonamide drug commercially available in the market used to treat high intraocular pressure in patients with open angle glaucoma. It inhibits carbonic anhydrase II by the interaction of sulfonamide group to the active zinc ion of carbonic anhydrase II.

The target of this research is to get not only CA II binders but also an inhibitory aptamer. To achieve this goal, we have to make sure that the aptamers binds to the active site of carbonic anhydrase II and inhibits its activity. Therefore, a negative selection was done with Brinzolamide. At first the CA II and Brinzolamide was incubated for an hour at 37°C to allow the active site of CA II to be occupied by Brinzolamide. Then the aptamers were added to the mixture and allowed to incubate for one more hour at 37°C. The aptamers will bind to CA II other than the active site. The binders were discarded and the unbound aptamers or active site binders were collected and amplified. This negative selection was done twice to make sure we have eliminated all the non-active site binders. Few more positive selections were done with these active site binders to get higher affinity and more specific binding aptamers.

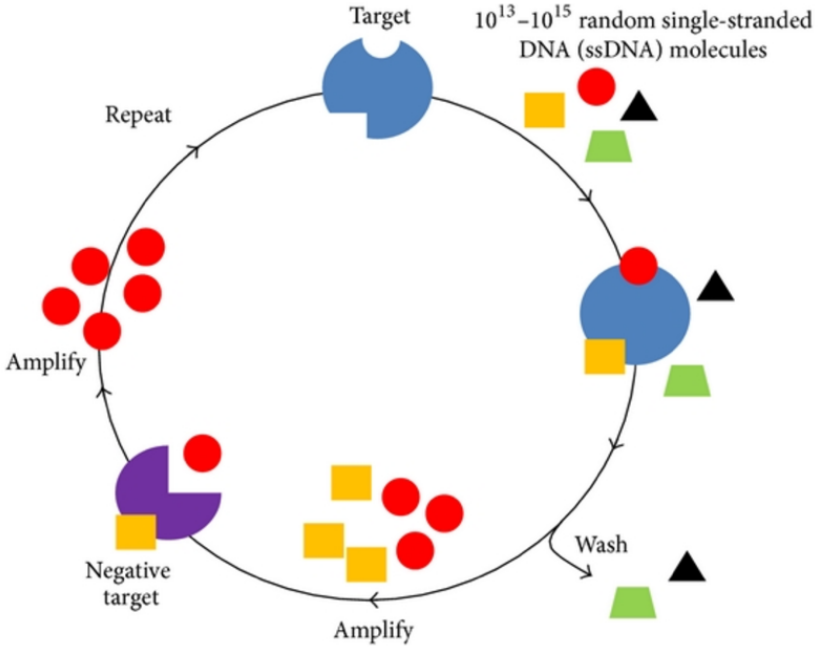

Figure 3.5: Schematic representation of negative selection in SELEX method [92].

3.4 Evaluation of aptamers by Next Generation Sequencing

Next Generation Sequencing is a great platform where thousands to millions of DNA molecules can be sequenced. NGS enables sequencing of multiple individuals simultaneously. NGS facilitates the scientist to perform a wide variety of applications and study biological systems with its ultra-high throughput, speed and scalability. NGS overcomes the limitations of Sanger sequencing.

After 10 rounds of positive and negative selection, the selected aptamers were sequenced by Next Generation Sequencing (NGS) from the company called Novogene. Next generation sequencing requires high quality product. The symmetric PCR product after 6th positive selection round was sent to Novogene for sequencing of the library. The table below shows the requirements of the PCR sample for NGS.

Sample Type	Remarks	Amount (Qubit)	Volume	Concentration	Purity
PCR products	Strongly Recommended	⩾3 μg	≥20 μL	≥50 ng/μL	OD260/280=1.8-2.0 No degradation or RNA contamination
	Required	⩾1.5 μg			

Figure 3.6: Requirements of quality and quantity of PCR sample for NGS

During NGS the quality of the crude PCR sample was determined. Once the DNA sample passes the quality control test, the qualified DNA is cut into fragments by restriction enzyme. The construction of the DNA library (Fig 3.7) is done through the process of end repairing, adding A to tails, purification, PCR amplification and etc. libraries are sequenced by Illumina high-throughput sequencer with paired-end sequencing strategy. The qualified libraries are fed into sequencers after pooling according to its effective concentration and expected data volume.

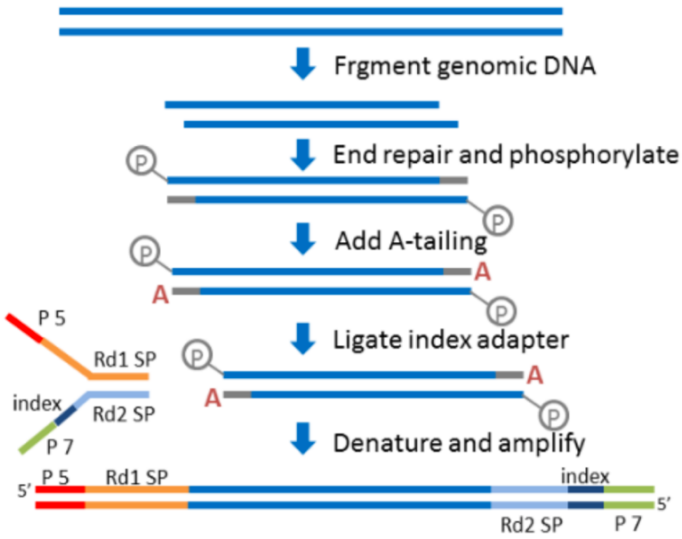

Figure 3.7: Principle of library construction in NGS.

3.5 BIOPHYSICAL ASSAYS

3.5.1 Enzymatic assay of Carbonic Anhydrase II

CA activity assay was performed using Wilbur-Anderson method. The assay was carried out at 0°C using CO_2 as a substrate following the pH variation due to the catalyzed conversion of CO_2 to HCO_3^-. CO_2 saturated water was made by adding CO_2 from a tank into a 250ml Erlenmeyer flask filled with ddH2O. The flask was placed in an ice bath. The enzyme was suspended in 3ml of 20 mM Tris buffer (pH 8.3) in a vessel that contained a pH electrode. The time taken for the pH to change from 8.3 to 6.3 was measured on the addition of 2 ml ice cold CO_2-saturated water. A blank reaction was performed by adding ice-cold water instead of the enzyme into 3 ml ice-cold Tris buffer followed by the addition of the substrate CO_2. A negative control reaction was carried out by adding the enzyme CA II in 3ml Tris buffer followed by CO_2 substrate. A positive reaction was carried out by adding an inhibitor Brinzolamide with CAII, Tris buffer and finally CO_2 substrate. A test reaction was done by the aptamers with CA II in Tris buffer and the CO_2 substrate.

CHAPTER 4: RESULTS

4.1 CLONING, EXPRESSION AND PURIFICATION OF HUMAN CARBONIC ANHYDRASE II

The hCAII _pcDNA3.1+/C-(K) DYK construct (Genscript), was PCR amplified using Q5 high-fidelity DNA polymerase (NEB) with restriction enzymes specific primers. A gradient PCR was set up to ensure the annealing temperature of the primer. Restriction digestion using Nde I (NEB) and Sac I (NEB) enzymes was performed for the PCR amplified product and ligated to the corresponding sites in pET28a vector using 2X Instant sticky ligase Mix (NEB). The ligation product was transformed in the DH5α bacterial cells using electroporation method. Further, the colonies were inoculated and checked for the positive clones by performing restriction digestion using Nde I and Sac I.

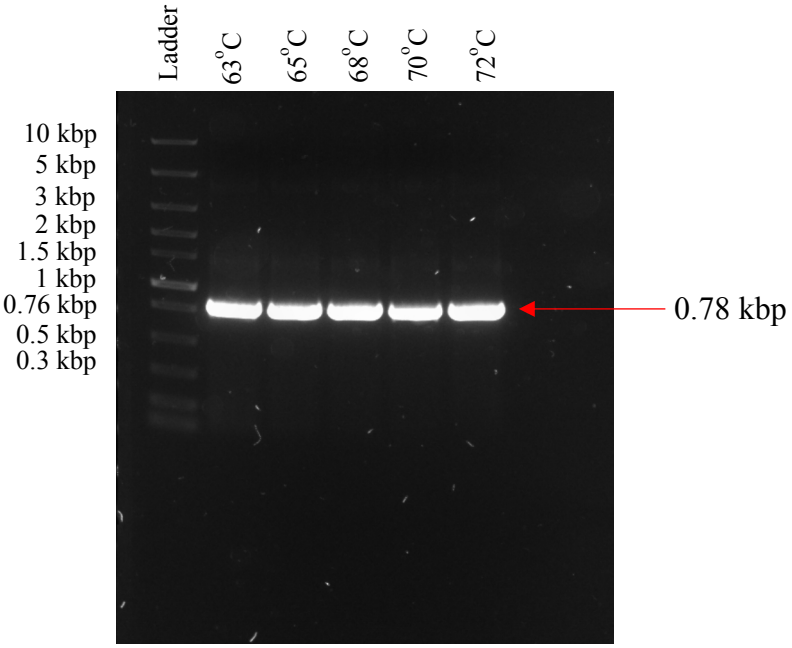

Figure 4.1: PCR amplification of Nde I – hCAII – Sac I.

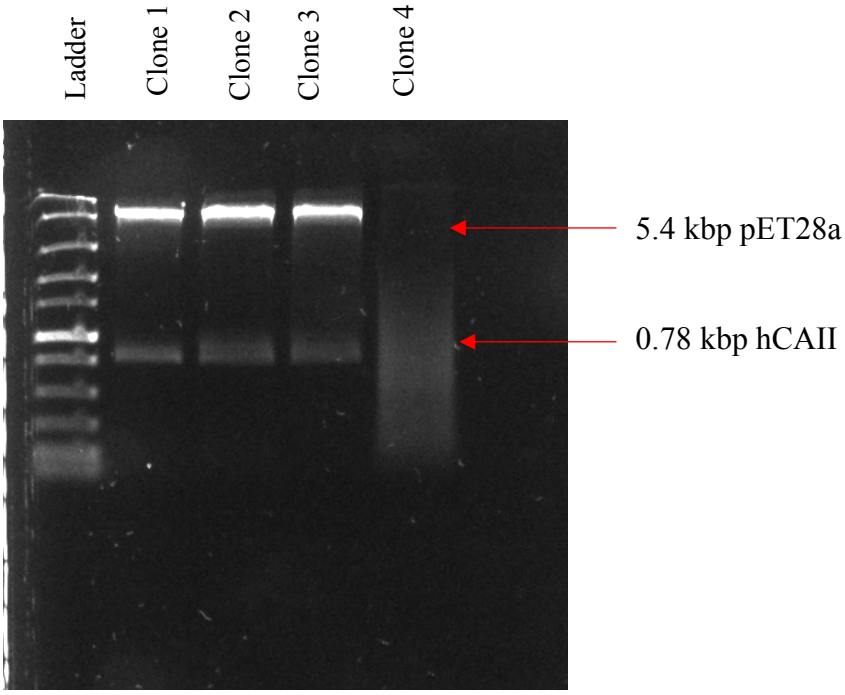

Figure 4.2: Restriction digestion of hCAII pET28a using Nde I and sac I.

The hCAII_pET28a construct was transformed into BL21De3 cells by electroporation method of transformation. The transformed cells were plated onto LB-Agar-Kanamycin plate and incubated at 37°C overnight to form colonies. A single colony was inoculated into 10 ml of LB media along with 50 μg/ml of kanamycin, 0.5 mM $ZnSO_4$ at 37°C for overnight at 200 rpm. Overnight grown primary 10 ml culture was added along with kanamycin and 0.5 mM $ZnSO_4$ into 1 liter secondary culture. Cells were grown at 37°C until 0.6-0.8 at $O.D._{600}$ and further induced with 0.1 mM IPTG at 18°C for 16 hours. Cells were harvested for 20 minutes at 6000 xg. and the cell pellet was resuspended into phosphate buffer and centrifuged again at 3700 xg for 15 minutes. The final pellet was thawed and resuspended in 30 ml of lysis buffer and sonicated for 5 minutes with 10 seconds on pulse followed by 10 seconds off pulse. The cells were centrifuged at 38,759 xg for 45 minutes at 4°C and the supernatant was loaded onto the 1 ml crude FF-His column pre-equilibrated with loading buffer at 0.5 ml/min flow rate. Further, 15 column volume of loading

buffer wash performed, followed by the elution step using elution buffer. Elution was performed using 50 mM, 100 mM and 250 mM imidazole concentration. hCAII protein was eluted in all the three concentrations of imidazole, with the purest in the 250 mM imidazole fraction. The hCAII fractions were pooled and concentrated using 10 kDa cut-off concentrator. hCAII protein was analyzed for its size as well as purity by performing gel electrophoresis using 15% SDS PAGE gel. The gene hCAII was successfully cloned in pET28a vector. It was expressed, soluble and successfully purified in 250 mM imidazole concentration.

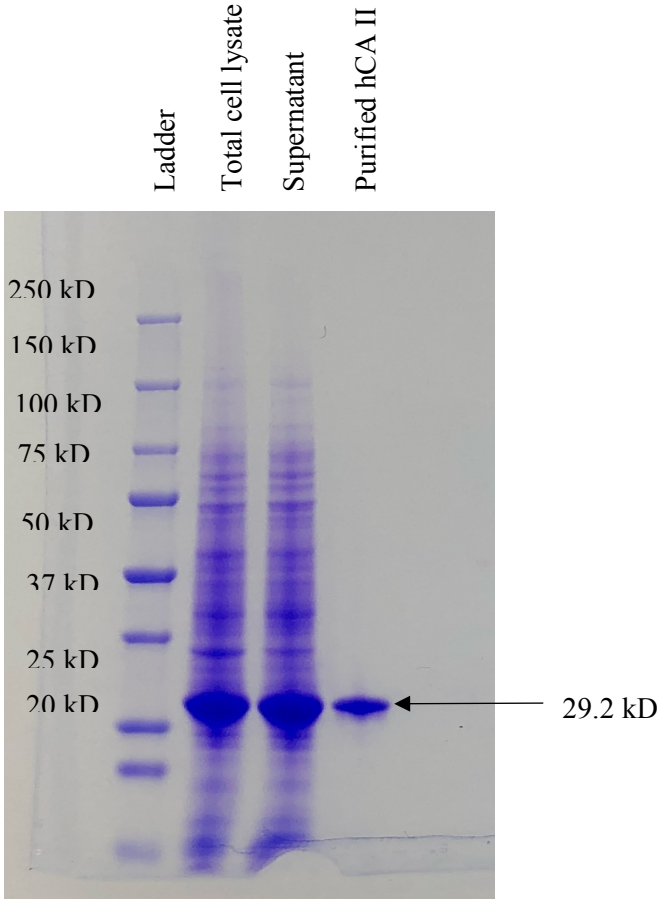

Figure 4.3: Expression and purification of hCA II_pET28a.

4.1.1 Determination of hCAII activity

After expression and purification of hCA II, it was very important to determine the catalytic activity of hCA II to make sure that the expressed hCA II is active. The catalytic activity of human CA II was monitored by the enzymatic assay of Wilbur Anderson method. The assay was performed at $0^{o}C$ using CO_2 as a substrate following the pH variation due to the catalyzed conversion of CO_2 to HCO_3^-. CO_2 saturated water was made by adding CO_2 from CO_2 cartridges into a 250ml Erlenmeyer flask filled with ddH2O. The flask was placed in an ice bath. The enzyme was suspended in 3ml 20 mM Tris buffer (pH 8.3) in a vessel that contained a pH electrode. The time taken for the pH to change from 8.3 to 6.3 was measured on the addition of 2 ml ice cold CO_2-saturated water. A blank reaction was done where water was used instead of the enzyme CA II and negative control reaction was performed with Brinzolamide (BRZ) which is supposed to inhibit the catalytic action of CA II and finally a test control reaction was carried out with CA II at different concentrations to determine its catalytic activity. All the reactions were repeated three times. The results (table 4.1) showed that increase in concentration of CA II decreases the time taken for the pH to change from 8.3 to 6.3. The concentrations used was 1, 10, 50 and 100 pmol. The lowest concentration (1 pmol) of CA II took 55 seconds to change the pH from 8.3 to 6.3 whereas the highest concentration (100 pmol) of CA II took only 19.4 seconds to bring the pH down to 6.3 when compared with the blank reaction of 69.8 seconds. This assay confirmed that the hCA II was catalytically active.

Table 4.1: Carbonic anhydrase activity assay at different concentrations of CA II

No. of reactions	Name of the reaction	Reaction	Average time (seconds)
1	Blank	$CO_2 + H_2O$	69.8 ± 1.6
2	Test control	CO_2+ CA II (1pmol)	55.0 ± 0.8
3	Test control	CO_2+ CA II (10 pmol)	48.0 ± 1.4
4	Test control	CO_2+ CA II (50 pmol)	23.9 ± 1.2
5	Test control	CO_2 + CA II (100pmol)	19.4 ± 0.4
6	Negative control	CO_2+ CA II + BRZ	65.3 ± 1.2

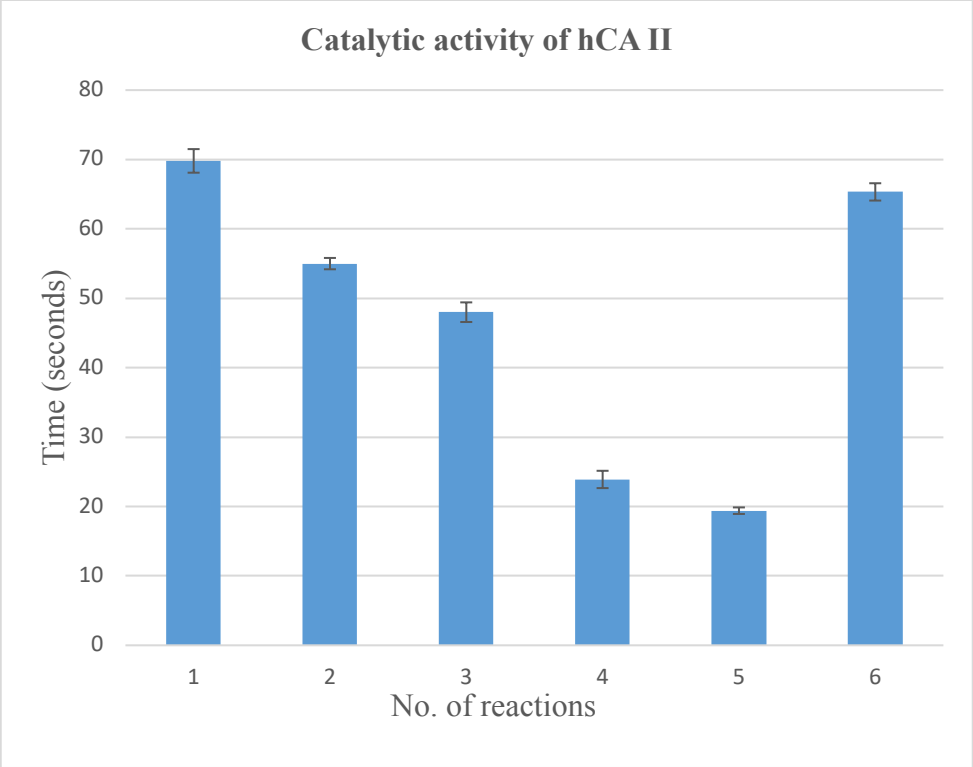

Figure 4.4: Determination of catalytic activity of hCA II activity.

4.2 IN VITRO SELECTION OF APTAMERS

A ssDNA library with random region of 40 was used for the selection of aptamers against CA II. The random region was flanked by two primer binding sites of 23 nucleotide for PCR amplification shown below:

5' - TAG GGA AGA GAA GGA CAT ATG AT - (N_{40}) - TTG ACT AGT ACA TGA CCA CTT GA - 3'

3'- AAC TGA TCA TGT ACT GGT GAA CT - 5'

Reverse primer

3' – ATC CCT TCT CTT CCT GTA TAC TA – N_{40}) – AAC TGA TCA TGT GGT GAA CT -5'

5' – TAG GGA AGA GAA GGA CAT ATG AT – 3'

Forward primer

The first selection cycle was started with 10^{24} sequences of aptamer library. Total of 6 rounds of positive selection was carried out with the target CA II and 4 rounds of negative selection was done with CA I, CA IV and Brinzolamide. Aptamers after 10^{th} round was isolated and sequenced by next generation sequencing and the most abundant aptamers from the pool of sequences were screened for inhibition assay.

4.2.1 Positive selection cycle

The first round of selection cycle was started with 1 nmol of ssDNA library incubated with 400 pmol of CA II. At the beginning of each cycle the ssDNA library were denatured and renatured to allow folding of the DNA into a stable tertiary structure. The library was refolded in the binding buffer (2 mM HEPES – NaOH, pH 7.5, 3 mM $MgCl_2$, 100 mM NaCl) at 95°C for 10 minutes.

Positive selection cycles were carried out by incubating the target CA II with the library for 30 minutes at 37°C. The nitrocellulose filter was introduced into the reaction mixture to allow the CA II-ssDNA complex to bind to the nitrocellulose filter and the unbound ssDNA will remain in the solution which is meant to be discarded. Several numbers of washes were done to eliminate loosely bound aptamers and finally the bound aptamers were eluted with an elution buffer (400 mM Na-acetate, 5 mM EDTA, 7M urea) from the CA II-ssDNA complex. The eluted ssDNA was concentrated by ethanol precipitation and a PCR amplification was performed to convert ssDNA into a double stranded DNA. To convert the dsDNA to ssDNA, an asymmetric PCR was done followed by streptavidin magnetic bead isolation to obtain purified ssDNA without any by-products. The results (Fig 4.5, 4.6, 4.7) of 6 positive selection cycles displayed bright bands of dsDNA and ssDNA for symmetric PCR and asymmetric PCR respectively in a gel electrophoresis image. Finally bright bands of ssDNA were obtained after magnetic bead isolation. The gel images confirms the production of ssDNA aptamers after each cycle and proves that all the selection cycles were successful.

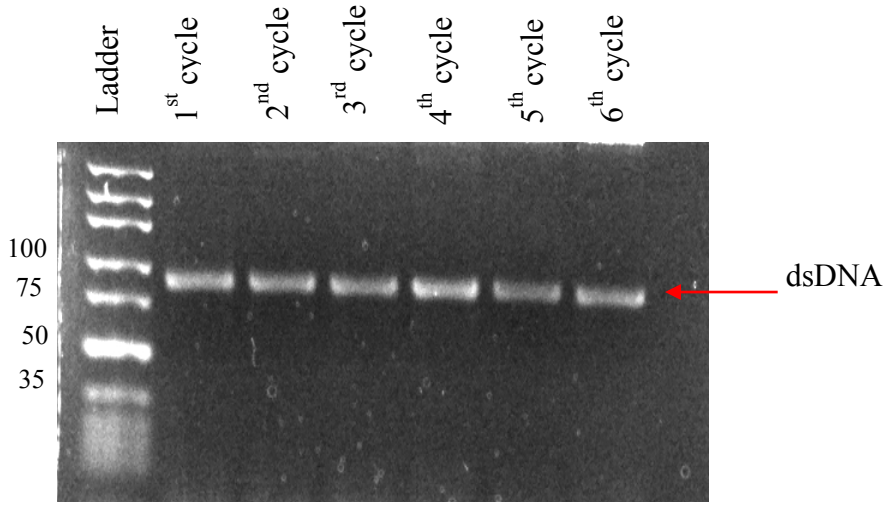

Figure 4.5: Symmetric PCR amplification of 6 positive selection rounds.

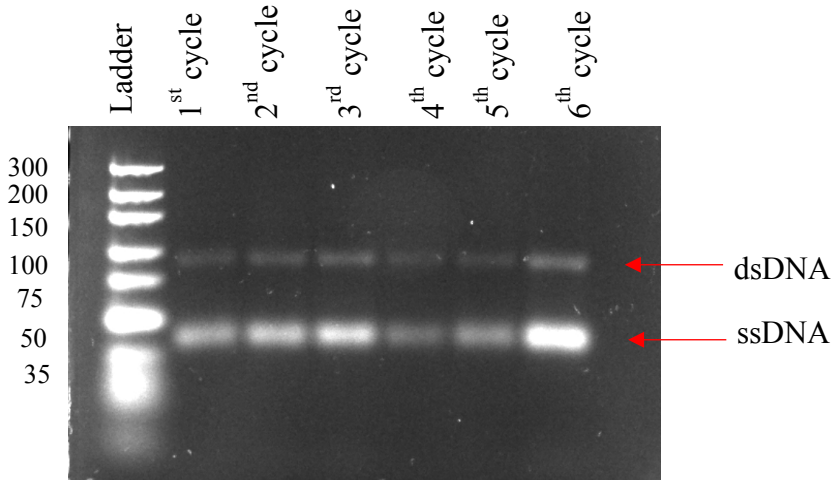

Figure 4.6: Asymmetric PCR of 6 positive selection cycles.

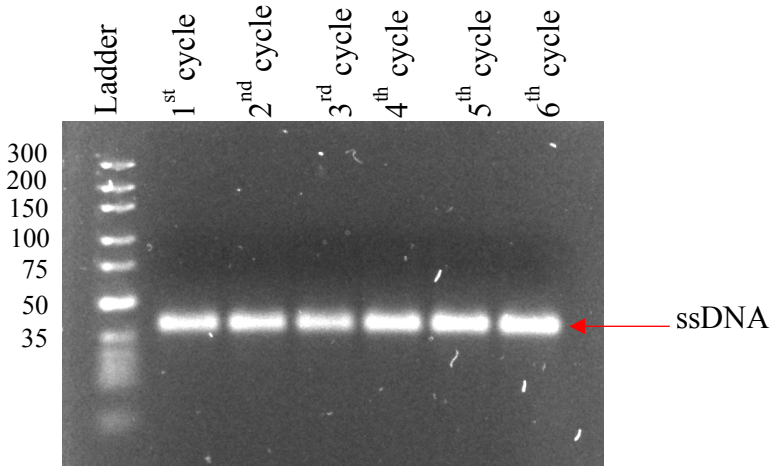

Figure 4.7: Streptavidin magnetic bead isolation

4.2.2 Negative selection cycle

Negation selection was done with the negative targets CA I, CA IV and Brinzolamide. The purpose selecting against CA I and CA IV is to make the aptamers CA II isozyme specific. In this selection round the binders of CA I and CA IV were eliminated and the unbound aptamers were

considered as CA II specific binders and were collected to continue the cycle. The reason of selecting against Brinzolamide is to obtain CA II active site inhibitors. This selection cycle ensures that the CA II binders specifically binds to the active site of CA II and inhibits its activity. Similar to positive selection cycle, the negative selection cycles were started with refolding the ssDNA library at the same condition. The library was incubated with the negative targets (CA I, CA IV, Brinzolamide) and the negative target binders were eliminated and the unbound aptamers were collected followed by PCR amplifications (symmetric and asymmetric PCR). The ssDNA product after asymmetric PCR was purified by streptavidin magnetic beads. Each negative selection was repeated twice.

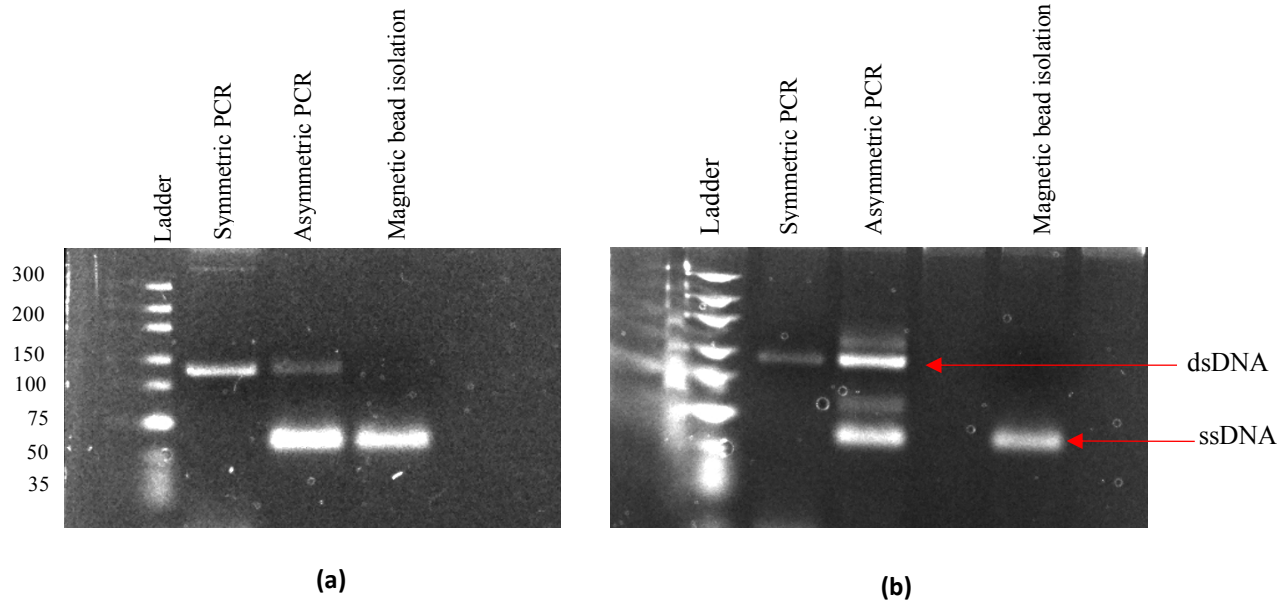

Figure 4.8: Negative selection cycle.

(a) Negative selection cycle with CAI and CAIV. The products in magnetic bead isolation step are ssDNA aptamers which specifically binds to CA II only. (b) Negative selection with Brinzolamide. The ssDNA in magnetic bead isolation are the aptamers which binds to the active site of CA II and making the CA II inactive.

4.2.3 Optimization of PCR amplification

Aptamers are selected from libraries usually containing up to 10^{15} different sequences by an iterative process of binding, washing, elution, amplification and purification. It is important to note that amplification of large oligonucleotide library with random region should be optimized very carefully since over amplification may produce non-specific products and may lead to reduced yield.

In PCR, DNA is amplified by a series of polymerization cycles comprising of three temperature-dependent steps which includes denaturation of DNA strands, annealing of primers and synthesis of DNA by the enzyme DNA polymerase. The quality and the yield of the products depend on several parameters such as annealing temperature and number of PCR cycles. Primers tends to bind non-specifically if the annealing temperature is lower than the optimum temperature. However, primers are not even able to bind to the template if the temperature is significantly high. Optimization of annealing temperature is critical especially when the ssDNA is treated with lot of washing and elution buffer during selection rounds, making the DNA impure which ultimately produces nonspecific products during PCR amplification. In order to obtain high quality and quantity of PCR product, the annealing temperature was optimized by using gradient PCR. A gradient of 53°C to 64°C was set for 30s. A PCR reaction was run under those selective temperatures and then gel electrophoresis was performed to ensure the optimum annealing temperature.

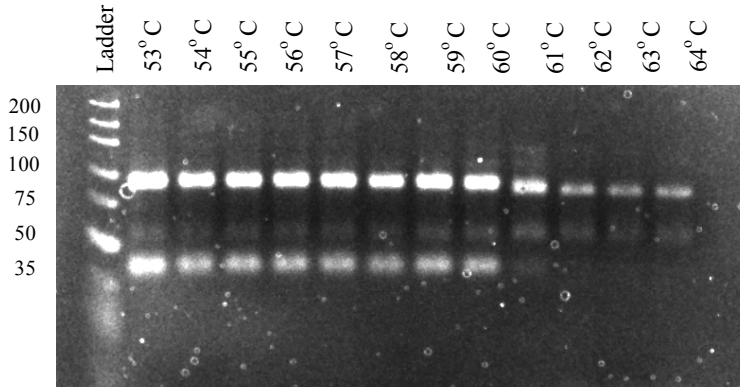

Figure 4.9: Optimization of annealing temperature.

The gel image (figure 4.9) displayed that the temperatures below 62°C produced some non-specific amplification. In order to get rid by-products and to obtain pure dsDNA, 62°C was selected as an optimum annealing temperature. Another round of PCR was performed with annealing temperature of 62°C to make sure no by-products are formed at this temperature.

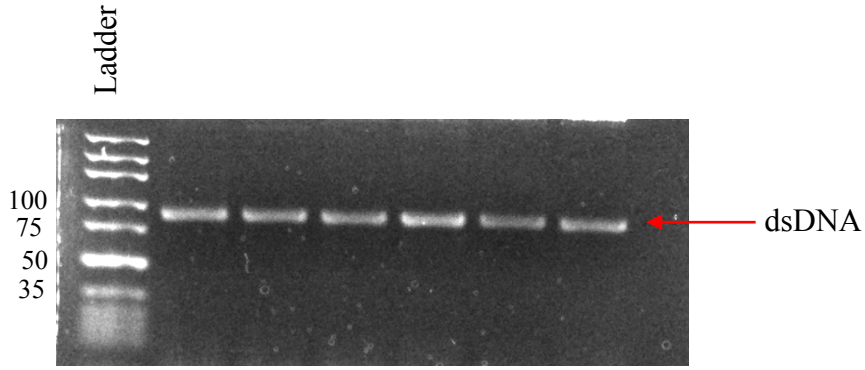

Figure 4.10: PCR at optimum annealing temperature of 62°C.

The gel image (fig 4.10) of second round of PCR at annealing temperature of 62°C showed no non-specific amplification. Therefore this temperature was selected as an optimum annealing temperature for every selection round. Optimization of total number of PCR cycle is one of the important parameter for successful PCR. Excessive cycling increases the opportunity for

nonspecific amplification and errors. In order to achieve optimum number of PCR cycle, a gradient PCR was set up using different number of PCR cycles such as 10, 12, 15, 20, 25, 30, 35 and 40.

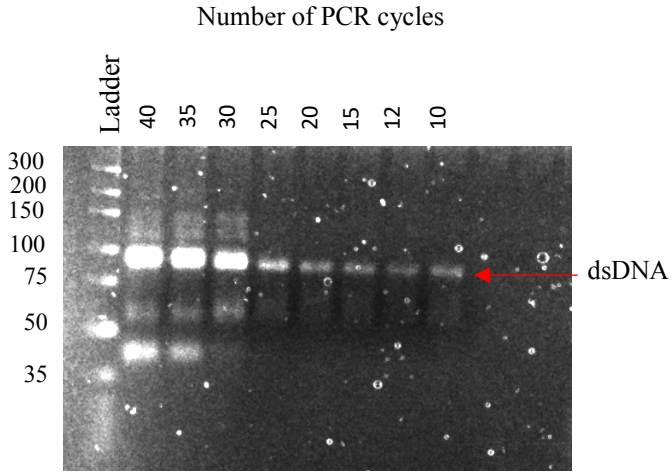

Figure 4.11: Optimization of total number of PCR cycle.

The result shows that at cycle number 40 high yield of dsDNA is obtained but it also produced high level of non-specific amplification. Cycle number 35 and 30 showed the same behavior. From cycle 25 to 10, the formation of non-specific amplification is greatly reduced but it also produced less yield of dsDNA. The aim is to obtain high yield as well as good quality of dsDNA product. To achieve this goal, 25 number of PCR cycles were selected for amplification. This will ensure the product has no non-specific amplification and it will produce reasonable yield.

4.2.4 Optimization of generation and purification of ssDNA

The quality and yield of ssDNA plays a vital role in aptamer selection. The method of purifying the PCR product by either gel extraction or PCR clean up by mini spin columns which uses the silica membrane technology is not an efficient process as it leads to loss of PCR product and the purity ratio (A_{260}/A_{230} ratio in spectrophotometer) of ssDNA is decreased. The generation of DNA aptamer requires the conversion of dsDNA to ssDNA. There are various method to produce

to ssDNA from dsDNA such as magnetic bead separation with streptavidin coated beads, lambda exonuclease digestion and asymmetric PCR. Magnetic bead isolation method involves the binding of dsDNA to the surface of magnetic beads with alkali, causing the aptamer tertiary structure to be lost and impairing the efficiency and specificity of the aptamers.

The lambda exonuclease digestion method is designed to digest dsDNA tagged with a phosphoglycerate-probed primer which is recognized by lambda exonuclease to remove the complementary strand. This method provides very low yield of ssDNA. The drawback of asymmetric PCR is that the ssDNA should be purified by gel extraction which leads to loss of PCR products as well.

An indirect purification method was developed in order to achieve high yield and quality of ssDNA. At first symmetric PCR was done to produce dsDNA where biotinylated forward and reverse primers were used. Secondly, an asymmetric PCR was carried out using the symmetric PCR product as a template with an excess non biotinylated forward primer and very limited amount of biotinylated reverse primer. Asymmetric PCR produces a mixture of large quantity of ssDNA and very less quantity of dsDNA. The dsDNA and the by-products after asymmetric PCR carried biotin. Finally the dsDNA and the by-products were eliminated by streptavidin magnetic beads.

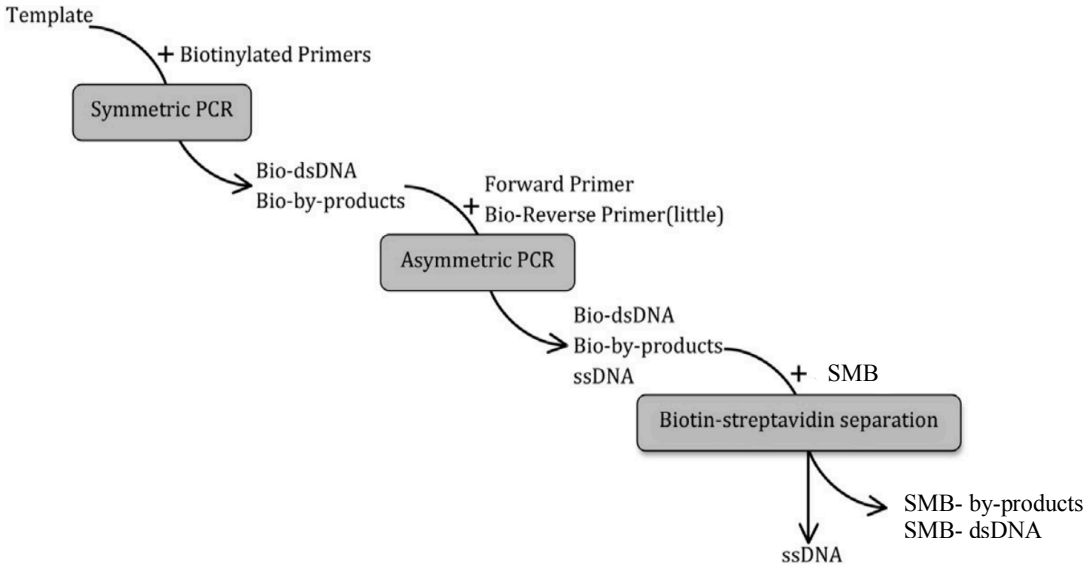

Figure 4.12: A schematic representation of the indirect purification method [93].

The result (figure 4.13) of indirect purification method presents no by-products or dsDNA after magnetic bead isolation step. This method was carried out at every cycle of SELEX round to obtain high quality and quantity of ssDNA.

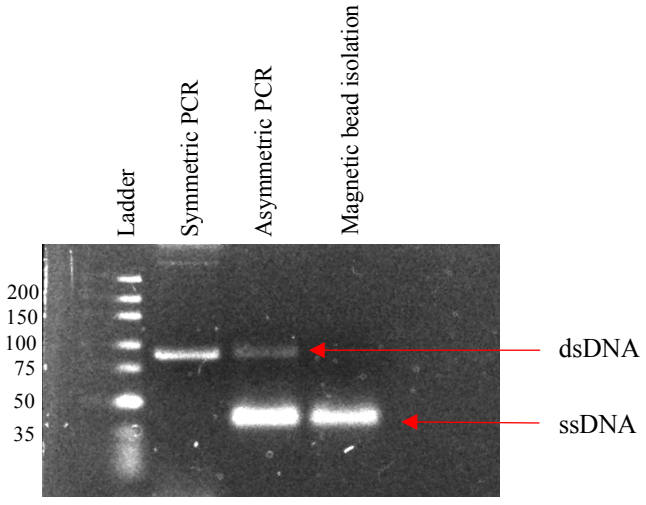

Figure 4.13: PCR product purified by magnetic beads

4.3 NEXT GENERATION SEQUENCING

4.3.1 Analysis of Next Generation Sequencing

The aptamers obtained from 10th round of selection was sequenced by next generation sequencing (NGS). The analysis of the sequences provided total of 7 million sequences. Out of 7 million sequences, 6 millions were unique sequences. Sequences with 86 nucleotides were 450 K and among 450 k , 388 k sequences have both constant primer region. From the pool of 388 k, 26 k sequences have random region of N_{40}. As our initial library was 86 nt with N_{40} random region, we decided to screen sequences from the pool of N_{40}. The table 4.2 shows the 11 most abundant sequences and the 11 aptamers were later screened for inhibition assay in order to find out the CA II inhibitor.

Table 4.2: Aptamer sequences after 10th round of selection cycle

Name of sequences	Frequency	N_{40} variable sequence
A1	7	CAACATCCCCGGCAATGGGTAACTCAAGCCATCTATAACC
A2	7	CATCCTGGACCCATACATTTACCTCATTATCACCGGCTGG
A3	6	GCCTGTTTTTGTAGATCATTGATAGCATACCCCCGGAGGG
A4	6	CACCCCTCTCAACTGTTATTACAACTGGCCGGTATCCGGA
A5	6	CACCGCATTGTAGATCCTATAGCCACTGTGTTTGATTGTG
A6	5	CCACACCACCTATAAGCACATAACTTAGCTATAATTCGGC
A7	5	GCACCCACCCAAATACCCTTAATCCAATTTTATTCCGGCG
A8	5	CGTAGCTATGATATTGATTGTTTTTAAATCCACCCGTGCA
A9	5	ACCGTACACCTCACATTGGTATTTAGCACTCTCCGATCCAT
A10	5	CACCCCTCGGACGTACCAGTATCGCTTTTATCGCTACCCA
A11	5	GCGATTACAACGTTCTTTACCTGTGACCCCACCGTTGCCA

4.3.2 Carbonic anhydrase II inhibition assay with aptamers

The aptamers A1 to A11 were screened for inhibitory activity of CA II by the same method of Wilbur Anderson electrometric assay. The time was measured for the pH to change from 8.3 to 6.3. A blank reaction was carried out with water and a test control reactions of aptamers (A1 to A11) and a positive control with Brinzolamide (BRZ) were performed followed by a negative control with CA II. Each of the experiments were repeated three times. An average time (seconds) was calculated for the pH to change from 8.3 to 6.3. The concentration ratio of CA II : BRZ and CA II: Aptamer used was 1:2.

Table 4.3: Screening of inhibition by 11 Aptamers

No. of reaction	Reaction	Average time (seconds)	CA II inhibition
1	CO_2	70.2 ± 1.9	
2	CA II	19.2 ± 0.8	
3	BRZ	68.9 ± 0.9	+
4	A1	21.0 ± 1.6	-
5	A2	19.6 ± 1.2	-
6	A3	19.2 ± 1.6	-
7	A4	20.0 ± 1.2	-
8	A5	38.0 ± 1.4	+
9	A6	18.9 ± 0.9	-
10	A7	22.0 ± 1.2	-
11	A8	51.0 ± 0.8	+
12	A9	19.1 ± 0.4	-
13	A10	21.0 ± 0.9	-
14	11	21.0 ± 1.6	-

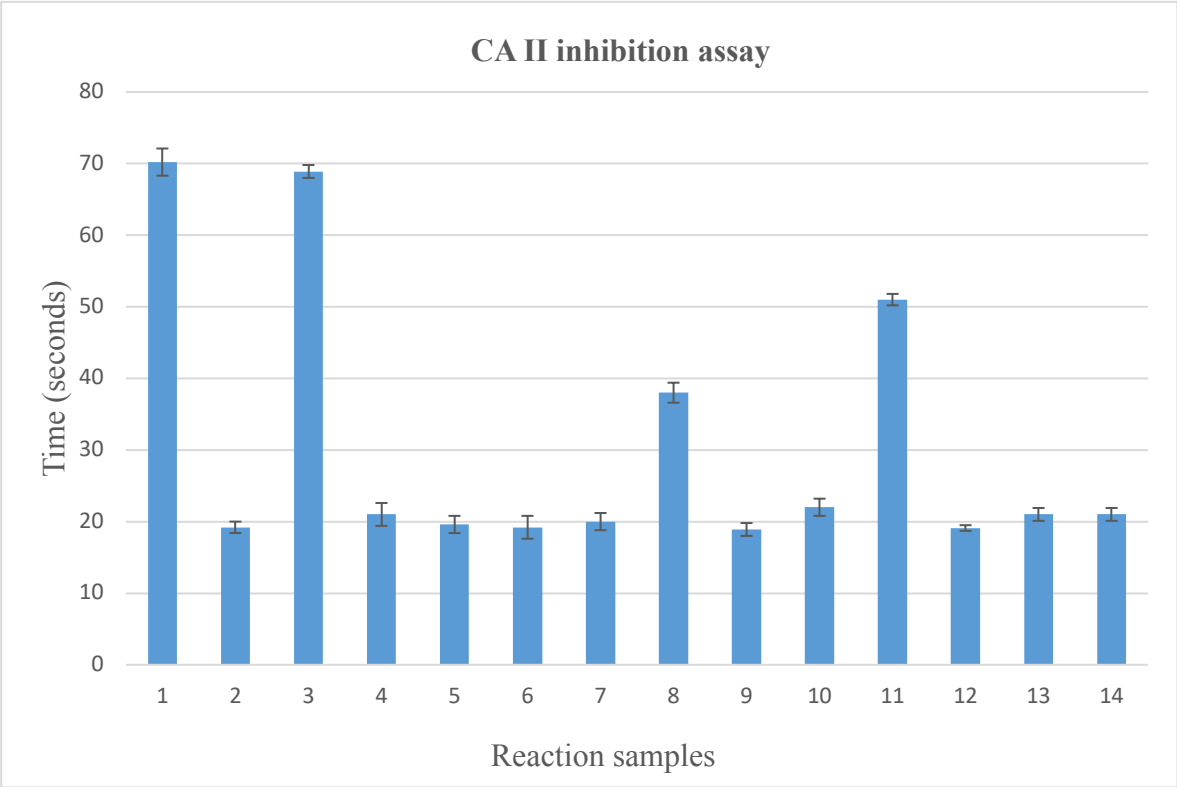

Figure 4.14: Inhibition assay of Aptamers against CA II

When the 11 aptamer sequences were tested individually, two aptamers, A5 and A8 was found to inhibit CA II. The time taken for Aptamer 5 to change pH from 8.3 to 6.3 was found to be 43.0 ± 1.4 seconds and Aptamer 8 took 51.0 ± 0.8 seconds to drop the pH from 8.3 to 6.3 whereas the commercial drug Brinzolamide takes 66.7 ± 0.9 seconds to bring the same change. A5 and A8 were further tested for concentration dependent inhibition assay to make sure that both the aptamers are inhibiting CA II with increasing concentration.

4.3.3 Concentration dependent inhibition assay of A8 and A5

In order to screen the concentration dependent inhibition of A5 and A8, different range of ratios of CA II:A5 and CAII:A8 were set from lower to higher concentrations of A5 and A8. The

assay started with a blank reaction where no CA II and inhibitor was added. The set of ratios were 1:0, 1:0.5, 1:1, 1:2, 1:4, 1:8 and 1:16.

Table 4.4: Concentration dependent inhibition assay of A8 and A5

No. of reactions	CAII : A5	Average time (seconds)	CAII : A8	Average time (seconds)
1	Blank	70.8 ± 1.2	Blank	71.4 ± 0.6
2	1:0	33.0 ± 0.9	1:0	19.6 ± 0.9
3	1:0.5	31.0 ± 0.5	1:0.5	40.0 ± 0.8
4	1:1	35.8 ± 1.2	1:1	43.0 ± 1.6
5	1:2	38.0 ± 1.9	1:2	50.6 ± 0.5
6	1:4	34.2 ± 2.0	1:4	66.3 ± 0.2
7	1:8	34.7 ± 0.5	1:8	70.6 ± 0.5
8	1:16	35.2 ± 0.9	1:16	71.6 ± 1.2

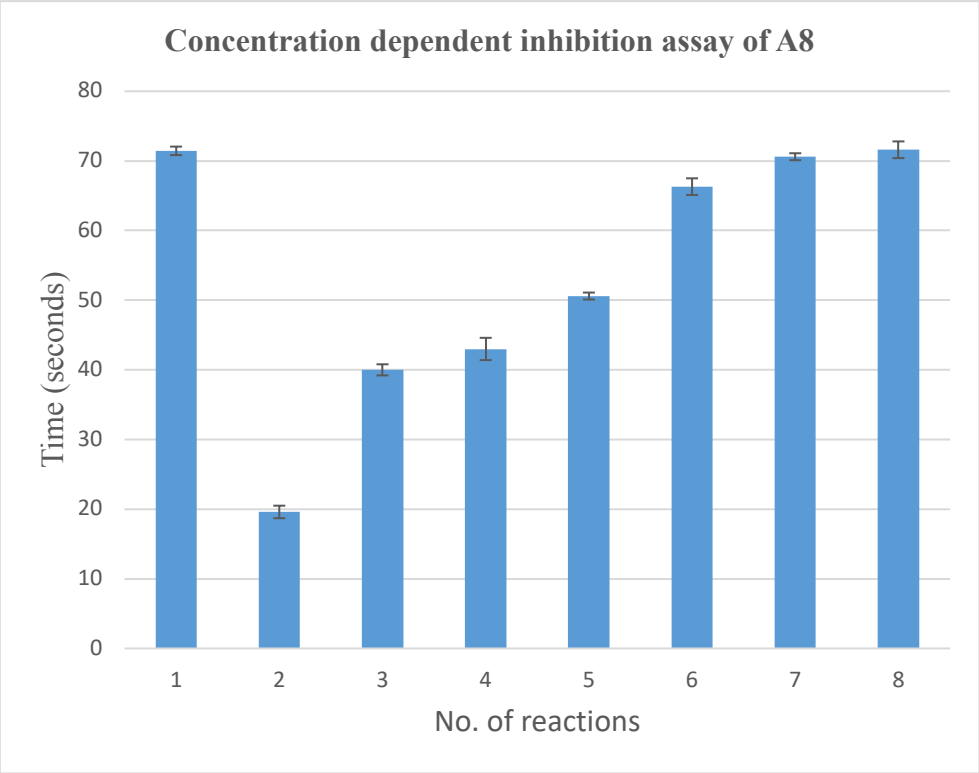

Figure 4.15: Concentration dependent inhibition assay of A8.

The concentration dependent inhibition assay elicited increase in inhibition with increasing concentration of A8. At zero concentration A8 did not show any inhibition and the reaction was very fast due to the presence of enzyme CA II. When the concentration was increased from 0 to 0.5, the average time increased for pH to drop from 8.3 to 6.3. A great increase in time was noticed at highest concentration of A8. This result showed that increase in concentration of A8 decreases the rate of reaction catalyzed by the enzyme CA II because A8 binds to CA II and inhibits its catalytic activity. On the other hand, A5 did not show a consistent increase in inhibition with increasing concentration. So A5 was considered to be a poor binder and an inhibitor of CA II and A8 was considered as a CA II inhibitor. Therefore A8 was selected for further investigations.

4.3.4 Isozyme specific inhibition assay of A8

Aptamer 8 was found to inhibit CA II as a result of positive selection cycles. To make sure that the aptamer specifically inhibits CA II but not CA I and CA IV, negative selections were done to select out CA I and CA IV binding aptamers. Therefore the aptamers collected after 10 rounds of positive and negative selection is considered to be CA II specific aptamer. To determine the specificity of A8 toward CA II, an inhibition assay was performed with A8 against CA I and CA IV. In this assay, we expect the aptamer to not to bind to CA I and CA IV and not to affect the rate of reaction in presence of A8. The assay was started with a blank reaction where no enzyme and inhibitor was added. A positive control was carried out with CA IV only without any inhibitor and a negative control reaction with Brinzolamide was performed followed by the test control in different concentration ratios of CA IV: A8 such as 1:0, 1:2, 1:4, 1:10. The same protocol was followed for assay with CA I as well.

4.3.5 Inhibition of isozyme CA IV by A8

Inhibition assay was performed with A8 against CA IV where a positive and a negative control reaction was carried out along with a blank reaction. In this assay CA IV enzyme was used to catalyze the hydration of CO_2. In presence of CA IV the reaction is very fast. We expect the aptamer to be isozyme specific, so on the addition of A8, the rate of reaction will remain unchanged.

Table 4.5: Isozyme specific inhibition assay of A8 against CA IV

No. of reactions		Ratio	Average time (seconds)
1	Blank		71.3 ± 2.05
2	CAIV:A8	1:0	16.3 ± 1.7
3	CAIV:A8	1:2	14.3 ± 1.2
4	CAIV:A8	1:4	16.6 ± 1.7
5	CAIV:A8	1:10	17.7 ± 2.05
6	CAIV:BRZ	1:2	64.3 ± 1.6

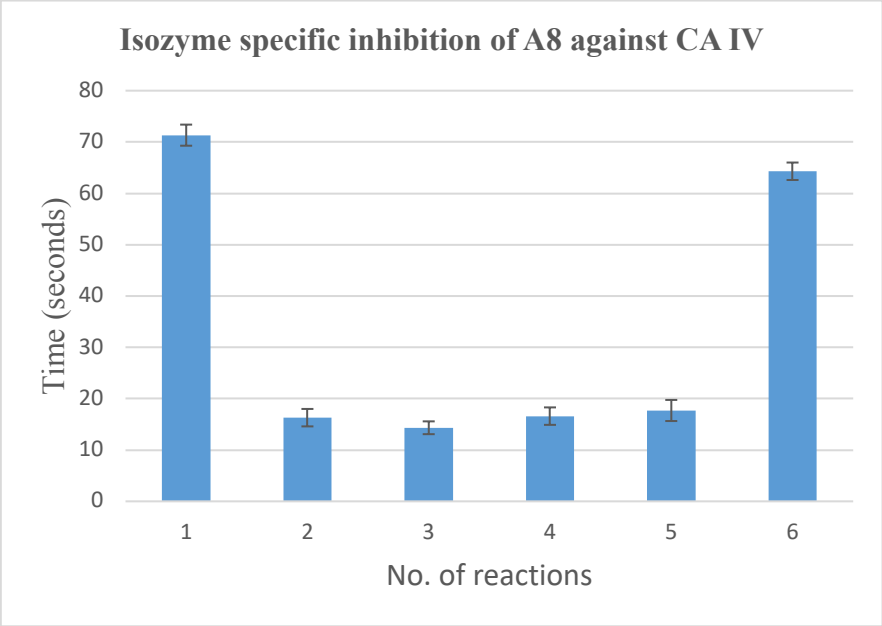

Figure 4.16: Isozyme specific inhibition assay of A8 against CA IV

4.3.6 Inhibition of isozyme CA I by A8

An inhibition assay was performed with A8 against isozyme CA I following the same protocol. Different concentrations of CA I: A8 was used like the previous assay with CA IV and A8. The concentration ratios were 1:0, 1:2, 1:4, and 1:16. A negative control reaction was carried out with Brinzolamide similar to previous assays.

Table 4.6: Isozyme specific inhibition of CA I by A8

No. of reactions		Ratio	Average time (seconds)
1	Blank		70.6 ± 1.8
2	CAI:A8	1:0	17.6 ± 1.2
3	CAI:A8	1:2	16.3 ± 1.2
4	CAI:A8	1:4	17.3 ± 1.3
5	CAI:A8	1:10	16.7 ± 1.6
6	CAI:BRZ	1:2	62.6 ± 1.2

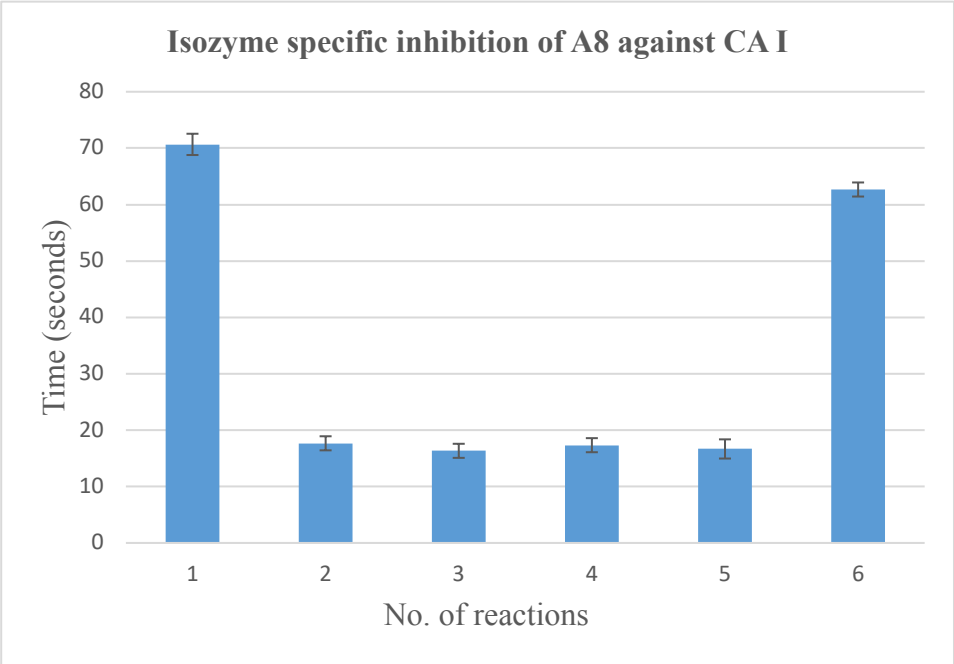

Figure: 4.17: Isozyme specific inhibition of CA I by A8

The data (fig 4.17) above explains there's no inhibition of isozyme CA I by A8 when it is compared with the positive control reaction. The rate of reaction did not changed at all on the addition of A8. The time taken for pH to drop to 6.3 at highest concentration was 16.7 seconds which shows similar behavior as positive control reaction. On the other hand, the time taken for the negative control was 62.6 seconds. This assay confirms that A8 is very specific toward CA II

but not CA I and CA IV. A8 doesn't inhibit isozyme CA I and CA IV. Therefore A8 is considered to be isozyme specific CA II inhibitor.

4.4 SECONDARY STRUCTURE PREDICTION OF A8

The secondary structure of the potential aptamer A8 was predicted by MFold algorithm (developed by Dr. Michael Zuker). MFold predicts optimal and suboptimal secondary structures of RNA and DNA using the energy minimization technique of Zuker. The varied conditions entered into the Mfold programme were matched with selection criteria of the aptamers. Prior to selection the aptamers were denatured and snap-cooled at 25°C to produce ssDNA aptamers. At this step the aptamers folded into three-dimensional structure based on the sequences. Thus a temperature of 25°C was entered into the Mofold programme to match the temperature of the snap cooling process. During the denaturation process, the aptamers were in a binding buffer which contained ions that may contribute to the aptamer structure. To match the buffer conditions, Mg^{2+} and Na^{+} concentrations of 3 mM and 100 mM were entered into the Mfold algorithm respectively. The N_{40} variable region of the aptamer (A8) was inputted into the Mfold algorithm to predict the secondary structure. The sequence of A8 with its primer binding region is:

5'-TAGGGAAGAGAAGGACATATGATCGTAGCTATGATATTGATTGTTTTTAA
ATCCACCCGTGCATTGACTAGTACATGACCACTTGA -3'

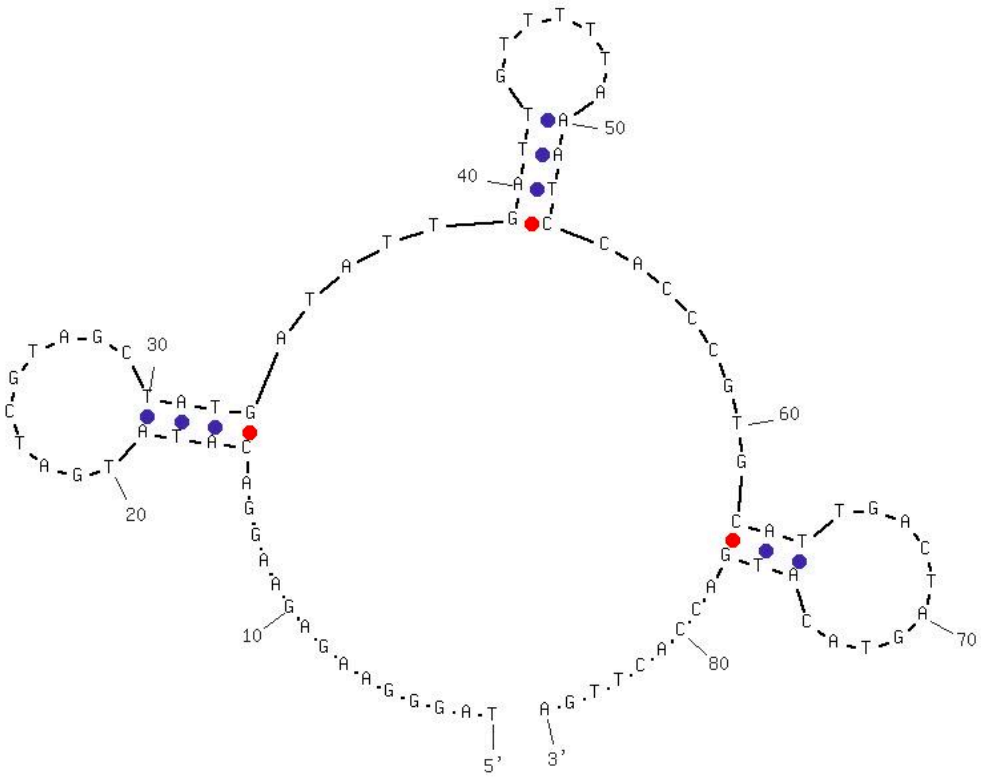

Figure 4.18: Secondary structure prediction of Aptamer 8

CHAPTER 5: DISCUSSION

Carbonic anhydrase inhibitors are widely used therapeutic agents in the management and prevention of many diseases. This is mainly due to the wide distribution of the 14 vertebrate CAs in many cells , tissues and organs where they play crucial role in many physiological functions. Still, the available pharmacological agents are not perfect . They possess many side effects due to their lack of selectivity toward different CA isozymes. One of the example is sulfonamide as antiglaucoma agents which has susceptibility toward different CA isozyme. Thus development of CA isozyme-specific inhibitor would be highly beneficial for obtaining novel types of drugs and devoid of major side effects. Still, prospects for achieving such goals are not very optimistic due to high similarity between the isozymes. The most similar isozymes are tend to be CA II and CA IV which seems to be the predominant and most wide-spread isozyme in many tissues in which specific inhibition is required.

In this research project, *in vitro* selection was carried out to identify ssDNA aptamers that bound to Carbonic anhydrase II specifically and inhibited its catalytic activity. The ssDNA aptamers were selected by SELEX method in which nitrocellulose filter partitioning was used to separate ssDNA-protein complexes from unbound ssDNA. The pre-treatment of the filters with a binding buffer effectively decreased the non-specific binding of ssDNA to the filters. The negative-selection with nitrocellulose filter before the selection round removed the matrix binders from the library.

There is no standard protocol for SELEX which can be suitable for all targets. Therefore the conditions of the selection cycles were optimized according to the target properties and the requirements of the selected aptamers. One of the important feature of oligonucleotide library design is the length of the random region. The single-stranded oligonucleotide motifs can be built

starting from 30 nucleotides [39]. Usually longer random region contributes to greater structure complexity which is very important in target recognition and binding [79]. However, from previous literatures it was found that the efficiency of aptamer selection decreases with random region longer than 70 nt [80]. Therefore, in this research project ssDNA with random region of 40 nt was used for the selection of ssDNA aptamers against CA II. N_{40} random region was considered to be ideal length as the complexity of the library is sufficient and the chemical synthesis of such library is cost-effective.

Analysis shows that there is no significant difference between RNA and DNA aptamers in terms of affinity and specificity [39]. Moreover, DNA aptamers have certain advantages over RNA aptamers. The selection of DNA aptamer is much faster and easier than RNA aptamers. DNA aptamers exhibit higher stability than RNA aptamers in a broad range of conditions including biological systems which makes them more suitable for clinical applications. Thus, ssDNA library was chosen instead RNA library.

The first round of selection cycle was started with 10^{24} sequences with 40 nt randomized region. After 10 rounds of positive and negative selection and amplification, CA II-specific targeted aptamers were isolated and were sequenced by next generation sequencing. After analysis of raw data of NGS, the most abundant sequences were selected. The screening of aptamers for enzymatic assay was started with 11 abundant sequences. The enzymatic assay of carbonic anhydrase was performed by following the pH method (Wilbur-Anderson Unit). This method was chosen to do the carbonic anhydrase enzymatic assay because this method resembles the real biological system where the catalysis of CA takes place in the reaction of interconversion of carbon dioxide and water to bicarbonate ion. At first the aptamers were tested for inhibition of CA II. One of the aptamer (A8) showed inhibitory activity toward CA II out of 11 aptamer sequences. Another

set of experiment was performed with A8 to determine its specificity toward CA II. Therefore A8 was introduced in the hydration reaction of CO_2 where CA I and CA IV was the catalyst. No effect of A8 on the activation of CA I and CA IV was observed. The reaction with A8 didn't bring any change in time for the pH to drop from 8.3 to 6.3 when compared with the negative control Brinzolamide.

The most active anti-CAII aptamer had a random central sequence 5'-CGT AGC TAT GAT ATT GAT TGT TTT TAA ATC CAC CCG TGCA-3' and was designated as CA II specific inhibitory aptamer (CAII-A8). This sequence can be folded into a common hairpin loop-stem motif by secondary structure prediction. Therefore this research project offers the possibility to generate anti-CAII aptamer which is an isozyme specific inhibitor and can have implication in the treatment of glaucoma.

CHAPTER 6: SUMMARY & FUTURE PROSPECTS

Carbonic anhydrase inhibitors are widely used therapeutic agents in the management of many diseases. This is because of their wide distribution of CA isozymes in many cells, organs and tissues where they play significant role in many physiological functions. The available CA inhibitors possess many undesired side effects due to lack of their specificity toward different isozymes. Thus development of isozyme-specific inhibitor would be highly beneficial for obtaining drugs, devoid of major side effects.

This research study focused on antiglaucoma agents, developing an isozyme specific inhibitor which would inhibit CA II specifically in order to decrease IOP by reducing the inflow of aqueous humor. The challenge was to discriminate between the isozymes CA I, CA II and CA IV. Toward this goal, ssDNA aptamers were selected by SELEX method. Few rounds of negative selection was done against the negative targets CA I and CA IV to make the aptamers isozyme CA II specific. After 10 rounds of selection and next generation sequencing, aptamers were screened for inhibition assay and one aptamer was found to inhibit CA II specifically. pH dependent enzymatic assay (Wilbur Anderson) was done to determine the inhibitory activity and isozyme specificity. To validate the inhibitory activity and isozyme specificity, further investigations should be done such determining the binding affinity of A8 toward CA II by SPR. To make sure A8 doesn't have any binding affinity to CA I and CA IV, Kd value should be determined by SPR as well. Once the Kd value determines its inhibitory activity and specificity we can find out the mode of inhibition of A8 whether it is a competitive inhibitor or an uncompetitive inhibitor. Further investigations such as X-ray crystallography can be done to study the molecular structure of the aptamer-CAII complex. This will give as the opportunity to find out the inhibitory mechanism of A8 to CA II . Also more aptamer sequences can be screened for inhibition and specificity to find

out an aptamer which may have better inhibitory activity than the commercial drug Brinzolamide. The conclusion is that SELEX method can be employed to investigate aptamers of different enzymes and can make it isozymes specific which may contribute to development of novel drugs for may therapeutic applications.

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