

Selection and characterization of an aptamer to a thiazole orange derivative for use in a fluorescence-based biosensor

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Author's Declaration

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Abstract

Aptamers are short single-stranded DNA or RNA sequences that have been selected *in vitro* to have a high binding affinity to a specific target molecule. One class of aptamers can bind to small non-fluorescent molecules to result in a dramatic fluorescence enhancement, once bound. These aptamer sequences, along with their cognate fluorogenic molecules, are used as light-up probes. RNA light-up probes are used to monitor sub-cellular localization of RNA in cell with the RNA aptamer Mango I being notable for having a high affinity along with excellent fluorescence enhancement. The usage of RNA aptamers in biosensing application is limited due to the fact that the degradation of RNA due to the pervasive RNase is a key factor in the sensors shelf life. For this reason, DNA aptamers are the choice for usage in biosensing applications.

In chapter 2, a new DNA aptamer, named 02a, that binds to the target TO1-biotin was isolated after nine rounds of Systematic Evolution of Ligands by EXponential enrichment (SELEX). The selection was performed on streptavidin coated beads with the target bound to the surface with free dye in solution in higher selection rounds to select for slower off-rate binding. Using Next-Generation Sequencing (NGS), the library after the 4th and 9th rounds of selection were sequenced to identify enriched sequences. The 02a sequence family emerged showing superior fluorescence enhancement, 136.5 times more than unbound TO1-biotin, as well as high affinity, with a K_D of 98.7 nM, for the target, when compared to other families obtained by NGS analysis.

In chapter 3, several sequence families were studied to discern structural information to better understand the binding interaction. Using several primary and secondary sequence analysis tools, a three-tiered G-quadruplex motif for the families was predicted. For the 02a aptamer, it was found that binding and fluorescence enhancement was attenuated in the absence of K^+ and that the

region outside the predicted quadruplex gives access to potassium-dependent fluorescence enhancement conformers.

In chapter 4, biosensing approaches were evaluated for their use in the detection of the biomarker IgE. An aptamer for IgE, D17.4, was used in several sensing platforms such as Localized Surface Plasmon Resonance (LSPR), Electrophoretic Mobility Shift Assay (EMSA), Förster Resonance Energy Transfer (FRET), and Lateral Flow ImmunoAssay (LFIA) as well as in a modular aptamer configuration and was used to evaluate the detection of isolated IgE and IgE in human serum. Although the detection of IgE was possible in most set-ups, complex optimization steps are needed for optimization for commercial use, especially when using human serum samples.

Chapter 5 summarizes all of the findings and future work is discussed. Overall, *in vitro* selection was used to select for a DNA aptamer for the thiazole orange derivative TO1-biotin which was then characterized to have a quadruplex motif. This novel aptamer, 02a, is a useful fluorescent module for biosensing applications and has superior characteristics than other published light-up DNA aptamers.

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List of Abbreviations

BHQ-2.....	Black Hole Quencher®-2
cDNA	complementary DNA
CDS.....	circular dichroism spectroscopy
CE-SELEX.....	capillary electrophoresis SELEX
DFHBI.....	3,5-difluoro-4-hydroxybenzylidene imidazolinone
DFHO.....	3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime
DNA.....	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ESI-MS	electrospray ionization-mass spectroscopy
FISH.....	fluorescence <i>in situ</i> hybridization
FMN.....	flavin mononucleotide
FNA.....	functional nucleic acid
GFAAS	graphite furnace atomic absorption spectroscopy
GFP	green fluorescent protein
HBI.....	4-hydroxybenzylidene imidazolinone
ICP-MS	inductively coupled plasma-mass spectrometry
IgE.....	immunoglobulin E
IUPAC.....	International Union of Pure and Applied Chemistry
LSPR.....	localized surface plasmon resonance
MEME.....	Multiple Expectation maximizations for Motif Elicitation
MGA	malachite green aptamer

mRNA.....	messenger RNA
NADH.....	nicotinamide adenine dinucleotide
NGS.....	next-generation sequencing
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PCR.....	polymerase chain reaction
QCM	quartz crystal microbalance
QGRS.....	Quadruplex forming Guanine-Rich Sequences
RFP	red fluorescent protein
RFU.....	relative fluorescence units
RNA	ribonucleic acid
RPM	reads per million
rRNA.....	ribosomal ribonucleic acid
RT-PCR.....	reverse transcription polymerase chain reaction
SELEX	systematic evolution of ligands by exponential enrichment
snRNP	small nuclear ribonucleoproteins
SPR	surface plasmon resonance
ssDNA.....	single stranded deoxyribonucleic acid
THF	tetrahydrofolate
TMPyP4.....	5,10,15,20-Tetra(N-methyl-4-pyridyl)porphine
tRNA.....	transfer ribonucleic acid
UTR.....	untranslated region
μIVC	microfluidic-assisted <i>in vitro</i> compartmentalization

Chapter 1 | Introduction to Aptamers in Biosensors

It is clear, today more than ever, that the need for accurate testing of biological markers is essential. The role of nucleic acid, especially DNA, in this capacity cannot be understated as the use of PCR as a diagnostic tool is at the forefront of people's minds (1). Certainly, the growing market for direct-to-consumer DNA testing shows that there is interest in specific DNA sensing away from the bench (2). Typically, biosensors use proteins as the biological component in the form of antibodies or enzymes as is the case for many point-of-care detection strategies for COVID-19 (3), pregnancy (4) and blood glucose detection (5). Due to several advantages of nucleic acids (6), there is growing interest in using nucleic acids that have similar functions, like aptamers and nucleic acid enzymes, in sensing applications (7).

1.1 Functional Nucleic Acids

DNA has a rich and illustrious history that has its structure becoming the most recognizable icon in biology today when the Nobel Prize in Physiology or Medicine was awarded to James Watson, Francis Crick and Maurice Wilkins for the discovery of the double helix in 1962 (8); the birth of the field of molecular biology occurred when the genetic code was cracked by Robert W. Holley, Har Gobind Khorana and Marshall W. Nirenberg, resulting in another Nobel Prize in Physiology or Medicine in 1968 (9). The “central dogma” (10) has long relegated RNA to a biopolymer whose canonical role is to act as a messenger between DNA which stores the genetic information, and proteins which play both structural and functional roles in the cell. Up until the late 80s, RNA was not thought of as having function outside of its roles as messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA) and small nuclear ribonucleoproteins (snRNPs) (11). Discovery of RNA with novel function had sparked renewed interest in discovering

the interplay between structure and function as well as giving credence to a world before proteins coined the “RNA World”(12). Several types of functional nucleic acids (FNA) have been discovered since, namely RNA (13) and DNA (14) with enzymatic activity and binding capabilities (15).

1.1.1 Nucleic Acid Enzymes

Ribozymes are RNA with catalytic activity, able to catalyze reactions like its proteinaceous cousin, the enzyme. Ribozymes were discovered independently by both Thomas R. Cech’s (16) and Sidney Altman’s (17) groups who in 1989 shared the Nobel Prize in chemistry for “their discovery of catalytic properties of RNA.” The types of catalysis natural ribozymes can perform are usually limited to nucleic acid cleavage, ligation and replication, thus the types of substrates are usually limited to nucleic acids (18). Compared to proteinaceous enzymes, this repertoire may seem limited, but many protein enzymes involve the use of nucleic acid derivatives as cofactors to perform catalysis such as nicotinamide adenine dinucleotide (NADH), flavin mononucleotide (FMN) and tetrahydrofolate (THF). Although natural ribozymes have a shallow pool of chemical diversity, which may limit the breadth of catalysis, *in vitro* approaches allowed for the development of ribozymes able to catalyze reactions such as Michael-additions (19), Diels-Alder reactions (20) and phosphoamide bond formation (21).

Unlike in RNA, there are currently no known naturally occurring deoxyribozyme (DNAzyme) (22). Compared to ribozymes, DNA almost exclusively exists in the double helix form *in vivo* and there is less chemical competency available due to the lack of the 2’-hydroxyl group unlike in RNA (23). Despite these restrictions the Breaker group was able to create the first DNAzyme by using the same selection principles as with synthetic ribozymes, creating a

DNAzyme that cleaved an RNA strand in a Pb^{2+} dependent manner (24). Since the discovery of the first RNA cleaving DNAzyme in 1994, the repertoire of catalysis for DNAzymes has expanded to include reactions such as DNA cleavage (25), Diels-Alder cycloaddition (26) and porphyrin metalation (27).

1.1.2 Aptamers

Aptamers are oligonucleotides selected *in vitro* to bind to a target ligand. Much like ribozymes, the discovery of aptamers is credited to two groups who discovered them separately; Jack W. Szostak's and Larry Gold's groups independently developed the technique for selection of RNA that is able to bind a chosen target. The Szostak group used the term *in vitro* selection (28), whereas the Gold group used the term systematic evolution of ligands by exponential enrichment, or SELEX (29); although both groups use different names, the approach is similar. Through the use of *in vitro* selection, aptamers can be raised against a very large range of desired targets; the nature of these targets can range from a single metal ion (30), protein biomarkers (31), all the way up to something as large as a whole cell (32). The application of aptamers can range from sensing (33), targeted drug delivery (34) and even as a therapeutic drug (35). Used in a sensing application, aptamers can behave much like an antibody in that a labeled reporter can be conjugated onto the sequence that can indicate the presence of the target analyte. Used in a drug delivery application, the aptamer with high affinity to a specific cellular epitope can deliver the toxic therapeutic in a more targeted manner, thus lowering the dosage of the drug needed. The first commercially available aptamer drug, brand named Macugen, is used to treat macular degeneration and contains an anti-vascular endothelial growth factor aptameric moiety.

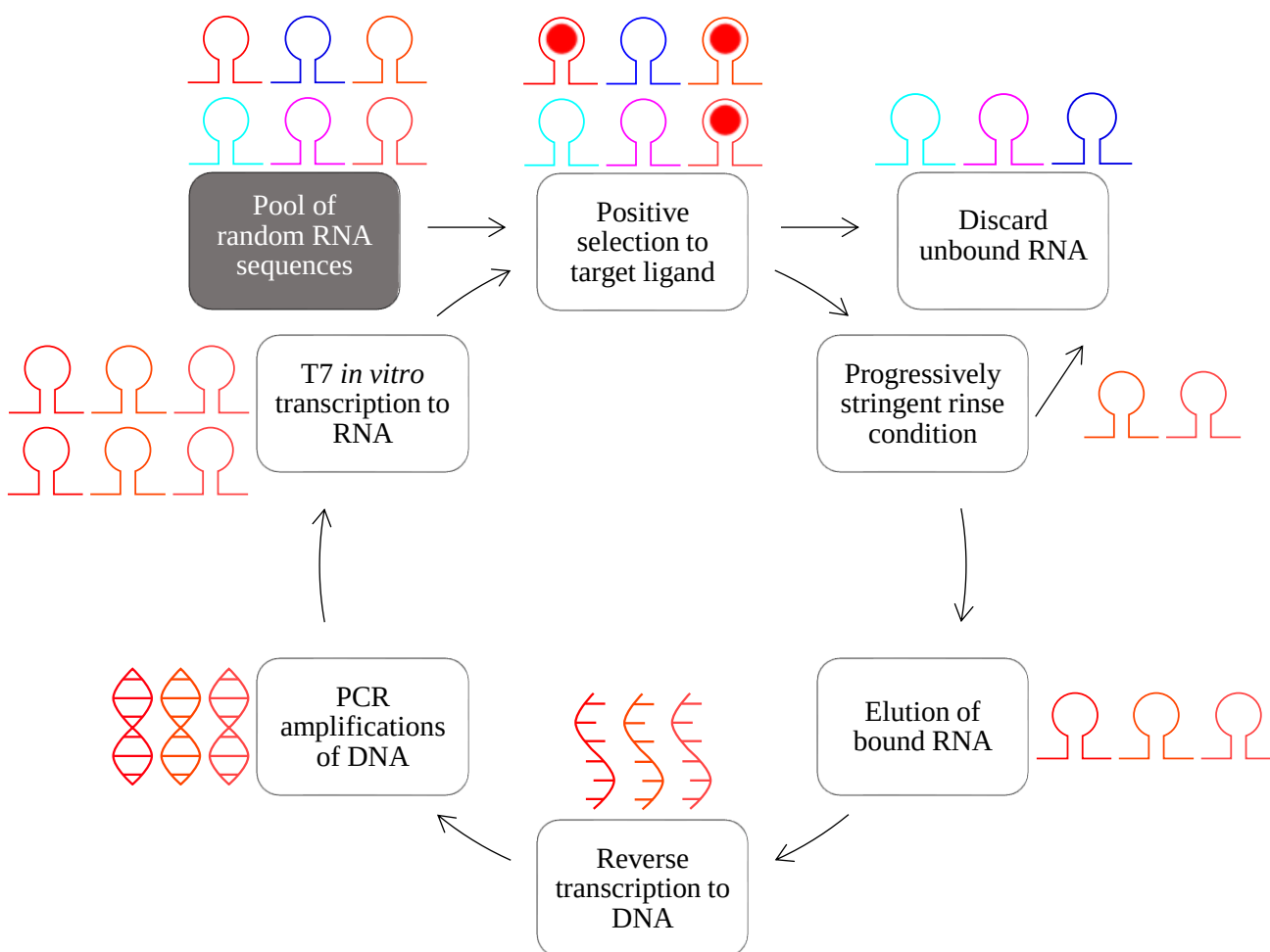


Figure 1.1 | In vitro selection of an RNA aptamers

A schematic of the *in vitro* selection process is shown. The pool of RNA is challenged to bind to the red target and unbound sequences are washed off. The sequences with some binding attribute, characterized with some degree of red color, is eluted, reverse transcribed and then amplified. Transcription of the amplified DNA results in a pool of enriched RNA sequences with higher affinity to the red target. With more stringent wash conditions, sequences without high affinity to the target are removed from the selection cycle, shown by the removal of the less red sequences. With enough iterations of stringent conditions, the population of sequences converge onto high affinity binders.

The method for the development of a new RNA aptamer involves an iterative approach, shown in Figure 1.1, in which a partially random pool of single stranded RNA is screened and then enriched with sequences with higher affinity, deemed aptamer candidates (36). A round of selection for an RNA aptamer involves the screening of a large random library, typically between

10^{14} to 10^{15} sequences, for oligonucleotides that bind to a selected target, which is frequently immobilized on a solid support. Unbound oligonucleotides are washed off and the bound RNA is eluted and converted to complementary DNA (cDNA) by reverse transcription. cDNA is amplified via polymerase chain reaction (PCR) and then the DNA is converted back into RNA via *in vitro* transcription. This will form a new pool that is now enriched with aptamer candidates that have tighter binding interactions with the target. Several rounds of selection are done with more stringent binding conditions to promote the population in the library to converge on sequences that have a high affinity for the target.

The strategy for the selection of DNA aptamers is relatively similar however the interconversion between RNA and DNA is not needed. On the other hand, the generation of single stranded DNA (ssDNA) now becomes the challenge since the amplification step produces double stranded DNA (dsDNA) (37). Typical strategies for the production of ssDNA, shown in Figure 1.2, include asymmetric PCR (38), size-based separation (39), enzyme digestion (40), or streptavidin assisted solid-phase separation (41). Asymmetric PCR involves the preferential amplification of one strand over another by using molar excess of the preferred strand; once exponential amplification exhausts the primer with the lower concentration, linear amplification of the desired strand occurs. Size-based separation generates ssDNA by using a chemically elongated primer for the complementary strand that is separated from the target strand using urea-denaturing polyacrylamide gel electrophoresis (PAGE). A typical enzyme used for the generation of ssDNA is the lambda exonuclease; a 5' phosphorylated primer is used for the complementary strand which is selectively digested by the enzyme after generating dsDNA from PCR amplification. The most widely used method for generating ssDNA involves the use of

streptavidin-coated magnetic beads; a biotinylated primer is used for the complementary strand which is captured by streptavidin and the target strand is released by chemical denaturation.

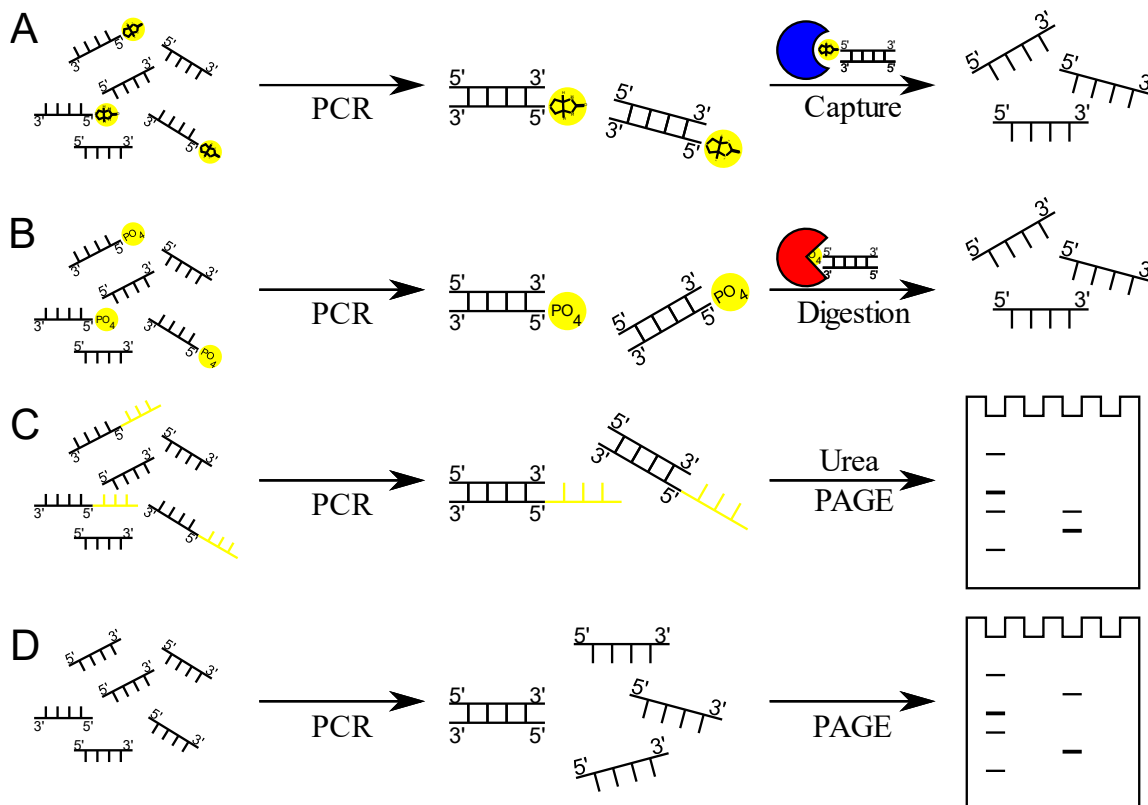


Figure 1.2 | Generation of ssDNA

A schematic of several methods used for the generation of ssDNA for use in SELEX generated using Inkscape is shown. Many of the methods in literature use a labeled reverse primer (yellow) in the PCR amplification step in order to isolate the desired forward strand through various means. Solid-phase separation, **A**, uses a biotinylated reverse primer that is captured onto streptavidin coated beads, shown in blue, followed by a denaturing step to separate the strand of interest from the solid support. For the case an enzyme digestion approach using lambda exonuclease, **B**, a 5' phosphate labeled reverse primer is specifically targeted by the enzyme to selectively digest the undesired labeled strand to yield ssDNA. Size-based separation, **C**, uses an ethylene glycol linker of various lengths attached to a polyA extension to create a reverse primer that is much longer than the forward primer. When the PCR product is run on a denaturing polyacrylamide gel, the strand of interest travels farther through the gel and is separated due to size – excision from the gel yields ssDNA. Asymmetric PCR, **D**, is an unlabeled method of ssDNA generation that uses a molar excess of the forward strand that creates more of the strand of interest once the reverse primer is consumed in the mixture. The dsDNA is separated from the ssDNA using PAGE and the strand of interest is excised from the gel.

1.1.3 Aptazyme

Aptazymes are molecules that combine both the functions of an aptamer and nucleic acid enzyme into a single molecule resulting in allosteric regulation of the enzymatic function (42). The free aptazyme is in a state of inactivity, but when the allosteric regulator binds to the aptamer moiety, the resulting conformational change causes catalytic activity to become permissible (43). Engineered aptazymes were first designed by the Breaker group by combining an ATP-binding RNA to a self-cleaving ribozyme (44). This allows for the allosteric regulation of the RNA cleavage activity through the presence of ATP. Before their discovery, naturally occurring aptazymes were theorized to exist (45) and with the advent of whole-genome sequencing, the first naturally occurring aptazyme was later isolated by the Breaker group for the *glmS* ribozyme (46). The *glmS* ribozyme is present in Gram-positive bacteria where glucosamine-6-phosphate acts as a coenzyme which causes self-cleavage of the mRNA (47). Naturally occurring RNA aptazymes are called riboswitches and are present in the UTR of mRNA to play a key role in gene regulation (48). To date, natural riboswitches in mammalian cells have yet to be found (49), however designed riboswitches have been developed to modulate gene expression in mammalian cells (50).

The development of an aptazyme can be engineered in a rational manner through the fusion of the two optimized functional nucleic acids (51), typically via the insertion of the aptameric moiety in the stem-loop region of the ribozyme (52). However, it has been shown that engineered aptazymes, thus far, perform less efficiently than those found in nature (53). This may be due to the fact that, as opposed to the modular nature of an engineered aptazymes, the integration of the aptameric and catalytic domain into a single structural unit allows for more concerted activity. Alternatively, *in vitro* selection, with careful control of selection and separation, can also be used to generate an active aptazyme (54). With the use of next-generation sequencing analysis, RNA

structures more akin to natural bacterial riboswitches can be discovered (55), and are used as tools that can be used to upregulate or downregulate gene expression (56).

1.1.4 Selection Strategies

Conventional SELEX involves the immobilization of the target molecule onto a solid support and adding the nucleic acid library to the solution phase (57). Traditionally, for small molecules, immobilization is done by coupling the derivatized target onto a coated bead. Various tags can be added to the target to aid in immobilization and include histidine, glutathione S-transferase or biotin which have a high affinity for nickel-nitrilotriacetic acid, glutathione, and streptavidin, respectively, which are used as a coating. The bead can be made from various materials including agarose, sepharose and polystyrene or a paramagnetic material such as iron oxide; depending on the material, separation of the solid phase can be achieved via centrifugation, filtration or by using a magnet. Although this is the most widely used method, it is not without its drawbacks such as the derivatization of the target may not be possible or desirable.

In order to avoid using a modified ligand, a variation of this solid-phase selection method involves the immobilization of the library using a short complementary sequence bound to the solid matrix (58). When the underivatized target is added to solution, target-induced conformation changes results in the release of the nucleic acid from the solid support. The principle behind this process is due to the polymorphic nature of nucleic acids causing structure-switching; nucleic acids can exist in the hybridized double-stranded form or the bound single-stranded form. Although this allows for the selection using unmodified target molecule, formation of the aptamer-target complex can be hindered by sterics due to immobilization onto a solid support.

Solution-phase selection is possible using capillary electrophoresis SELEX (CE-SELEX). CE-SELEX is based on the mobility shift of the target-nucleic acid complex – perturbation occurs with the bound species resulting in slower mobility through the column (59). This unlabeled method allows for the target-nucleic acid interaction to occur without steric hinderance since neither species are immobilized onto a solid support thus off-target binding to the solid matrix can be eliminated.

After multiple rounds of selection, the next most crucial step of SELEX is to identify the sequences that exhibited the desired binding response through sequencing. The traditional method of aptamer identification involves cloning followed by Sanger sequencing of select sequences (60). Briefly, sequences are ligated into plasmids and transformed into cells, which are then plated. Tens of colonies are selected, and the plasmid is isolated then sequenced via the chain-termination sequencing method. This method is possible because after several rounds of stringent selection steps, the sequence space of the library is dominated by high binding sequences which would be represented in the relatively low amounts of colonies that are isolated. Although this method is advantageous because of its relatively low cost and generally accessible equipment required, much of the sequence space remains unexplored and highly efficient binders may be lost due to over-representation of sequences emerging due to PCR-bias and off-target binding. In order to address these drawbacks, next-generation sequencing (NGS) may be used to generate more than a hundred million sequence reads from the final pool of sequences (61). Due to the sheer number of sequences that are read, analysis of the sequence data can produce, not only individual aptamer sequences, but aptamer clusters and even binding motifs. With such a high sequence coverage available to this method, fewer rounds of enrichment are needed to identify high-binding aptamer sequences that emerge.

1.2 Light-up Aptamers

In the realm of molecular biology, seeing is believing. When using imaging and spectroscopic techniques, fluorescence is an attractive option, due to its sensitivity, safety, and ability to analyze multiple tags in one experiment. When green fluorescent protein (GFP) was discovered (62), it changed the world of molecular biology by allowing scientists to reliably monitor gene expression and protein interactions. A protein of interest can be tagged with GFP at the genetic level and when expressed, gene regulation and protein localization can be tracked within the cell in real time using this fluorescent label (63). The chromophore of GFP, 4-hydroxybenzylidene imidazolinone (HBI), is formed in the inner alpha helix of the protein after a spontaneous cyclization of three amino acids, serine, tyrosine and glycine (64), shown in Figure 1.3. Several mutants have been produced that can cause a red (65) or blue-shifted (66) excitation/emission spectrum that can now run the gamut of colors on the visible spectrum (67). It is due to these versatile, genetically-encoded, fluorescent tags that many discoveries about cellular structures and processes have been made possible (68).

Although nucleic acids with intrinsic fluorescence have yet to be discovered, aptamers that are able to bind fluorogenic molecules (non-fluorescent molecules that become fluorescent under permissive conditions) are used in applications similar to GFP (69). These RNA aptamers, sometimes referred to as light-up aptamer, are used in the field of molecular biology to study the transcriptome and their DNA counterparts can be used in biosensing applications as fluorescent modules (70, 71).

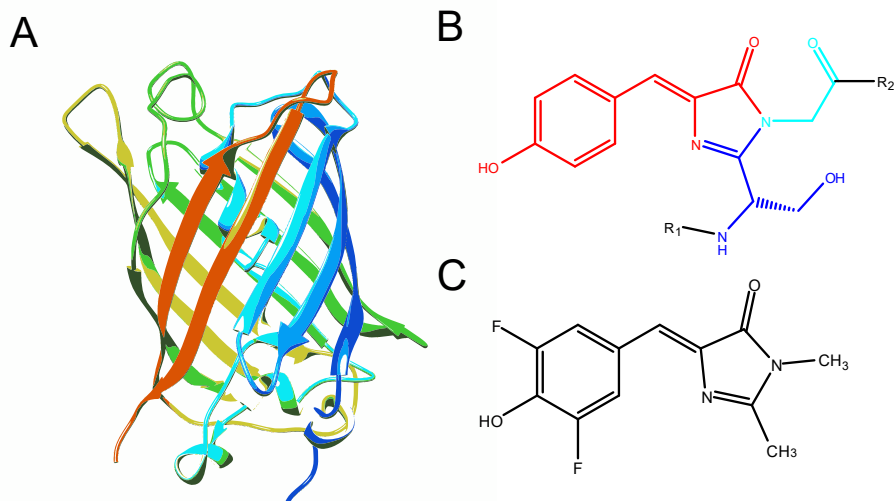


Figure 1.3 | Structure of GFP

The structure of GFP **A** (PDB ID: 1GFL), generated using UCSF Chimera, is dominated by a beta barrel which consists of 11 β -strands that enclose an α -helix. The internal α -helix contains the 4-hydroxybenzylidene imidazolinone (HBI) chromophore **B** that is formed from the cyclization of S65(blue)-Y66(red)-G67(cyan) in wild-type GFP. Currently there is no purely nucleic acid system that has innate fluorescence, however, there are aptamers that are designed bind a derivative of the GFP chromophore **C** 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI).

1.2.1 Light-up RNA Aptamers

The primary use of fluorescent RNA aptamers is to study differential subcellular localization of RNA (72). This type of application is analogous to the use of GFP in the study of proteins by labeling the protein of interest at the genetic level. Traditionally, fluorescence *in situ* hybridization (FISH) or the MS2-fluorescent protein system was used for RNA imaging (73), however there are several RNA aptamer-based fluorescent tags used to study RNA today. Notable light-up RNA aptamers include ones that bind malachite green (74), HBI derivatives, such as the DFHBI (75) and 3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime (DFHO) (76), as well as a thiazole orange derivative called TO1-biotin (77).

The malachite green aptamer (MGA) was the first example of an aptamer that, once bound to its cognate fluorogenic ligand, causes a marked increase in fluorescence. The 38-nucleotide

MGA was selected by the Wilson group for use in “laser-mediated, site-specific inactivation of RNA transcripts” (74). A gene of interest is tagged with MGA on the genetic level and expressed in the presence of malachite green. Subsequent irradiation with a 630 nm wavelength laser induces free radical production from the bound aptamer complex resulting in degradation of the proximal sequence thus silencing the gene. Although there is limited use of the aptamer for RNA imaging (78), due to the cytotoxicity of malachite green (79), it has been used in turn-on fluorescence aptamer sensors (80, 81).

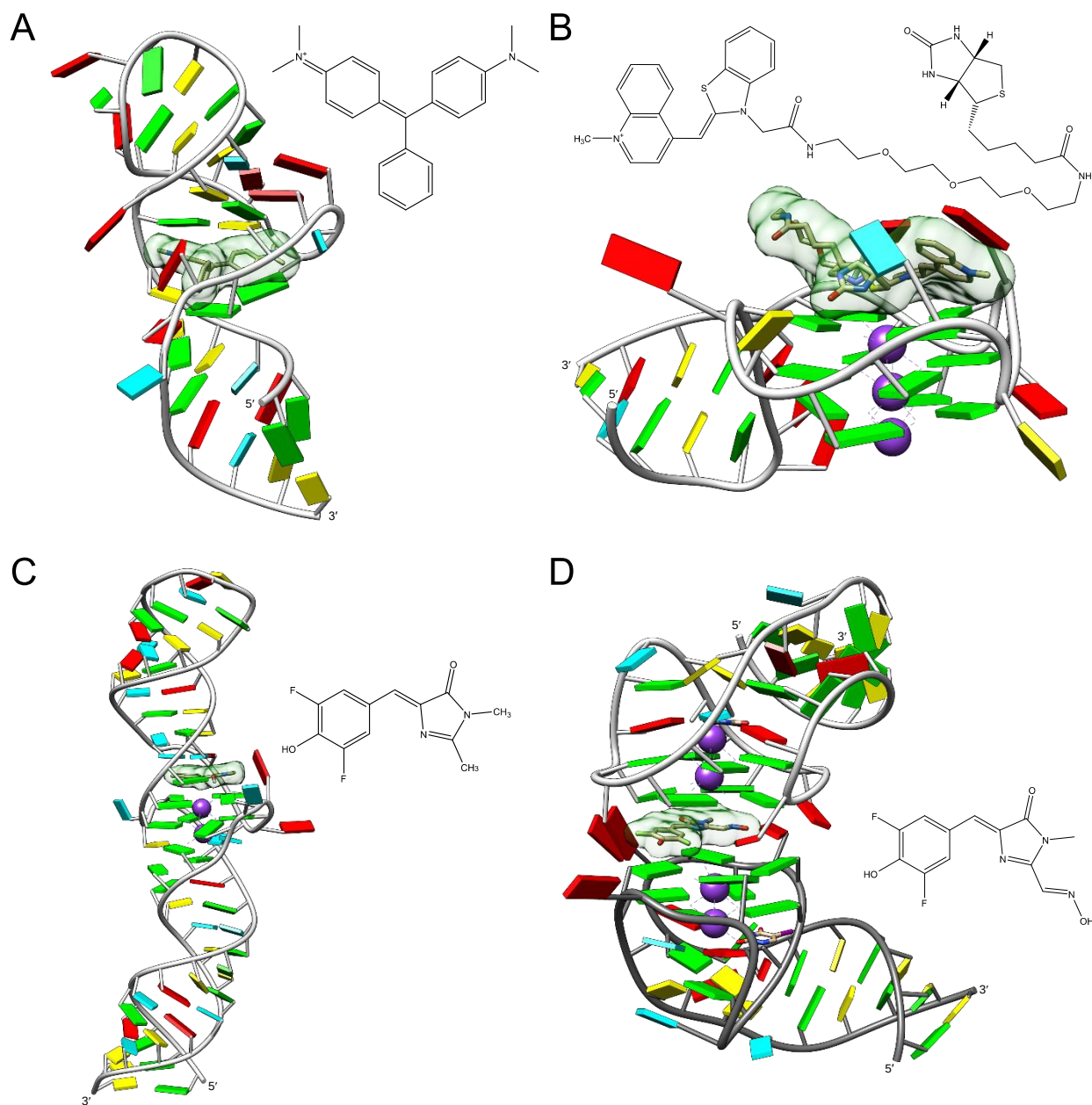


Figure 1.4 | Solved structures of light-up RNA aptamers

Some select 3D structures of light-up RNA aptamers are shown bound to their cognate ligand generated using UCSF Chimera is shown. The nucleobases guanine (green), adenine (red), thymine (yellow) and uracil (cyan) are represented by slabs, potassium ion (purple) is represented by orbs and the ligand is highlighted with a surface (green). **A** (PDB: 1Q8N) shows the solution structure for the malachite green aptamer bound to its cognate ligand, malachite green (82). The first solved structure of a bound light-up RNA aptamer is of the malachite green aptamer, albeit bound to tetramethyl-rosamine. **B** (PDB: 5V3F) features the crystal structure of the RNA Mango aptamer bound to TO1-biotin (83). The dye is sandwiched between a three-tiered G-quadruplex and held in place by two adenines. **C** (PDB: 4TS2) displays the crystal structure of the bound

Spinach aptamer to its ligand DFHBI (84). The elongated structure of the Spinach aptamer features a central two-tiered G-quadruplex motif that characterizes the binding domain. **D** (PDB: 5BJO) shows the crystal structure of the Corn RNA aptamer in complex with DFHO (85). The dye binds at the homodimeric interface between the two-tiered G-quadruplex of each monomer.

1.2.2 DFHBI Aptamer

The most widely used light-up RNA aptamer system are those that were selected to bind to GFP-derived fluorogens; the first such aptamer system being the Spinach-DFHBI system (75). The initially selected 98-nucleotide aptamer was used to visualize the localization of 5S rRNA in the cellular nucleus in real time as a proof of concept for *in vitro* imaging using an RNA aptamer module. DFHBI was used for in cell imaging due to good cell permeability, low cytotoxicity and low phototoxicity especially compared to malachite green for use in these applications; despite these advantages of the fluorogen, the aptamer was unstable *in vivo* due to poor thermal stability at 37°C and the complex would tend to undergo photobleaching which were areas for improvement of the aptamer system. Following the initial publication, several other aptamers with HBI derivatives such as Broccoli (86), Baby Spinach (84), and Corn aptamers (76) were created.

There are several solved crystal structures of the Spinach aptamer using different crystallization methods (84, 87, 88). The overall structure of the aptamer-dye complex remains the same between all of the solved structures, with the dye binding pocket located between a two-tiered G-quadruplex and a U-A-U base triple, seen in Figure 1.4 C. Using a structure-guided approach, the crystal structure was used to design the miniaturization of the sequence, resulting in the 51-nt Baby Spinach aptamer (84). In order to combat the poor thermal stability *in vivo*, reselection of a DFHBI aptamer assisted by a flow cytometry technique resulted in a 49-nt aptamer called Broccoli with enhanced *in vivo* fluorescence and thermal stability (86).

Another HBI derivative, (DFHO), was synthesized to mimic the fluorescent module of red fluorescent proteins (RFP). The selection of an aptamer against DFHO resulted in the 28-nt Corn aptamer. The Corn-DFHO system was more resistant to photobleaching than both the Spinach-DFHBI or Broccoli-DFHBI systems and was used to monitor RNA polymerase III transcriptional activity (76). The crystal structure of the Corn aptamer shows that the bound complex involves a homodimer of the aptamer with the dye binding at the dimer interface between two G-quadruplexes (85), seen in Figure 1.4 **D**.

The structures for the Spinach and Corn aptamers have been solved and show vastly different structures for both; however, the sequences converge on a G-quadruplex motif that confers structural and functional significance which emphasizes the importance of this motif in RNA-induced-small-molecule fluorescence. There currently are only a few solved structures of RNA aptamers bound to fluorogenic molecules; typically dyes have close contacts with a G-quadruplex or base triple resulting in restricted rotation allowing for resonance to be maintained (89–91).

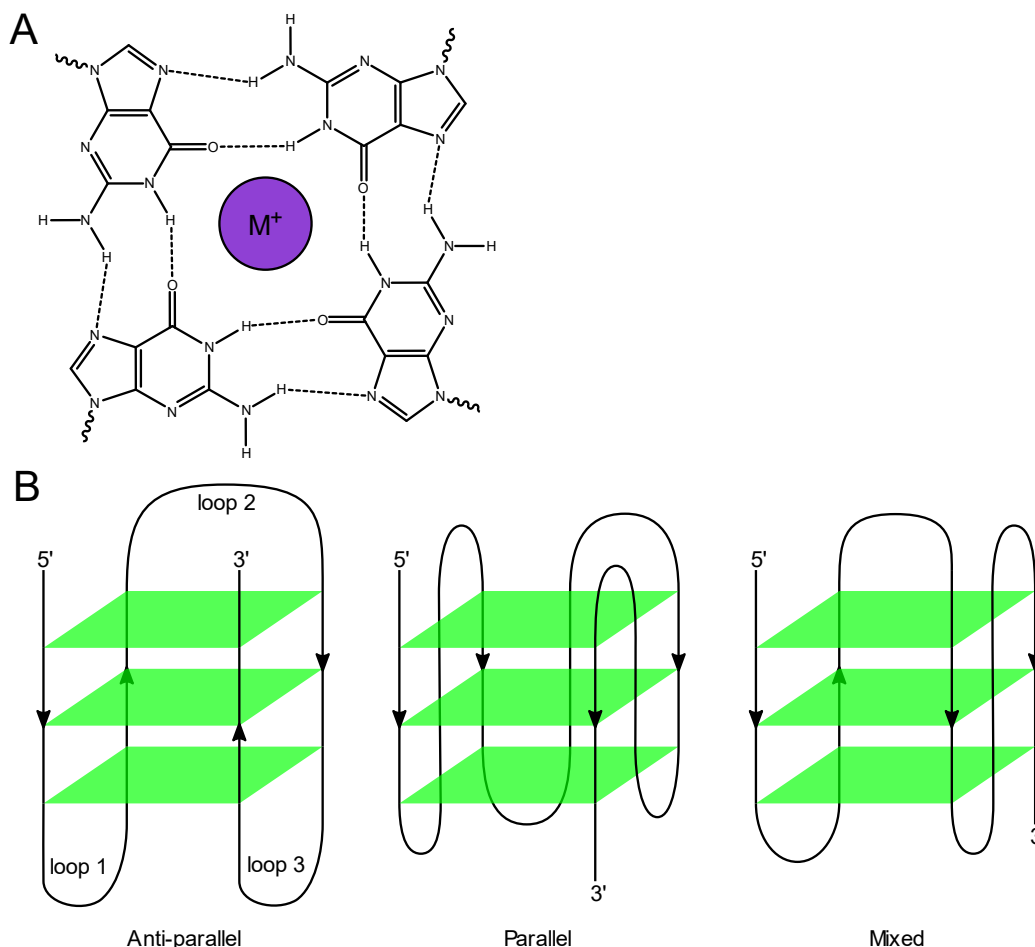


Figure 1.5 | G-quadruplex

G-quadruplex motifs consists of at least two layers of G-quartets where the guanines form a square planar structure shown in **A**. G-quartets are formed from four guanines that are cyclically bonded through Hoogsteen interactions in a planar structure (92). Stabilization of the quartet and quadruplex is mediated via monovalent cations, typically potassium ion. Examples of typical unimolecular G-quadruplex topologies are shown in **B** generated using Inkscape. The strand orientation of the quadruplex can result in a number of topologies with varying loop configurations which can be classified as anti-parallel, parallel or mixed (93).

1.2.2 Mango RNA Aptamer

Thiazole orange is a cyanin dye that is routinely used to stain reticulocytes, immature red blood cells, in hematological studies (94). It has been shown to intercalate in both DNA (95) and RNA (96) but has a much higher affinity to double stranded than single stranded nucleic acid.

When bound to dsDNA, an increase in fluorescence is observed; this phenomenon led to the development of several commercially available stains used to visualize DNA in place of ethidium bromide, namely SYBR safe (97). Not only is thiazole orange and its commercially available derivatives less mutagenic than ethidium bromide, the detection of the DNA from gel electrophoresis can be done using non-destructive blue light, which lowers the risk of damage by UV radiation to the DNA of interest (98). Although thiazole orange has a significant amount of fluorescence enhancement, the lower affinity of the unmodified thiazole orange limits wide usage in place of ethidium bromide in gels for lower DNA concentrations (99).

The Unrau group wanted to create a bright and high affinity complex for use in RNA labeling that could rival that of the Spinach aptamer; to this end they developed the RNA Mango aptamer (77). The main focus during the selection process was to choose conditions that would promote a slow k_{off} thus favoring the bound state resulting in a lower K_D . Successful isolation of the Mango aptamer resulted in a light-up module that had a higher fluorescence efficiency than previously isolated light-up aptamers. In the pursuit of aptamers with higher affinity and selectivity, microfluidic-assisted *in vitro* compartmentalization (μ IVC) was used to select enhanced aptamers from the final selection pool, Mango II, III & IV (100). The resulting reselection gave rise to aptamers with much higher brightness with quantum yields four times that of the originally selected aptamer with affinities in the same low nanomolar range. To date, the crystal structures for Mango I – IV, have been solved and all feature a G-quadruplex on one face of the dye that is held in the binding pocket with nucleotides in various configurations (83, 101–103).

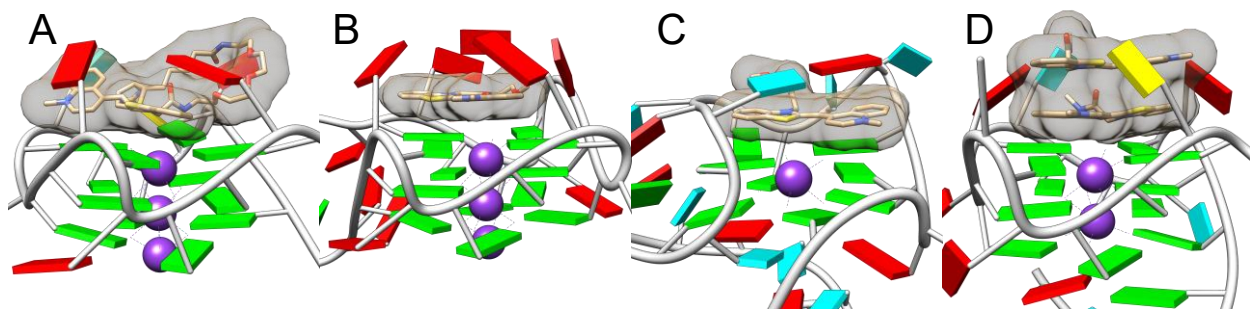


Figure 1.6 | Mango binding pocket

Visualization of the binding pocket of Mango I – IV obtained from X-ray diffraction crystallography generated using UCSF Chimera is shown. The nucleobases guanine (green), adenine (red), thymine (yellow) and uracil (cyan) are represented by slabs, potassium ion (purple) is represented by orbs and the ligand is highlighted with a surface (green). The binding pocket of the Mango I aptamer **A** (PDB: 5V3F) shows the dye being held between a 3-tiered G-quadruplex and two unpaired adenines with an unpaired uracil interacting with the biotin moiety (83). The binding pocket of Mango II **B** (PDB: 6C63) has a very similar binding pocket with a 3-tiered G-quadruplex and three unpaired adenines having the same roles (101). The binding pocket of Mango III **C** (PDB: 6E8S) has the dye being held between a 2-tiered G-quadruplex and a U-A-U base triple (102). The binding modality of Mango IV **D** (PDB: 6V9B) is quite different in that the structure is an asymmetric homodimer with two distinct binding pockets (103). The binding stoichiometry is 3 with two stacked dyes in one binding pocket and a single dye in the other. In both binding domains, the pocket is located between a 3-tiered G-quadruplex and four unpaired nucleotides.

1.2.3 Light-up DNA Aptamers

Unlike in RNA, differential localization of DNA is limited and is typically locally constrained in the nuclei of the cell (104). Since a large driver for the development of light-up RNA aptamers is to study RNA processes *in vivo*, there are currently fewer selected DNA aptamers for fluorogenic dye molecules. By no means does that mean that there is no place for fluorescent DNA aptamers; application for such molecules is in DNA based fluorescent probes used in biosensors (105). RNA based biosensors can be cost prohibitive and prone to degradation due to RNase presence, however making sensors based on DNA can help mitigate these drawbacks (106). Selection for DNA aptamers that bind malachite green (107), crystal violet (108) and dimethyl indole red (109) have been conducted, however the resulting aptamers have a marked decrease in

fluorescence enhancement compared to their RNA analogues (110). Although the main use for these light-up DNA aptamers is in *in vitro* sensing (111), labeled ssDNA aptamers can be used for *in vitro* sensing – especially on the cell surface (112).

Table 1.1 | Characteristics of fluorescent aptamers

Aptamer		Fluorogen	K _D (nM)	Ex/Em (nm)	Reference
RNA	MG aptamer	Malachite green	117	630/650	(113)
RNA	Spinach	DFHBI	540	469/501	(114)
RNA	Corn	DFHO	70	505/545	(76)
RNA	Mango	TO1-biotin	3	510/535	(83)
RNA	DIR aptamer	Dimethylindole red	86	600/646	(115)
DNA	MG aptamer	Malachite green	2430	630/650	(107)
DNA	CV aptamer	Crystal violet	490	580/640	(108)
DNA	DIR aptamer	Dimethylindole red	650	600/646	(109)

1.3 Biosensing

A chemical sensor is a device that is able to detect chemical information, such as composition and concentration, and can give a signal that can be quantitative, semi-quantitative or qualitative (116). Chemical sensors are composed of a receptor and a transducer. The receptor is selective for the chemical of interest and, through specific interaction with the target, produces a signal that the transducer can detect. The transducer is then able to transform one form of the signal into another, analytically useful, form, but is not selective like the receptor. The detection of biological compounds can be difficult with traditional chemical sensors due to the lack of specificity for the large range of biologically relevant analytes; this challenge can be overcome using a biosensor.

The definition of a biosensor has evolved over time, with the broadest sense of the word being a chemical sensor containing a biological component. The IUPAC definition of a biosensor

is, “a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals (117)”. One of the first well documented uses of a primitive biosensor was by coal miners in the early 20th century, who brought caged canaries into mining shafts in order to detect the presence of toxic gasses, such as carbon monoxide (118). Since the canary is more sensitive to the presence of the toxic gasses than humans, the formation of carboxyhemoglobin resulting in death can be considered the receptor and transducer. A more prototypical early biosensor is the glucose sensor used by those afflicted with diabetes which uses the action of the enzyme glucose oxidase as the biological component (119). The receptor is composed of the protein enzyme, glucose oxidase, which detects glucose and oxidizes it. This redox reaction is able to be detected by the transducer, which transforms the redox reaction into an electronic signal that is displayed onto a reader.

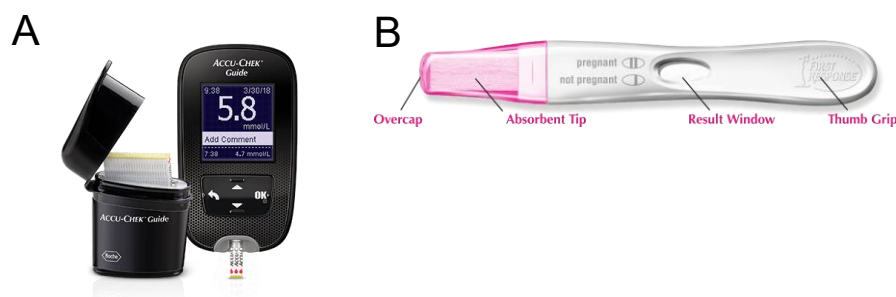


Figure 1.7 | Commercial biosensors

Examples of technologies that are dominant in the commercial point of care biosensing market. **A** is a typical glucose meter that is able to measure the blood glucose levels by leveraging the activity of the enzyme glucose oxidase (119). The redox reaction produces a signal that is interpreted by the device to give a digital reading to the user. **B** is a typical pregnancy test that detects the presence of the human chorionic gonadotropin (hCG) using a sandwich lateral flow assay approach (120). Gold nanoparticles conjugated with anti-hCG antibodies are captured at the test line if there is presence of hCG in the sample resulting in a red line due to localization of the nanoparticles.

1.3.1 Detection of Small Molecules

Heavy metal testing for environmental samples is important as these heavy metals (Hg^{2+} , Pb^{2+} , Cd^{2+} , Ag^+ , Cu^{2+} , etc.) have been shown to be toxic to humans. Health Canada approves of two standard methods of testing for heavy metals in drinking water: inductively coupled plasma-mass spectrometry (ICP-MS) and graphite furnace atomic absorption spectroscopy (GFAAS) (121). Although these methods can provide accurate results, these methods of instrumental analysis are limited to laboratory settings as they are expensive to operate and cannot provide real-time information. Due to increased awareness of heavy metal contamination in ground water, lakes, and oceans, which can result in poor health outcomes in humans, there is a need for fast, accurate and simple methods for the testing of drinking water. Biosensors are able to fulfill the need for cost-effective, fast, and reliable sensing in environmental samples. The bioreceptor component of the biosensor can include whole-cells (122), enzymes (123), antibodies (124) or functional nucleic acids (125–127). The binding of the target to the bioreceptor results in a transduction of binding event to one that is measurable, typically electrochemical or optical, and to a lesser extent thermal or mass-based (128).

1.3.2 Detection of Protein Biomarkers

A biomarker is a biological molecule that, once measured, can give insight into the biological state of the system; either normal or abnormal. Generally, the discovery of protein biomarkers whose change in state or expression levels correlates with the risk of contraction, the presence, or the stage of disease (129). Prominent examples of biomarkers that are studied include proteins that are associated with a variety of cancers (130), neurological diseases (131) as well as auto-immune diseases (132). Typically, analysis and discovery of biomarkers are found in samples

obtained from serum (133), plasma (134) and urine (135). Other biomarker sample origins include saliva (136), sweat (137) and nasal mucosa (138) sampling. Traditionally, analysis of biomarker samples is done using enzyme linked immunosorbent assay (ELISA) (139), mass spectroscopy (140) or protein microarray (141). These rigorous analysis techniques require the expertise of trained technicians and specialized equipment to obtain results. Point of care devices would be the holy grail for disease state diagnostics; they would provide on-site fast and accurate results that can lead to faster treatment (142). A surging market for such point-of-care testing include the lateral flow test (143) which can give rapid results outside of the laboratory.

1.4 Nucleic Acid Sensors

In the realm of biosensors, antibodies have dominated the area as the preferred biomolecule to provide specificity in sensing applications. This is, to some degree, due to the standardization of methods for antibody discovery and application since their commercialization was not as restricted as functional nucleic acids, which was under patent until 2013. There are, however, many cases of published works that use nucleic acids in a sensing capacity which can be seen from the number of publications related to aptamer biosensors. This is due to the fact that there are many different ways that functional nucleic acids can be used in a sensing capacity.

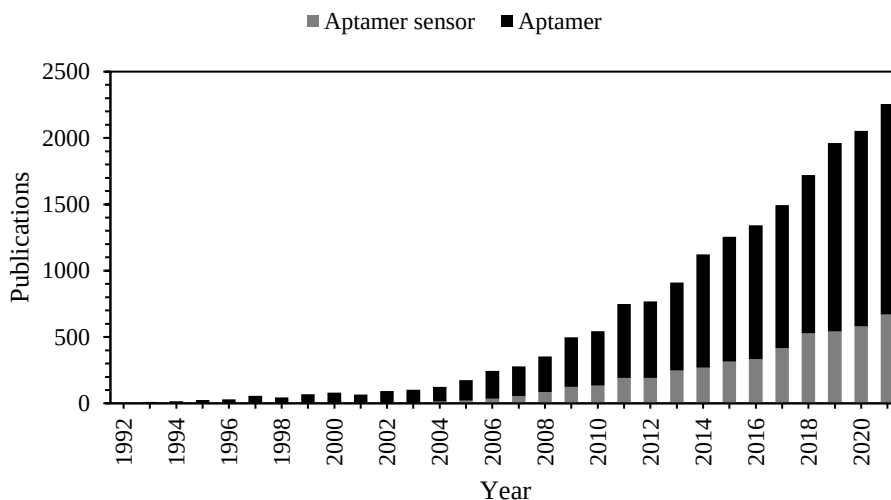


Figure 1.8 | Number of publications related to aptamer sensors

An analysis of the publication trends using Web of Science and the search terms “aptamer” or “aptamer sensor” is shown. The first published work using the term aptamer is found in a journal issued in 1992. After the initial paper, there is steady increase in publication with the first aptamer sensor appearing in a publication from 1998. The publication record show that this subject matter is currently a growing area of interest in the field, especially after the initial patents expired in 2013 (US005567588A).

1.4.1 Detection Methods using Nucleic Acids

There are several detection methods that are compatible with the use of functional nucleic acids in sensors. In many cases aptamers can be used in place of antibodies in applications that use them as the recognition element in the sensor (33). Like in a Western blot, reporters can be directly linked to nucleic acids for use in the detection of target molecules (144). Nucleic acids can be easily chemically modified for surface chemistries in detection methods involving electrochemical (145) and surface plasmon resonance (SPR) (146) approaches. Spectroscopic methods such as colorimetric (147), or florescence (148) which require less or sometimes no instrumentation are also appropriate for FNA.

An electrochemical approach for the detection of an analyte usually involves the transduction of a biochemical event, such as an enzyme-substrate reaction or receptor-ligand

interaction, into electrical signals (149). A notable example of a biosensor that uses an electrochemical approach is the first blood glucose sensor (150). Novel biosensors that use this approach involve the immobilization of the biomolecule onto sophisticated nanomaterials such as carbon nanotubes (151), metallic nanoparticles (152), and silicon nanowires (153). This method is very accessible but it may be hard to differentiate contaminants in a complex sample (149).

Surface plasmon resonance, SPR, is a physical characteristic of metal surfaces, such as gold, that is exploited to measure the change of mass on the metallic surface. This optical method involves the excitation of the wave that propagate the surface, known as the surface plasmon, with light and reading the angle of reflection in which the surface plasmon absorbed light energy, known as the surface plasmon resonance angle. An increase in signal is indicative of an increase of mass onto the surface of the metal (154). Typically, a nucleic acid sequence that can recognize the target is bound to the sensor surface and once the sample containing the target is injected into the system, the binding event results in a signal on the sonogram indicating the presence of the target in the sample. This capture method for target detection is very sensitive for the target, however the requirement for specific instrumentation and training limits the wide usage of this sensitive and powerful method.

Colorimetric methods involve the change in color of the sample due to the presence of the analyte. An advantage of this type of method is that a spectrum of available response types is available, ranging from qualitative to quantitative. This can allow for a quick method of detection with results easily understood by the human eye. The nucleic acid in this case is often directly labeled with a colorimetric label that results in a localization of the reporter if the target is present in the sample indicating a positive response. In order to achieve a quantitative measure of the

signal, spectroscopic methods must be employed to quantify the spectral intensity which mitigates the advantage of naked eye detection.

Fluorescent methods of detection in functional nucleic acid sensors largely involve the use of fluorophores in a molecular beacon-like configuration. A molecular beacon has two components, the fluorophore, and the quencher, typically covalently bound to the 5' and 3' end of the sequence. When the target is bound by the labeled aptamer, the fluorophore, and quencher are brought spatially closer and loss of signal indicates presence of the target. This method allows for high sensitivity for the detection of the sample.

1.4.2 Nucleic Acid Enzyme Sensors

Nucleic acid enzyme-based sensors have more limitations than its proteinaceous brethren as, typically, the catalytic activity is done in a single turnover manner (155). This by no means indicates that ribozymes and DNAzymes cannot be used as sensors; much to the contrary, nucleic acid enzymes are able to detect the presence of certain metals very accurately with a high level of precision (156). Typically, the presence of the cofactor, the metallic analyte, mediates the cleavage of the substrate strand with a ribonucleotide at the cleavage site. The resulting cleavage can lead to a fluorescent signal due to the separation of the fluorophore-quencher pair (157) or coulometric change of gold nanoparticles due to disaggregation of conjugated nanoparticles (158). A non-optical detection method that utilizes an electrochemical approach (159) typically has the DNAzyme conjugated to the solid phase electrode on one end and a reporter on the other end. The hybridization of the substrate strand to the DNAzyme attenuates the redox signal, however when the target is present and the substrate strand is cleaved, the reporter is able to make close contact with the electrode producing an increased signal response.

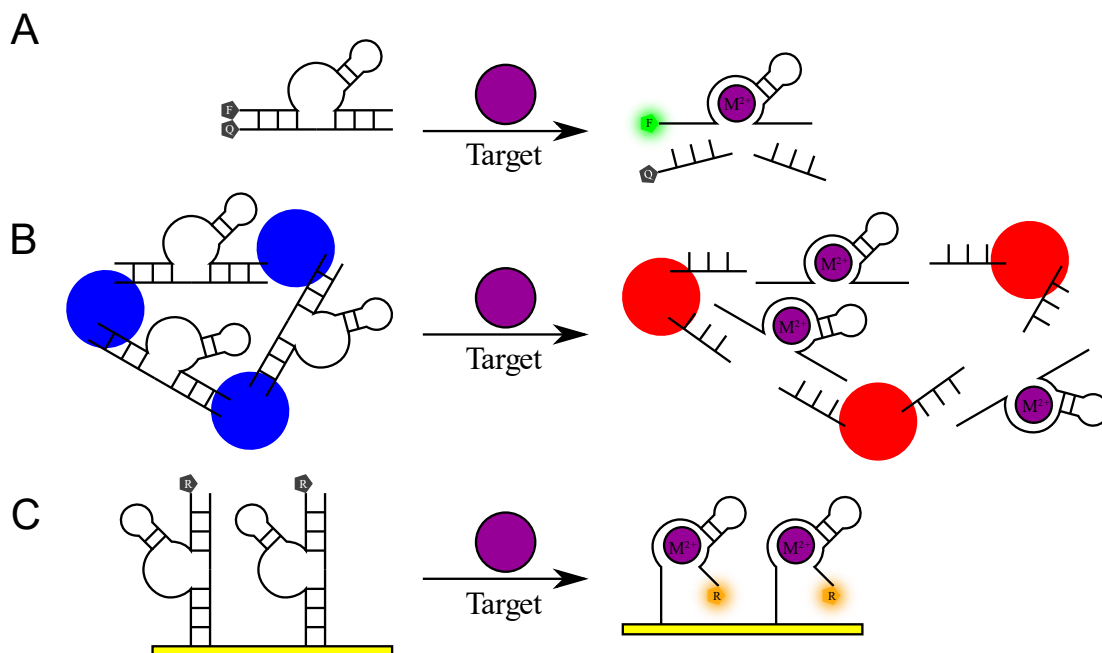


Figure 1.9 | Nucleic acid enzyme sensor detection methods

Schematic of typical ribozyme and DNAzyme sensor designs used for the *in vitro* detection of metal ions generated using Inkscape. A turn-on fluorescence approach is shown in **A** that utilizes a fluorophore-quencher probe for detection (157). Cleavage of the substrate strand is induced when there is the target metal in the sample causing the separation of the quencher thus producing a measurable signal. A colorimetric approach using gold nanoparticles is shown in **B**. Crosslinking of the nanoparticles due to hybridization of the substrate strand and the nucleic acid enzyme causes low dispersion of the nanoparticles resulting in a blue color (158). When the target metal ion is present in the sample, cleavage of the substrate strand causes disaggregation of the nanoparticles resulting in a red-colored solution due to shifts in the surface plasmon resonance. An electrochemical approach using a redox reporter is shown in **C** (159). When the metal target is present in the sample, cleavage of the substrate strand allows for the redox reporter, typically methylene blue, to move closer to the electrode resulting in a measurable change in electrical potential.

1.4.3 Aptamer Sensors

Aptamers can be used in sensors in the same way antibodies are as they both are able to bind and capture the desired target molecule. A distinct advantage that aptamers are able to provide is that they are able to be easily modified chemically and the predictable conformational change

of the aptamer, once bound, can be used to design novel sensors. The direct binding of the target can be measured when coupled to a detection method that can measure the change in mass such as quartz crystal microbalance (QCM) or optically with SPR; fluorescent, colorimetric, and electrochemical approaches have also been used in conjunction with an aptamer sensor.

QCM and SPR mediated aptamer sensors involve similar surface chemistry; the aptamer is immobilized onto the gold coated quartz crystal sensor chip, for QCM, or gold surface sensor chip, for SPR. This is done using a thiolated nucleic acid derivative which spontaneously forms a covalent bond with the gold surface. When the target binds to the aptamer, the increase mass results in a change in signal; attenuation of the vibrational frequency and an increase in SPR angle for QCM and SPR spectroscopy, respectively. The use of gold nanoparticles over the traditional gold films have been shown to enhance the signal, and have been found to detect concentrations as low as 0.78 aM (160).

Although aptamers cannot perform redox reactions directly, they can still be used in an electrochemical approach for detection of a target. Typically, one end of aptamer is conjugated onto the electrode surface, commonly gold, and the other end is conjugated to a redox reporter, often methylene blue (161). When the target is bound, the reporter is brought close to the electrode, and the difference in potential can be discriminated between the bound and unbound state (162). Another common approach can be the use of an aptamer to attenuate the redox reaction when bound to the target molecule by shielding the target from an oxidizer (145).

With the use of a molecular beacon approach, aptamers can use fluorescence for the method of detection. By taking advantage of the conformational change of an aptamer when it binds to the target, labeled aptamers can increase the distance between the quencher and fluorophore when bound to the ligand (163). The use of fluorescence in conjunction with aptamers is not limited to

just the use of molecular beacons; the change in quantum yield of a bound dye can be exploited to indicate an increase in analyte (164).

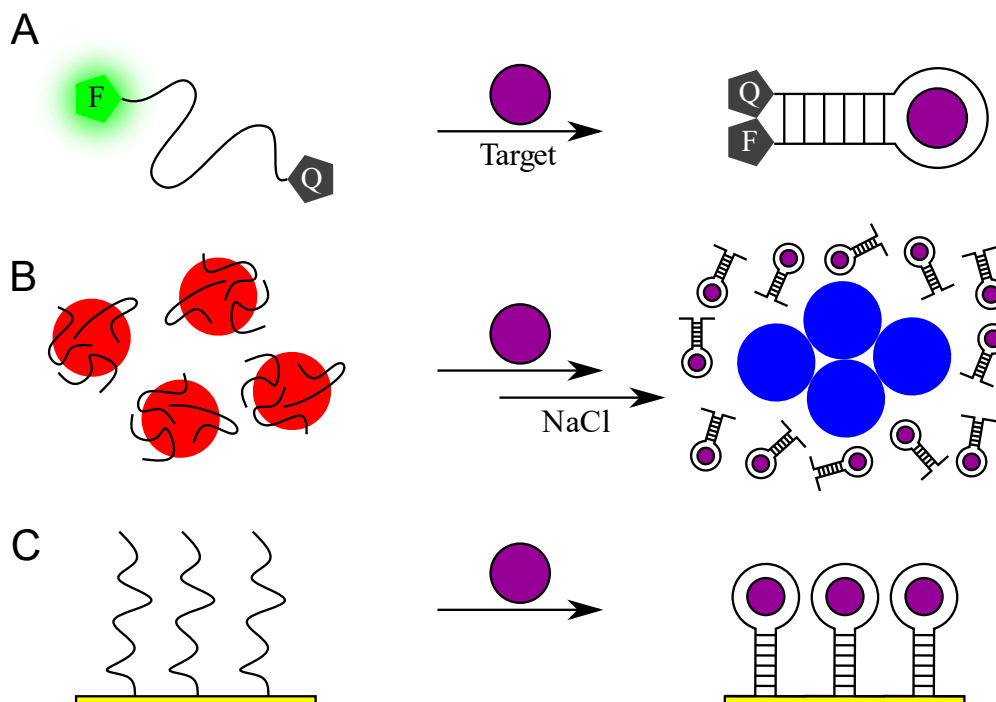


Figure 1.10 | Aptamer sensor detection methods

Schematic of typical aptamer sensor design generated using Inkscape. In **A**, the aptamer sensor is designed using a molecular beacon-like approach (165). The aptamer is labeled with a fluorophore and quencher, typically at the 5' and 3' ends. When the target molecule is bound to the aptamer, the quencher is brought close to the fluorophore resulting in a loss of signal indicating the presence of the target in the sample. **B** is a schematic of a typical colorimetric biosensor using unmodified gold nanoparticle probes (166). The unstructured aptamer adheres to the surface of the gold nanoparticle allowing for dispersion of the particles resulting in a red colour. When the target molecule is bound by the aptamer and under high ionic conditions, aggregation of the nanoparticle occurs resulting in a blue-shift due to a change in the surface plasmon resonance. **C** shows a typical example of surface immobilization of aptamers for use in diagnostics (167). The gold nanofilm is modified with a monolayer of aptamer which is able to bind to the target changing the surface plasmon resonance character. A positive response curve is correlated to the increase of mass on the gold surface indicating presence of the target. Figure adapted and modified from (7).

1.4.4 Aptazyme Sensors

Natural aptazymes, namely riboswitches, function in the cell as ligand-mediated gene regulation modules therefore the use of synthetic aptazymes for the sensing of small molecule ligands *in vivo* can be used. A readable detection of an output signal can be produced either via the suppression or production of a gene product due to riboswitch-mediated gene regulation; typically, the reporter gene used is for one that encodes for GFP (168) or β -galactosidase (169) resulting in a concentration-dependent fluorescent or colorimetric signal respectively. The aptazyme module is inserted proximal to the reporter gene on the genetic level and when the target molecule for the aptameric moiety of the aptazyme is present, it folds into either a transcriptionally permissible or non-permissible state which results in a turn-on or turn-off signal (170).

Aptazymes can make use of the detection methods used by many *in vitro* nucleic acid enzyme sensors but with greater control conferred by allostery. The most prominent method of *in vitro* detection utilizing aptazymes is a fluorescent method. Target mediated activation of the ribozyme moiety occurs through the binding of the target to the aptameric portion. Signal occurs due to the presence of exogenous nucleic acid molecular beacon-like substrates and allows for separation of the fluorophore-quencher pair, resulting in increased fluorescence (171). Due to the modular nature of aptazymes, rational design of aptazyme sensors is possible.

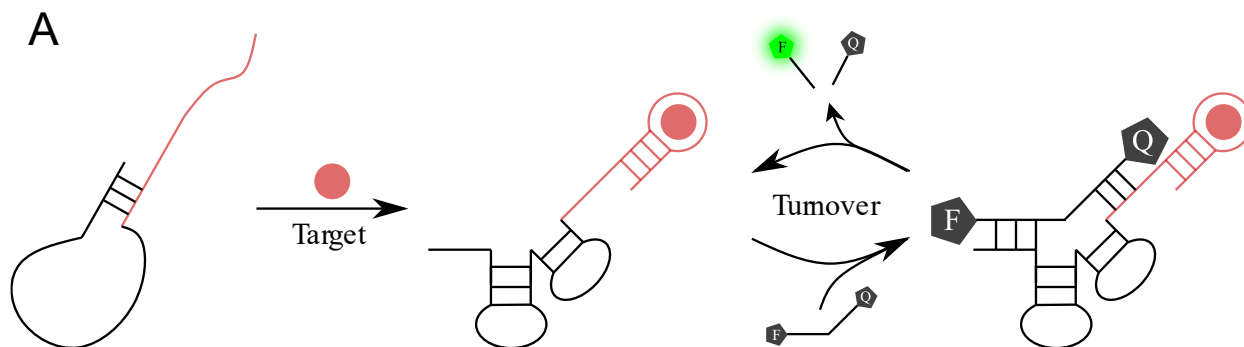


Figure 1.11 | Fluorescent aptazyme sensor

A schematic of a rationally designed aptazyme sensor is shown above (148). The fusion of an aptamer (red) and DNAzyme (black) results in a hairpin structure that does not allow for the DNAzyme activity in the absence of the aptamer target. Aptamer-target induced structural change results in a permissive state for DNAzyme activity. Cleavage of the DNAzyme target results in fluorescence due to the quencher-fluorophore separation of the labeled oligonucleotide target. Figure adapted and modified from (148).

A colorimetric detection method is also suitable for use in the *in vitro* detection of target molecules by leveraging the aggregation dependent color change of gold nanoparticles. When the aptamer moiety is not bound, the DNAzyme is not able to act and the solution remains blue due to the proximity of the gold nanoparticles (172). Once the aptamer binds to its cognate partner, the DNAzyme is activated and cleaves the substrate strand which releases the crosslinked gold nanoparticles, leaving the solution red. A DNAzyme and aptamer are conjugated onto gold nanoparticles which are held together with a strand of DNA that is complementary to portions of both the other sequences and acts as a substrate for the DNAzyme in a three-way junction. Another interesting method involves visible aggregates of aptazyme-tethered gold nanoparticles (173). The aggregates are formed when the aptazyme self-cleaves in the presence of the ligand.

1.5 Aptamers as Diagnostic Tools

Many of the sensing methods that utilize fluorescence as a detection method mentioned thus far utilize labeled nucleic acid strands, typically with a fluorophore and quencher conjugated to the ends of sequences. A change in fluorescence response due to the presence of the target molecule is due to a change in distance between the fluorophore and quencher. Although these labeled fluorescent sensors have their uses, label-free fluorescent sensors may be desired due to their advantages (174). The conjugation of the fluorescent reporters may interfere with the binding of the target molecule, the cost of conjugation of a fluorophore and quencher can be avoided, and background fluorescence of unquenched fluorophore can be reduced. In a label-free approach, a fluorogenic dye molecule can be used in a displacement assay where the dye exhibits sequence independent intercalation resulting in fluorescence enhancement – target-induced conformational change displaces the dye molecule, resulting in a turn-off signal (175).

A more interesting label-free approach takes advantage of the target-induced self-assembly of cleaved aptamer fragments to reconstitute the active site (176–178); a cleaved sequence of the light-up aptamer is fused to an aptamer for the target of interest. Briefly, the sequence for the aptamer of the fluorogenic dye is split into fragments and joined to an aptamer for the target of interest via a linker region. The light-up moiety is in a non-permissive binding configuration until the target of interest binds its aptamer, after which, the tertiary structure of the dye-aptamer complex is allowed to form due to proper strand orientation. The cleavage and target-mediated reconstitution of functional nucleic acid active sites is well documented, especially for well-studied aptamers such as the ATP (179), thrombin (180) and cocaine (181) aptamers. Self-assembly of bifurcated light-up aptamers for the Spinach (182), Broccoli (183) and malachite green RNA

aptamers (184) have shown a decrease in fluorescence, however the limit of detection remains relatively similar due to low background fluorescence (185).

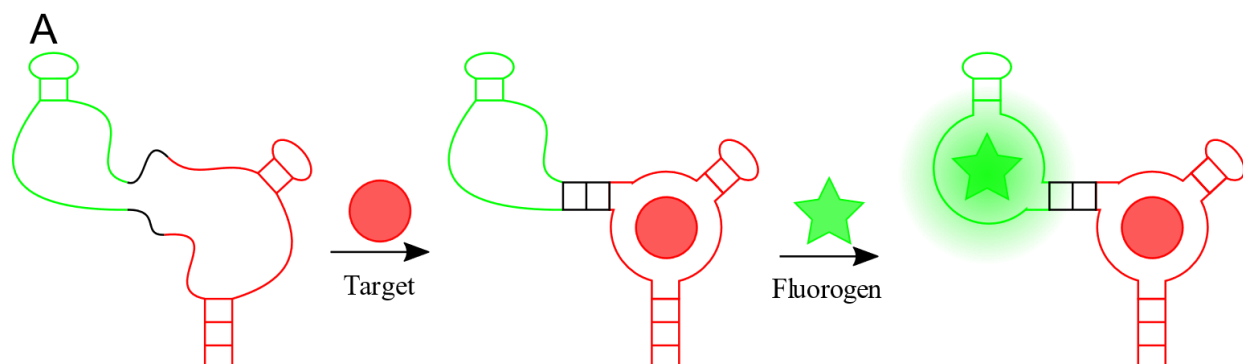


Figure 1.12 | Modular aptasensor

A schematic of a typical modular fluorescent aptasensor is shown above. Two aptamer that have been selected separately are fused with a linker region, shown in black. One aptamer is used to provide the specificity for the sensor, shown in red, and the other is to act as the reporter, shown in green. The fused aptasensor is in a non-permissive conformation for the reporter when the target ligand for the red aptamer is absent. When the red aptamer binds its target, the red circle, a target induced conformational change occurs in which the reporter aptamer is now able to bind the fluorogen, green star, to produce a fluorescent signal. The intensity of the fluorescent signal is proportional to the concentration of the red target. Figure adapted and modified from (184).

Research Objective

Aptamers are an interesting tool for use in biosensors as they allow for the development of modular sensors. The flexibility of DNA to be chemically modified during synthesis allows for DNA aptamers to be used in a plethora of sensing platforms. Typical biosensors contain a biological ligand-binding domain, which provides selectivity to the sensor, and a reporter domain, which provides sensitivity to the sensor, both of which can be fulfilled with an aptamer. Since aptamers are able to bind to a large range of targets, the rapid development of many different ligand binding domains with only a few rounds of SELEX can be achieved. A class of aptamers that are used as reporters in sensing applications are RNA aptamers that bind to fluorogenic dyes, thus producing a strong fluorescence response. One such aptamer is the RNA Mango I aptamer which,

upon binding to its cognate dye target, TO1-biotin, provides a bright light-up module with high affinity. RNA, however, is prone to degradation due to RNase, and thus is not well suited for use in biosensors. Therefore TO1-biotin is an appealing target for the selection of a DNA aptamer that can be used in biosensing applications. To this end, the research focused herein is to select for a tight binding and bright DNA aptamer for the TO1-biotin dye. This will be achieved with the assistance of next-generation sequencing to elucidate the convergence of the sequence library onto tight binding sequence families that emerge after several rounds of selection. The prime candidate will then be characterized to identify key structural information that can help guide rational sequence minimization. Preliminary work on a biosensor for the detection of immunoglobulin E (IgE) using a fluorescent module can then be initiated. Several detection methods are then assessed for their abilities to detect isolated IgE as well as IgE in human serum. The use of a selected DNA aptamer for TO1-biotin as a fluorescent module of a novel IgE biosensor provides an interesting endeavor moving forward.

2.1 Introduction

The use of fluorescence in biosensing allows for higher sensitivity as well as a larger dynamic range than traditional colorimetric detection methods (186). Nucleic acids do not have a functional group that allows for native fluorescence, unlike the proteinaceous green fluorescent protein, however, there have been RNA aptamers that bind to fluorogenic molecules which causes enhancement in fluorescence (187). These light-up RNA aptamers have historically been used to monitor RNA sequences *in vivo*, however their use in biosensing is limited by the fact that RNA molecules are sensitive to degradation due to the ubiquity of RNases (72).

Although the first light-up RNA aptamer discovered could be credited to the malachite green aptamer (74), the most widely used light-up aptamer for *in vivo* studies is the Spinach:DFHBI system (75). Of the fluorescence enhancing RNA aptamers, the Mango:TO1-biotin system has the highest affinity with a correspondingly high fluorescence enhancement (90). When compared to the Spinach:DFHBI system, Mango has two orders of magnitude higher affinity to its cognate ligand with only a 35% reduction in fluorescence enhancement. These traits make the selection of a DNA aptamer for TO1-biotin an attractive target for use in sensing applications.

The use of DNA in a biosensor is appealing due to the high stability of DNA as a biopolymer, however the selection and characterization of fluorescent DNA aptamers thus far have been limited (178, 188). In recent years, there have been some examples of DNA aptamer selection for previous fluorogenic dye binding RNA aptamers (107–109); however there has yet to be a DNA aptamer that binds TO1-biotin.

The SELEX process can be seen as a black box as, traditionally, only a handful of sequences of the final round of selection is analyzed (189). With the use of NGS, both the likelihood of losing a key aptamer candidate due to sampling is dramatically decreased and the population dynamics of the sequence space can be explored (190). Since the sequence data does not fit into typical bioinformatics pipelines, several niche algorithms are developed to analyze the tens of millions of sequences produced from the sequencer to give a few key lead candidates (191).

In this study, we selected a ssDNA aptamer for TO1-biotin with streptavidin magnetic beads and wash conditions similar to those used in the selection of the ssRNA aptamer. Generation of the ssDNA library was adapted from studies for the selection for malachite green and crystal violet. NGS was used to identify key aptamer candidates which resulted in the discovery of a sequence with a sub-micromolar dissociation constant and good fluorescence enhancement. With the selection of a DNA TO1-biotin aptamer, a bright modular DNA based aptasensor can be made.

2.2 Results and Discussion

2.2.1 Selection Scheme

The method of *in vitro* selection involves a directed evolution-type approach to change the composition of a large and diverse oligonucleotide library to converge onto sequences that perform a desired function. In this work, the desired function was the binding of a DNA aptamer to a thiazole orange derivative, TO1-biotin, causing fluorescence due to the aptamer-bound complex for use in biosensing applications. In order to achieve this, the selection scheme used for the selection of the RNA aptamer for TO1-biotin and two DNA aptamers for light-up dye molecules were combined to generate suitable selection conditions as seen in Figure 2.1 below.

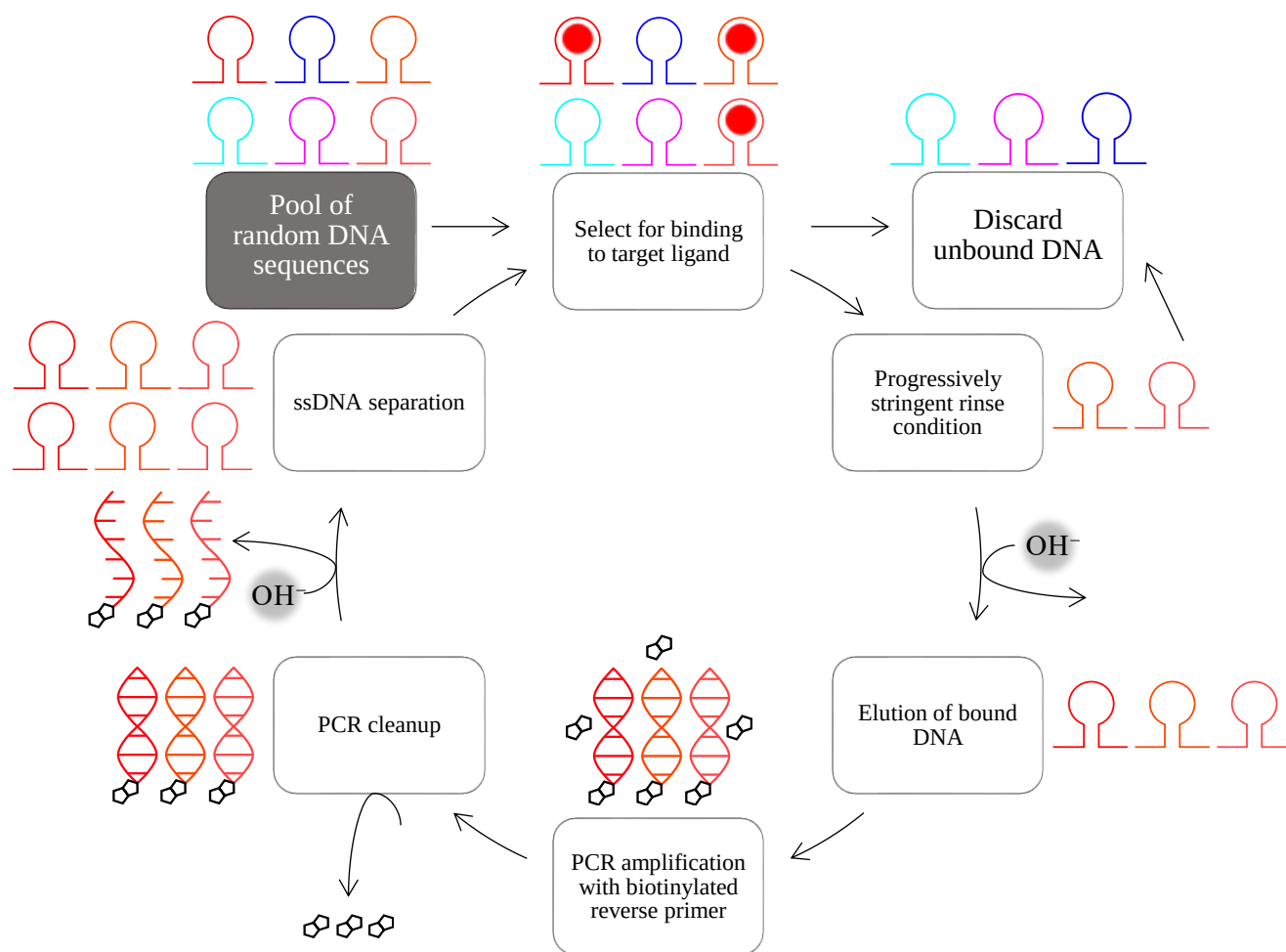


Figure 2.1 | Selection Scheme

Schematic of the selection protocol used for the selection of a DNA aptamer for TO1-biotin. The initial library is screened for binding to the target ligand bound to streptavidin magnetic beads. Non-binding and weakly bound sequences are washed off with increasingly stringent wash conditions as the selection progresses. Bound sequences are removed with potassium hydroxide and are amplified with biotinylated reverse primer. Excess labeled primer is removed via PCR clean up and the double stranded amplicon is captured onto streptavidin magnetic beads. The single stranded forward strand is released with potassium hydroxide for use in the next round of selection.

The length of the sequence and diversity of the library was designed using the published selection of the RNA Mango aptamer (77) which was 88 nucleotides in length at a diversity on the order of 10^{13} . Selection conditions were adapted from the protocol originally used for the selection of the RNA aptamer, however changes to the selection scheme were needed to accommodate

selection using DNA instead of RNA. For the RNA Mango aptamer, the selection was done against the biotinylated target bound to streptavidin coated magnetic beads, washed with various conditions that include being challenged with 12 μM target, elution of bound RNA using base, RT-PCR of the eluent followed by *in vitro* transcription to produce ssRNA. Selection progress was monitored by measuring binding affinity using the radiolabeled RNA.

Since the RT-PCR followed by transcription steps were not needed, the library generation steps was modeled on the selection of the published DNA aptamers for malachite green (107) and crystal violet (108). This protocol was selected since both DNA aptamers had similar sequence length and diversity to the RNA Mango aptamer, being at 78 nucleotide length and a diversity on the order of 10^{14} , and they were selected to bind dye molecules to be used as light-up probes which was the objective of this selection. The protocols differ between the RNA and DNA selections in various way, mainly in the production of the single stranded library from the eluant of selection. In the DNA aptamer selections, the eluent was amplified using a biotinylated reverse primer and the amplicons were bound to streptavidin-agarose beads and the ssDNA library was produced by denaturing the double strand using base, leaving the bound reverse strand on the column. The wash steps and elution were also different for the DNA selection in that there was no change in stringency for the DNA selection from the initial wash conditions and competitive elution was done using 100 μM target. This may have resulted in the generation of an aptamer with lower affinity than otherwise desired. Selection progress was monitored by gel electrophoresis of the PCR products of the wash and elution fractions; later rounds of selection have a weaker band for the wash steps compared to the elution steps.

In this study, the initial 88 nucleotide library consists of a theoretical sequence diversity on the order of 10^{14} unique sequences with a central region containing 54 random nucleotides flanked

by 17 nucleotides of known sequences on the 5' and 3' ends for use in PCR. Selection starts with the formation of fluorophore-conjugated magnetic beads that are used as the solid support for aptamer selection. The DNA library is denatured at 90°C and then applied to the derivatized beads. Washes are performed at various conditions, shown in Table 2.2, that increase in stringency as the selection progresses. The stringency is controlled in both the binding and wash steps by incubation time and in the wash step by dilution of the magnetic beads, ionic strength of the buffer, and presence of the free dye. Anti-selection was performed on the first 5 rounds of selection by first incubating the library with the unmodified streptavidin beads and collecting the flowthrough for selection on the derivatized beads. The bound DNA is eluted using base then amplified using a biotinylated reverse primer. After PCR cleanup, the dsDNA is captured with streptavidin magnetic beads and ssDNA is eluted to yield the library for the next round of selection. A sample from the library of each round of selection was incubated with TO1-biotin and the fluorescence was measured to monitor the progression of the selection.

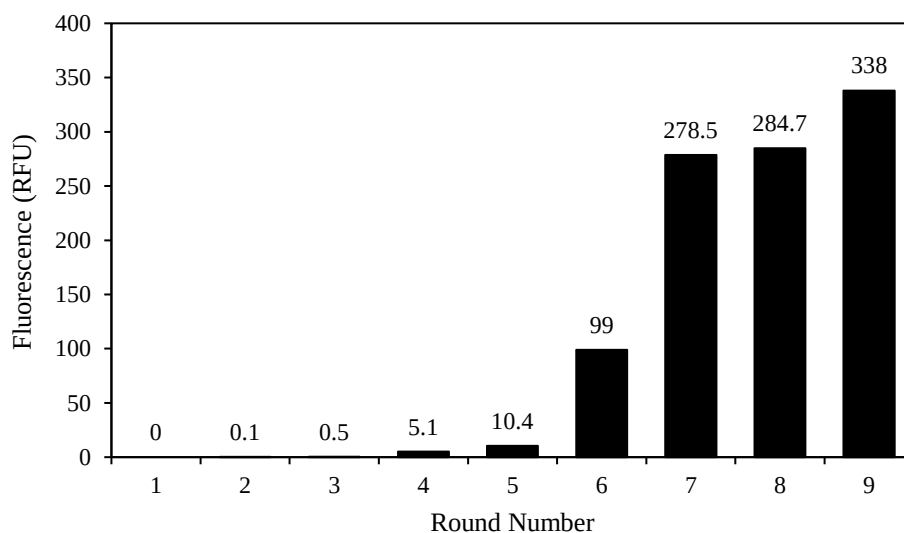


Figure 2.2 | Progression of selection

After each round of selection, the fluorescence enhancement of the library is measured to monitor the progression of selection. There is an increase in fluorescence of TO1-biotin with the incubation of the library after each round of selection with larger increases after some rounds more than others. Before and after the first round of selection, there is no measurable signal of fluorescence, however significant increases of enhancement are observed after stringency is increased on rounds 5, 7, 8 and 9. When competing ligand is added to the wash solution on round 7, a large increase in fluorescence is observed. Longer incubation time of the wash solution only resulted in a nominal increase in fluorescence on round 9, so further selection was stopped, and the library was sequenced.

Each step in the selection scheme resulted in an increase in fluorescence enhancement of the library, resulting in an apparent 3000-fold increase in fluorescence enhancement from the initial library as can be seen in Figure 2.2. The increase in fluorescence enhancement was slowest during the initial 4 rounds of selection when the wash only used the binding buffer, however this may reduce the risk of losing tight binders from sampling. The next 2 rounds resulted in a modest increase due to the dilution of the sample. This increases stringency by making the binding events more rare due to the decreased rate of collision of the DNA and derivatized beads. The 7th round of selection introduces free dye in the wash buffer which resulted in a large increase in fluorescence enhancement. This increase in stringency is due to the competitive binding of the free dye to

weakly bound DNA on the beads; this competitive binding promotes the convergence of the sequence space onto aptamer candidates that have a slower off rate. The next round of selection includes the decrease in ionic strength of the wash buffer which does not have much of an effect on the perceived fluorescence enhancement. The stringency is increased due to the reduction of the stabilizing ions in solution. The final round of selection was done with a longer incubation period with the wash buffer which resulted in a slight increase in fluorescence enhancement. The stringency is increased due to the increased time allowed for the competing free dye to sequester weakly bound DNA from the beads.

The rate of increase of fluorescence enhancement when using only the initial wash conditions was slow and would have required many more rounds of selection to reach a comparable level of enhancement. The very high affinity (3.2 nM) of the RNA TO1-biotin aptamer (77) can be partially attributed to the even more stringent conditions in the 12 rounds of selection. Conditions such as lower binding time, higher wash times and elevated temperatures were used to drive convergence onto fast on-rate, slow off-rate binders. With the selection of the DNA aptamers for malachite green (107) and crystal violet (108), more mild selection conditions can result in lower affinity aptamers; the 8 rounds and 11 rounds of selection resulted in micromolar to sub-micromolar affinity (2.43 μ M and 0.49 μ M respectively) with the same wash conditions every round. It may have been more expedient for the selection process to introduce more stringent selection conditions earlier to reduce the overall number of rounds for selection. Although the number of rounds is comparable to those used in similar studies, the use of NGS would be able to detect low copy number species that have been enriched in a more aggressively stringent wash schedule.

In total, 9 rounds of selection were performed and a sample from the final round, as well as the 4th round was sequenced using NGS to monitor the change in library composition and to monitor the convergence of the sequence space that allowed for fluorescence enhancement. The final clusters of the libraries can then be synthesized and tested as lead aptamer candidates for TO1-biotin fluorescence enhancement.

2.2.2 Next-generation Sequencing

Selection of aptamer candidates is traditionally done by cloning of the final library into a vector and choosing a few colonies for sequencing via the Sanger method (192), typically in the range of 20 – 25 sequences from the final round (109). Overrepresentation of sequences within the population due to enrichment will result in a higher likelihood of being chosen to be sequenced therefore the sequence of interest must dominate the sequence space. NGS lowers the chance that the aptamer is lost from random sampling due to the higher sequence depth inherent to the method which can sample on the order of $10^6 - 10^8$ sequences in the library (193). Sequence diversity can be probed at a much higher resolution and a more accurate picture of the sequence distribution can be established. NGS is originally developed for whole-genome sequencing and the workflow that has been developed for such applications are not suitable for use for analysis of the oligonucleotide pool obtained via SELEX therefore a different workflow must be adapted. To this end, several computational tools have been developed to help bench scientists handle the workflow needed for the analysis such as FASTaptamer (194), AptaCluster (195) and APTANI (196).

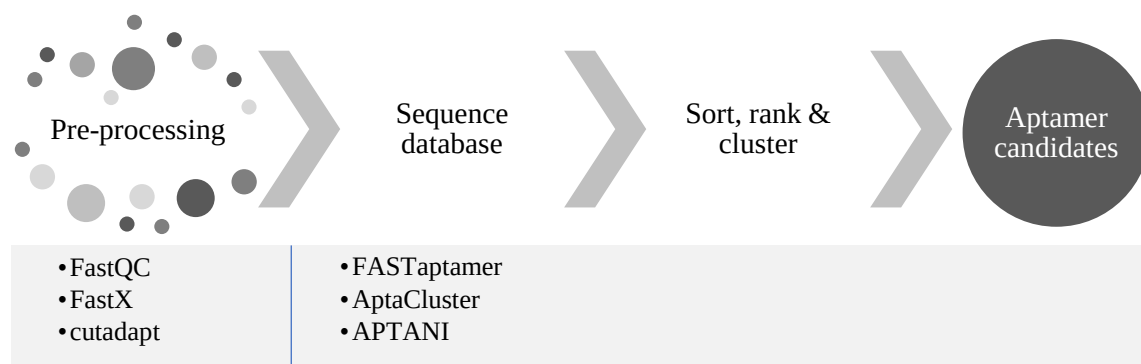


Figure 2.3 | NGS Workflow for SELEX

The general workflow for aptamer bioinformatics is shown above. FastQC is used to monitor the quality of reads, whilst FastX and cutadapt are used to manipulate the sequences to remove bad quality reads, Illumina adapter sequences and the primer sequences used for PCR amplification. After the raw sequence data has been pre-processed, downstream analysis tools such as FASTaptamer, AptaCluster or APTANI can sort, rank and cluster the database. High ranking clusters can be further analyzed as lead aptamer candidates.

FASTaptamer performs the tasks of counting, normalizing, ranking and sorting the abundance of each unique sequence in a population (194). Further analysis can be done by clustering sequences into sequence families based on Levenshtein edit distance, comparing sequence distributions for two populations, calculating round over round enrichment for each sequence in a population, and searching for user-defined nucleotide sequence motifs. Along with FASTaptamer, AptaCluster and APTANI are able to analyze pre-processed NGS data from SELEX but do so using different algorithms. AptaCluster groups and compares shorter segments of the sequence, deemed *k*-mers, allowing for faster computation compared to the full length pairwise comparison done with FASTaptamer (195); enrichment discovered this way can give insight into emerging motifs in the primary sequence. APTANI takes it a step further by predicting the secondary structure of each sequence and then grouping based on secondary structure motif (196). Although both AptaCluster and APTANI are able to process very large datasets, on the

order of tens of millions of sequences, at a much faster rate than FASTAptamer (197), the use of FASTAptamer is more widespread and established as can be seen by the published literature.

To prepare the sample for NGS, PCR amplification of the ssDNA library and PCR cleanup was done before being sent to the McMaster Genome Facility for sequencing on an Illumina MiSeq system for samples of round 4 and 9. Sequencing of the samples returned with 2.6×10^5 and 2.4×10^5 reads for round 4 and 9, respectively. Pre-processing was done using both the FastX-toolkit and cutadapt while the FASTAptamer-toolkit was used to analyze the resulting data and to cluster the unique reads into families of related sequences. Using this approach, round 4 had 1.2×10^5 unique reads, whereas round 9 had 8.5×10^3 unique reads, suggesting a convergence of the sequence space due to the selection conditions.

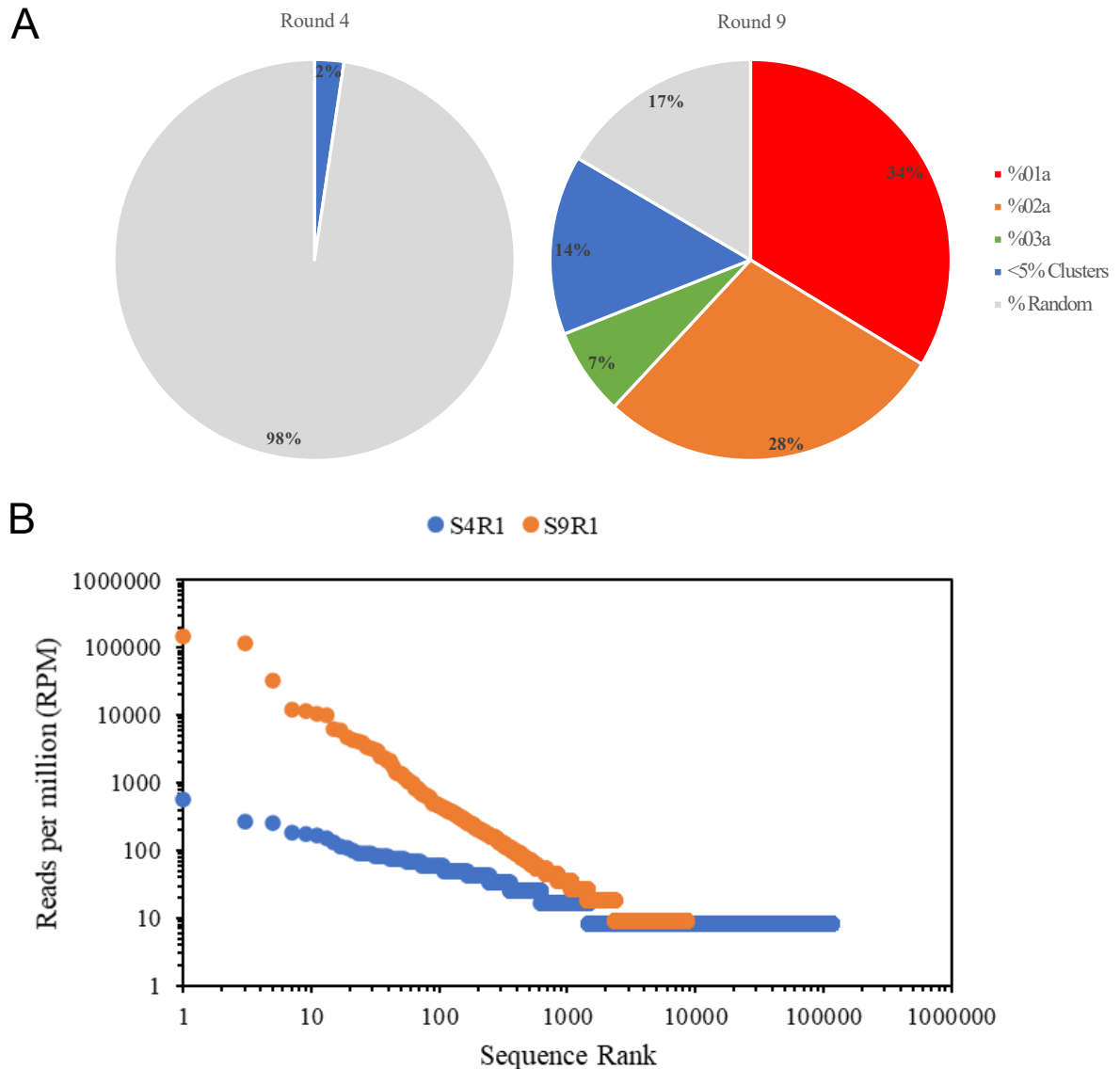


Figure 2.4 | Convergence of sequence space

The results of sequence analysis for the samples from round 4 and round 9 are shown above. The distribution of sequences that have been clustered using FASTAptamer **A** shows that at round 4, the sequence space is very diverse. There are only 2% of sequences that are able to be clustered into 5 families that each make up less than 5% of the total sequence space (blue) whereas most of the sequences, 98%, remains very distinct (grey) and not able to be grouped based on the clustering criteria. By round 9, several families of sequences start to dominate the sequence space with 01a (red), 02a (orange) and 03a (green) making up 69% of all reads, whilst an additional 14% of sequences can be clustered into 24 other families. The convergence of the sequence space can be seen when the normalized reads is plotted against each unique ranked sequence **B**. When compared to the later selection round, there is an order of magnitude larger diversity of unique sequences, but much lower enrichment of even the most abundant sequence. This change in population indicates a convergence of the sequence space and an enrichment of aptamer candidates.

Using the count function from FASTAptamer, the abundance of each unique sequence was enumerated and is visualized in Figure 2.4 **A**, above. The number of unique sequences that meet the 10 reads per million (RPM) threshold in round 9 was an order of magnitude less than round 4. The highest ranked unique sequence is two orders of magnitudes more abundant than from round 4. The change in profile of the two libraries shows a reduction of diversity and round over round enrichment of sequences demonstrating a successful selection scheme.

The cluster function shows that the selection conditions up to round 4 were not enough to have the sequences converge onto any clear families as can be seen in Figure 2.4 **B**. Of the sequences that were clustered based on edit distance, 98% of sequences were too diverse to form into families; however, there were 5 families of sequences that could be detected making up about 2% of all reads in the sample. The 9th round of selection resulted in three distinct families (1a, 2a and 3a) that dominate the sequence space, making up 69% of all sequences in the sample with an additional 14% of sequences forming 24 additional families for a total of 27 families forming 86% of the sequence space. This convergence of sequences from round 4 to round 9 shows that the more stringent conditions in the later rounds of selection were needed to drive the convergence of the population for traditional Sanger sequencing to work. However, of the 5 families present in round 4, 4 families persisted to round 9 (1a, 2a, 3a and 6a) with the dominant 3 sequences of round 9 represented in the round 4 clusters. Studies that use NGS to monitor the progression of SELEX show that small changes in the selection condition can result in a larger, usually unseen, change in the population (61). Without the use of NGS, dominant sequence families can be underrepresented due to sampling and some high binding sequences may be lost (193).

From the analyzed NGS SELEX data, various aptamer candidates of interest were synthesized for further testing for fluorescence enhancement including the top 4 families of round 9 (1a, 2a, 3a and 4a), which represent 71% of all reads in round 9, the next two most enriched families from round 4 (5b and 6b) to 9 and the two most abundant sequences in round 4 (7c and 8c).

2.2.3 Fluorescence Enhancement

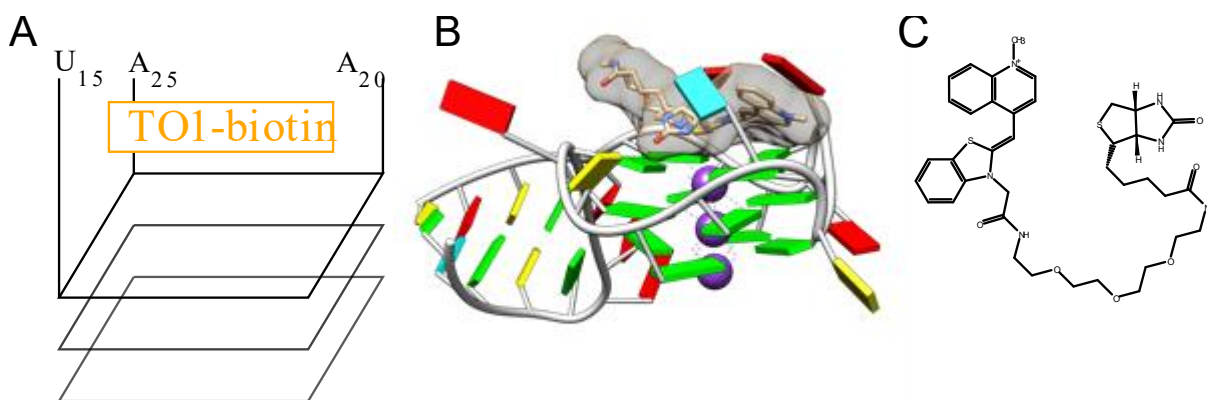


Figure 2.5 | RNA Mango bound to TO1-biotin

A schematic of the binding pocket of the RNA Mango aptamer bound to TO1-biotin is shown in **A** generated using Inkscape. The TO1-biotin dye binds to one face of the three-tiered G-quadruplex and is held in place with three loop nucleotides as can be seen in the crystal structure (83). **B** (PDB: 5V3F) features the crystal structure of the RNA Mango aptamer bound to TO1-biotin generated using UCSF Chimera. When bound, the dye adapts a ring-like conformation shown in **C** with the two adenines interacting with the two heterocycles and one uracil interacting with the biotin.

The target dye molecule used, TO1-biotin, is a thiazole orange derivative that was developed by the Unrau group for use in RNA tracking *in vivo* (77). The crystal structure of the RNA aptamer-dye complex shows that the dye binding pocket contains one face of a three-tiered G-quadruplex and three loop nucleotides; two adenines and a uracil hold the dye in place with the adenines interacting directly with the two heterocycles of the dye and the uracil interacting with

the biotin (83) shown in Figure 2.5. There are two excitation peaks centered at 260 nm and 510 nm with one emission peak centered at 535 nm for the RNA TO1-biotin aptamer. In order to test for fluorescence enhancement in the aptamer candidates selected in this work, similar conditions are used to test for fluorescence.

Fluorescence data was gathered using SpectraMax M5. Fluorescent titrations were performed on the aptamer candidates in the range 78.1 – 5000 nM DNA with the TO1-biotin dye at a concentration of 50 nM in PBS. Samples were heat denatured and annealed at room temperature before incubation with TO1-biotin. A wavescan of the complex at saturating conditions was obtained with two excitation peaks, one centered at 260 nm and one at 510 nm, and one emission peak centered at 535 nm as seen in Figure 2.6 **B**. For the fluorescence enhancement assays, the excitation wavelength of 488 nm was chosen to reduce noise due to overlap. The data was fitted to the quadratic form of a one-site binding curve using generalized reduced gradient non-linear regression in Microsoft Excel.

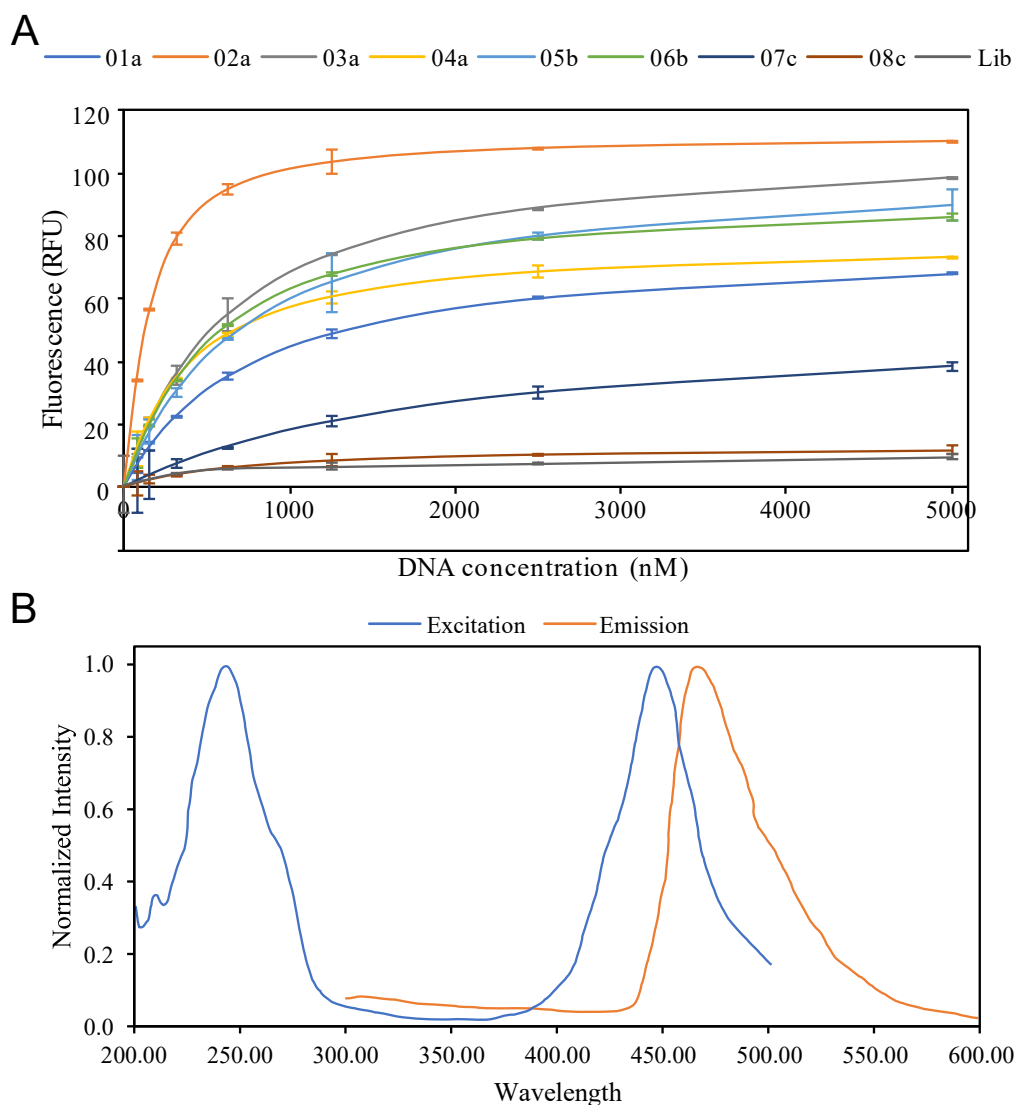


Figure 2.6 | Fluorescence enhancement of aptamer candidates

A shows fluorescent titration curves of a selection of aptamer candidates obtained from NGS. The 02a candidate has the highest fluorescence enhancement and the highest affinity for the TO1-biotin fluorogen. The 09c candidate has the lowest fluorescence enhancement and affinity with only a nominal increase from the initial selection library. **B** shows a normalized wavescan of the excitation and emission wavescans of the bound TO1-biotin. There are two excitation maxima located at 260 nm and 510 nm which both cause an emission with a maximum peak at 535 nm.

Below, Table 2.1, shows the aptamer candidate sequences tested for fluorescence enhancement signal with their normalized read frequency obtained from NGS, and binding affinity. The sequences 01a – 04a are the four most abundant clustered sequences from round 9

which make up 71% of the total sequence space. The sequences 05b and 06b are the families that have been significantly enriched from round 4, albeit less so than the initial four sequences. The final two sequences, 07c and 08c, are the most abundant sequences from round 4. The sequences with the highest affinities were 02a, 04a and 06b which were the 2nd, 4th and 13th most populated families respectively. The sequences with the highest fluorescence enhancement were 02a, 03a and 05b which were the 2nd, 3rd and 7th most populated families respectively. Surprisingly, the 01a candidate that has the highest read count, making up 34% of all round 9 reads, had a middling affinity at 711.0 nM and low fluorescence enhancement 78.0 RFU compared to the rest of the sequences tested. The sequence also contains a deletion resulting in a sequence length of 53 instead of 54 nucleotides compared to the other families. This suggests that the enrichment of the 01a sequence family during selection may be due to other factors such as PCR bias. The 2a candidate showed the highest affinity and fluorescence enhancement at 98.6 nM and 112.6 RFU, respectively and made up 28% of the reads in round 9. The enrichment of a sequence with good affinity and fluorescence enhancement indicating that the selection was successful.

Table 2.1 | Sequences and affinities from NGS

ID	Sequence	RPM	K _D (nM)	FE
01a	GGACTGGGGTGTGATGGGGTGGGGGGCAGTCTGCTGTTTGTATCAGCCTCTG	337035.4	711.0	56.1
02a	TATGTGGAGGGCAAGAGGGTGTGATGGGGTCGGTGGGTCCTGCCCTCTCATCG	282115.3	98.7	136.5
03a	TGATGACTTATCCGTGCGGGGTGTGATGGGGTGGATGGGCGCGTAGGGAAGTC	70650.0	706.8	107.3
04a	TCCCTGGAGAAGTTCGTGGGGTGTGATAGGGTGGGGGGCAGAACTTCCCGGCT	26081.9	351.9	77.6
05b	GACCGGCGAGGGACTGGGTGTGATGGGGTGGAGGGAGTACCGACGCCGCGGTG	14437.1	667.0	114.7
06b	CTAGTGTCATCTCCTGAGTGGGGGTGTGATGGGGTGGCGGGAATACCGATGGA	6123.2	425.5	97.3
07c	CACCGTCGAGGACCGTATAACCCCCACAGACGCGAGGGAGGGGTGAGGTGCTTG	1055.9	1910.0	45.4
08c	GACCGGCGAAGCTACTCACTGCGGGCAGGCGGTCTCGTTCGGCACCAACTCCG	352.0	721.9	14.4

Reads per million (RPM) is the normalized reads obtained from the 9th round of selection. The K_D was measured based on the fluorescence response curve when incubated with the TO1-biotin dye. Fluorescence enhancement (FE) was calculated by dividing the maximum fluorescence response by the signal obtained from the free dye.

The Pei group did several selections of DNA aptamers for fluorogenic dye molecules including malachite green (107), dimethylindole red (109) and crystal violet (108). The published K_D range from 2.43 – 0.43 μ M and fluorescence enhancement range from 8.17 – 140-fold after 8 – 14 rounds of selection. The fluorescence enhancement in these studies were calculated by dividing the maximal fluorescence in saturating conditions with the fluorescence of the unbound dye. In this work, the candidate 02a has a K_D of 98.7 nM and a fluorescence enhancement of 136.49 after 9 rounds of selection. However, when compared to the RNA Mango aptamer (77), the published K_D of 3.2 nM and fluorescence enhancement of 1100-fold leaves much to be desired. The much higher fluorescence enhancement can be attributed by the difference in method of calculating the value; fluorescence enhancement was calculated by comparing the quantum yield of the free and bound dye as opposed to comparing the fluorescence outputs. The reduction in affinity can be attributed to the harsher selection process employed with increased temperature, lower binding time and longer wash steps. Certainly the reduction in fluorescence enhancement and affinity is not isolated to this study alone, as the published RNA malachite green aptamer (198) also vastly outperforms the DNA counterpart. It is thought that RNA aptamers may have access to

more complex structures (199), however DNA is preferentially used in selections today due to the advantages it confers (200). By all measures the selection was successful in producing a high affinity and bright aptamer that can be further optimized.

2.3 Materials and Methods

2.3.1 Chemicals

The DNA library used for in vitro selection was custom synthesized from Eurofins (Huntsville, AL). The primers P1, P2 and P2 – biotin were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The sequences as used are listed in Table 2.3. The streptavidin magnetic beads were used to capture the target, TO1-biotin, and to separate the biotinylated reverse strand was manufactured by Thermo Fisher Scientific (Waltham, MA) and purchased from VWR (Radnor, PA). PCR was done using a standard Taq PCR kit from New England Biolabs (Ipswich, MA) and purchased from VWR. The small DNA fragments extraction kit and low molecular weight DNA ladder was from IBI Scientific (Dubuque, IA) was used to clean up the PCR after amplification was purchased from VWR.

2.3.2 In-vitro Selection of DNA Mango

Each round of selection started with washing the aliquot of streptavidin magnetic beads 3 times with buffer A (10 mM sodium phosphate pH 7.2, 140mM KCl, 1 mM MgCl₂, 0.05% Tween-20, 0.1 mg/mL heparin and 1 mg/mL HSA). To yield fluorophore conjugated beads, the rinsed beads were then incubated with a slight excess on TO1-biotin (3 nmol of TO1-biotin per 2 mg of beads) for 30 minutes at 25°C. Binding was confirmed by measuring the reduction in absorbance

of the supernatant after incubation using a Thermo Fisher Nanodrop 3300. Once the beads were fully saturated, the supernatant was removed, and the conjugated beads were washed three times with buffer A (2 minutes per wash). To prepare the library for in vitro selection, the library was heat denatured at 90°C for 2 minutes and cooled to 4°C in MQ to remove any intermolecular hybridization and added into buffer A. The prepared library is then incubated with the fluorophore conjugated beads for 30 minutes at 25°C. After incubation, the supernatant is removed, and the beads are washed 3 times according to wash conditions in Table 2.2.

. Bound DNA was eluted twice with 25 μ L of 50 mM KOH solution and immediately neutralized with buffer B (10 mM sodium phosphate pH 7.2, 140mM KCl and 1 mM $MgCl_2$). Counter selection was done by incubating the prepared library with rinsed streptavidin magnetic beads for 30 minutes at 25°C with the resulting supernatant being used in the positive selection step; counter selection was done on selection rounds 1 through 4. To select for aptamer candidates with high affinity and slow off-rates, the wash buffer was supplemented with 12 μ M TO1-biotin during the wash steps in rounds 5 and higher. In order to increase the stringency of selection, the time allowed for incubation of the DNA library with the conjugated beads was reduced from 30 minutes to 5 minutes and the wash steps incubation was increased from 2 minutes to 10 minutes.

Table 2.2 | Wash conditions on each selection round

Selection round	Wash 1	Wash 2	Wash 3
1	1 min 1 x volume WB buffer +heparin/BSA	1 min 1 x volume WB buffer +heparin/BSA	1 min 1 x volume WB buffer +heparin/BSA
2	“	“	“
3	“	“	“
4	“	“	“
5	1 min 2.5 x volume WB buffer +heparin/HSA	1 min 2.5 x volume WB buffer +heparin/HSA	1 min 2.5 x volume WB buffer +heparin/HSA
6	“	“	“
7	1 min 2.5 x volume WB buffer +heparin/BSA +12 μ M TO1	1 min 2.5 x volume WB buffer +heparin/BSA +12 μ M TO1	1 min 2.5 x volume WB buffer +heparin/BSA +12 μ M TO1
8	1 min 2.5 x volume WB buffer +heparin/BSA +12 μ M TO1	1 min 2.5 x volume 1/10 WB buffer +heparin/BSA +12 μ M TO1	1 min 2.5 x volume 1/100 WB buffer +heparin/BSA +12 μ M TO1
9	10 min 2.5 x volume WB buffer +heparin/BSA +12 μ M TO1	10 min 2.5 x volume 1/10 WB buffer +heparin/BSA +12 μ M TO1	10 min 2.5 x volume 1/100 WB buffer +heparin/BSA +12 μ M TO1

2.3.3 Polymerase Chain Reaction

After each round of selection, a portion of the quenched eluted sample is used to determine the optimal cycle number for amplification. 100 μ L PCR mixture is prepared using 2 μ L of the eluted aptamer candidate and aliquoted into 10 μ L portions for cycle testing. The 100 μ L PCR mixture is composed of 1X Standard Taq buffer, 1 mM dNTP, 1 μ M P1, 1 μ M P2-biotin, 1.25 units/50 μ L Taq polymerase. The DNA amplification was achieved using the following thermocycler settings, 95°C for 1 minute, (95°C for 20 seconds, 46°C for 20 seconds, 72°C for 20

seconds), 72°C for 1 minute, hold at 4°C. One 10 µL aliquot is removed from the thermocycler every 3 cycles to determine the optimal cycle number on a 2% agarose gel stained with 1 µg/mL ethidium bromide run at 120 V for 40 minutes. Once the optimal cycle number is determined, a 1 mL PCR mixture is prepared in 100 µL aliquots at the same concentrations and thermocycler settings as previously stated, with the optimal cycle number being employed for amplification.

Table 2.3 | List of DNA sequences used for PCR

Label	Sequence
N-54 Lib	CAG AAT CTC ACA CAG CCN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNG ATG GCT TGC GGG TTC C
P1	CAG AAT CTC ACA CAG CC
P2	GG AAC CCG CAA GCC ATC
P2 - biotin	/5Biosg/ GG AAC CCG CAA GCC ATC

The IBI Scientific small DNA fragment extraction kit was then used to clean up the PCR product. Briefly, binding buffer containing chaotropic salts is added to the PCR product before being loaded onto a silica fiber matrix. A wash buffer containing 75% ethanol is used to precipitate the full-length product whilst simultaneously washing off shorter primer sequences and other contaminants. The cleaned-up PCR product is dissolved and eluted from the matrix using TE buffer.

To obtain ssDNA from the dsDNA PCR product the DNA is captured onto streptavidin magnetic beads using the biotin modification on the complementary strand and eluted using base. Briefly, 2 mg of the streptavidin magnetic beads is washed 3 times with buffer B and incubated with the pooled cleaned up PCR product for 10 minutes. The bound DNA is then eluted twice with

25 μ L of 50 mM KOH to yield a ssDNA library for the next round of selection. The amount of ssDNA is quantified with a Thermo Fisher NanoDrop 2000 using 2 μ L of the pooled library.

2.3.4 Monitoring Selection

In order to monitor the progress of the selection, fluorescence enhancement of the library is measured using a Thermo Fisher NanoDrop 3300 Fluorespectrometer. A 0.5 μ M sample of the library is mixed with 100 μ M sample of TO1-biotin and the 2 μ L of the mixture in buffer B is loaded onto the fluorspectrometer. Using the white light LED source fluorescence emission was measured at 532 nm for each round of selection.

2.3.5 Next-generation Sequencing

To prepare the sample for NGS, round 4 and round 9 was prepared using the same PCR protocol as described in 2.3.3 Polymerase Chain Reaction, but with P2 in place of P2-biotin. Briefly, the optimal cycle number was determined, and amplification of the full-length product was achieved with the optimal cycle number. The PCR product was cleaned up with the IBI Scientific small DNA fragment extraction kit and the fragment length was confirmed via agarose gel electrophoresis. Sequencing is done using the Illumina MiSeq system with a 2% share of a MiSeq V3 2 $\times$ 300 flow cell.

2.3.6 Next-generation Sequencing Data Analysis

Total reads for the round 4 sample was 2.6×10^5 (268,334) and 2.4×10^5 (240,356) for round 9. Pre-processing of the FASTQ sequencing files was done using FASTX-toolkit (201) and cutadapt (202) in the Bioconda (203) channel of Anaconda in the Linux distribution BioLinux 8.

Since the sequencing was done using a shared sequencing scheme, 300 read cycles were performed on the samples. FASTX_trimmer was used to remove low quality read information and cutadapt was used to trim the known TruSeq adapter sequences from the 5' and 3' constant regions; any uncut sequences or sequences not between 85 - 95 length was discarded. FASTAptamer-toolkit (194) was used to count, generate clusters and calculate enrichment of sequences from round 4 to 9. The count function was used on the pre-processed FASTQ file to count, rank and normalize (to RPM) each unique sequence. The sample for round 4 had 115,906 unique sequences with the highest ranked sequence having 1,126.5 RPM whereas the sample for round 9 had 8,506 unique sequences with the highest ranked sequence having 300,071.8 RPM, suggesting a convergence of the sequence space, resulting in enrichment. The cluster function was used to group sequences into families of related sequences using the parameters of Levenshtein edit distance of 7 and elimination filter of at least 20 reads per family. Using these parameters, the round 4 sample had 5 families with the highest ranked family with 1106.2 RPM; the round 9 sample had 27 families with the highest ranked family with 337,035.4 RPM.

2.3.7 Fluorescence Enhancement

From the NGS data analysis, 9 sequences of interest as aptamer candidates were synthesized from IDT for further testing. All fluorescence data was obtained using a SpectraMax M5 Microplate Reader from Molecular Devices LLC. Each lyophilized sample was resuspended in MQ to the concentration of 100 μ M. A serial dilution of the DNA was added to a fixed concentration of TO1-biotin and buffer in a black bottom 96 well plate. Before adding to the well, the DNA was heated to 90°C for 1 min then cooled to 4°C and allowed to incubate with the TO1-biotin in the 96 well plate for 5 minutes before being read. The 200 μ L mixture had a final

concentration of DNA up to 5000 nM, TO1-biotin at 50 nM and PBS-M buffer with 10 mM Na₂PO₄ pH 7.4, 140 mM KCl & 1 mM MgCl₂. A wavescan of the complex was obtained with two excitation peaks, one centered at 260 nm and one at 510 nm, and one emission peak centered at 535 nm. For the fluorescence enhancement assays, the excitation wavelength of 488 nm was chosen to reduce noise due to overlap. The data was plotted on a Scatchard plot and the graphical K_D and B_{max} was used to fit the titration curve to the quadratic form of a one-site binding curve (204) (Equation 1) using generalized reduced gradient non-linear regression in Microsoft Excel.

$$F = \frac{([TO1] + F_{max} + K_D) - \sqrt{([TO1] + F_{max} + K_D)^2 - (4 \times [TO1] \times F_{max})}}{2}$$

Equation 1

2.4 Conclusions

With the help of the NGS analysis, an aptamer with good fluorescence enhancement and affinity was found from the selected group of sequences that were synthesized. The level of enhancement and affinity was similar to those found in literature; however, it does fall short of the RNA TO1-biotin aptamer. The decreased affinity between RNA and DNA aptamers is not atypical, in fact in many cases this is the reported norm due to more diverse and intricate structures available to RNA (205, 206). When compared to other DNA aptamers for fluorogenic molecules (107–109), the affinity and fluorescence enhancement in this work has a higher affinity and fluorescence enhancement.

The library after 4 rounds of selection resulted in very little fluorescence enhancement on the flurospectrogram and lacked convergence of sequence space from the NGS dataset. It is clear

that the read frequency of the 4th round of selection is not a strong predictor for enhancement since the sequence with the highest copy number has one of the worst fluorescence enhancement. However, the clustering of the sequences of the 4th round selection shows the emergence of some of the sequence families that would later dominate the 9th round of selection. The 01a, 02a and 03a families were present in the 4th round albeit composing only 0.29%, 0.45% and 0.38% of the sequences sampled respectively; after the 9th round of selection these sequences make up 34%, 28% and 7.1% respectively. With the use of NGS, the selection could have been stopped after the 4th round of selection and the discovery of the best performing candidate would have been achieved. Certainly, this would not be possible if traditional Sanger sequencing was used since the odds of sampling a sequence in the 02a family is quite small, especially if the sample size would be in the traditional range of 20 – 25.

That being said, having a longer selection scheme can allow sequences to become dominant from other factors besides binding affinity and result in overrepresentation in the sequence space. This can be seen by the fact that the largest populated family of sequences, 01a, is not the family that produces the highest fluorescence enhancement or with the highest affinity. The enrichment of the 01a can be due to factors such as PCR bias or off target binding with a large contributing factor being the shorter sequence length. These kinds of issues can be avoided with the use of shorter selection schemes to reduce PCR bias and harsher selection conditions to cause faster convergence of sequence space.

3.1 Introduction

High throughput sequencing can give a wealth of information on the potential motifs in the primary sequence that have developed due to selection. Emergence of structural regions induced by their sequence-dependent fold enriched by SELEX are assumed to contribute to the binding function of the aptamer (207). Discovery of motifs are typically done using programs such as GLAM (208) or MEME (209). The identification of multiple motifs in the sequence population can indicate divergent binding modality that can indicate the presence of aptamers within the library with differing structures (210).

Structural prediction methods are useful for streamlining the discovery of high affinity aptamers because, typically, structure determines function and as the sequence space converges, highly structured sequences emerge. The major driver that determines the overall folding energy of the three-dimensional structure of nucleic acids is the secondary structure which is dominated by strong interactions such as base pairing and base stacking (211). Typical secondary structure prediction tools like CentroidFold (212), Mfold (213), and RNAfold (214) make use of dynamic programming algorithms to compute a minimum free energy structure. Current computational methods for secondary structure prediction have a good success rate for short oligo aptamers, however the presence of pseudoknots and G-quadruplex motifs in the structure can result in inaccurate results (215).

G-quadruplex motifs are secondary structures that are formed in nucleic acid sequences that are rich in guanine; four guanines can associate via Hoogsteen hydrogen bonds to form planar structures called a G-quartet and two or more quartets can stack to form a quadruplex (216). The structure of the quadruplex can vary based on the differences in number of strands, loop length

and loop orientation that can result in different topologies described as parallel, antiparallel or mixed (217). Stabilization of the quadruplex structure is mediated by monovalent cations, especially K^+ , and changes in the environment can result in structural polymorphism (218). Naturally occurring G-quadruplex formation is typically found in telomeric regions (219), however non-telomeric G-quadruplexes are thought to be involved in gene regulation (220). The quadruplex motif occurs in many aptamers (221–223) with the most studied G-quadruplex containing aptamer being the thrombin binding aptamer (224).

Most approaches to characterize G-quadruplex structures have so far combined *in silico* predictions with *in vitro* biophysical evidence of G4 folding (225). Typically, *in silico* detection of quadruplexes is done by parsing the primary sequence and scoring algorithmically using programs such as QGRS mapper (226), G4Hunter (227) or G4RNA screener (228). Biophysical verification of G-quadruplexes can be done with via nuclear magnetic resonance (NMR) spectroscopy (229), UV melting (230) and circular dichroism spectroscopy (CDS) (231). CDS is notable in that it is a relatively simple method that is able to discriminate the topology of the quadruplex (232).

In this study the characterization of the aptamer candidates obtained from NGS was performed using computational methods to guide further biophysical testing and sequence minimization efforts. Sequence motif and structural prediction software are used to discover sites of interest for downstream testing. The presence of a G-quadruplex in a key aptamer candidate is probed with fluorescence and circular dichroism. Truncations show that the quadruplex is likely involved in the binding of TO1-biotin.

3.2 Results and Discussion

3.2.1 Primary Sequence Analysis

The sequences obtained from NGS were analyzed and grouped into families based on sequence homology as described in Chapter 2. In order to elucidate core binding regions that may indicate differing binding modes between families, the Multiple Expectation maximizations for Motif Elicitation (MEME) tool was used to discover motifs in the primary sequence of the 27 families present in the 9th round of selection as well as the aptamer candidates chosen for further testing. The default search settings were used for all of the sequences with the classic discovery mode, zoops motif distribution with 3 total motifs.

From the MEME analysis, there appears to be one consensus sequence that appears across all, but the last two families clustered from the 9th round of selection. This same motif is present in multiple lead candidates as shown in Figure 3.1 **A**; this demonstrates a convergence onto a single core binding motif based on the primary sequences tested. The motif, shown in Figure 3.1 **B**, is 21 nucleotides in length, is generally present in the middle of the sequence and dominated by many guanine triples; this may suggest the presence of a G-quadruplex in the secondary structure of the aptamer. The motif is highly conserved across all families when aligned using ClustalW, shown in Figure 3.1 **C**. Since thiazole orange has a known higher affinity for G-quadruplexes over duplexes (233), the presence of a G-quadruplex binding motif in this work should not be surprising. In fact, the selected RNA Mango aptamers I – IV all feature a G-quadruplex motif (100) in the solved crystal structures, with the binding pocket featuring the quadruplex motif contacting one face of the dye.

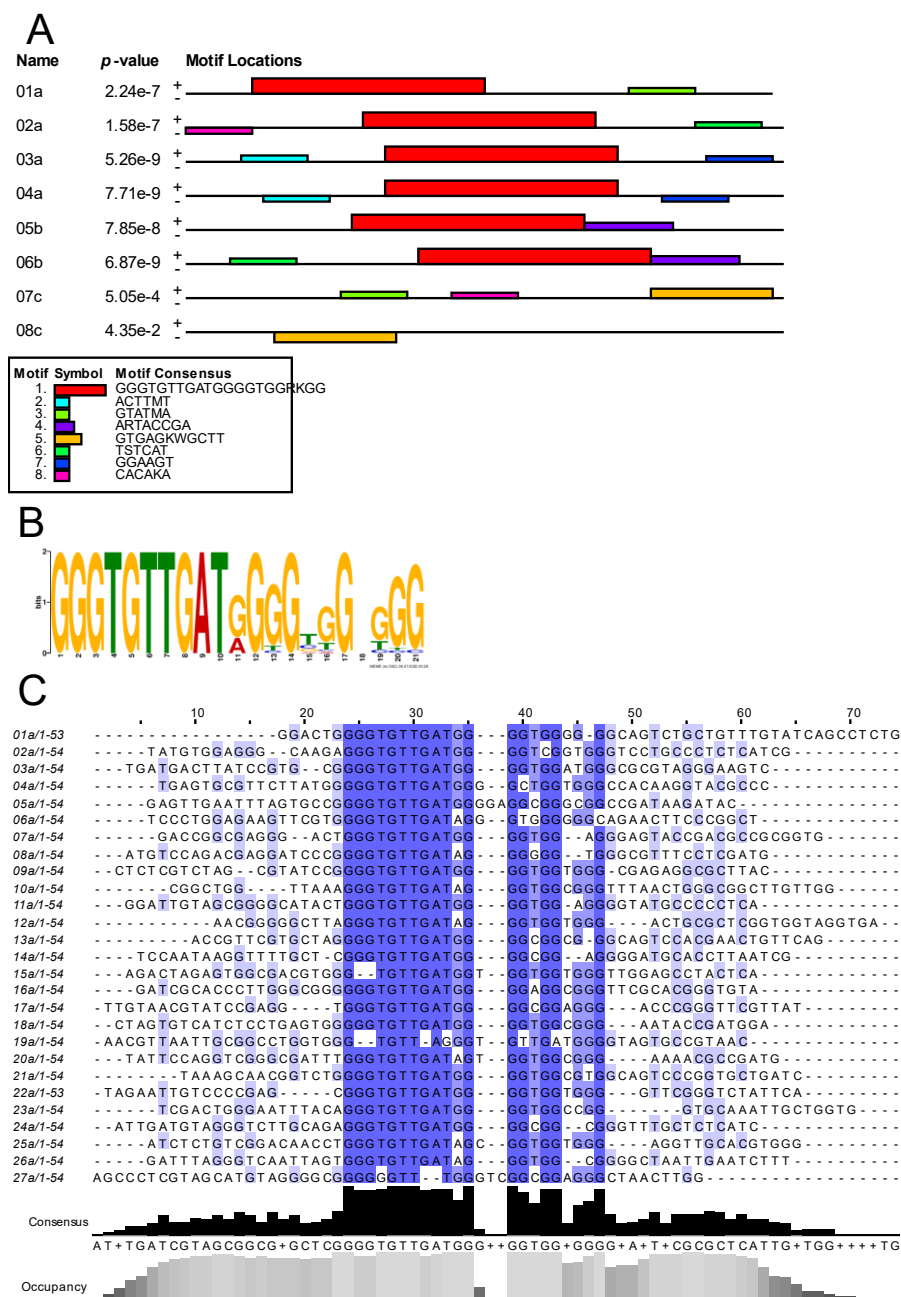


Figure 3.1 | Primary sequence analysis

The output from the MEME analysis is shown in **A**. Motif 1, shown in red, is present in the candidates that exhibit higher fluorescence enhancement and is located near the middle of most of the sequences that it is present in. A closer look at motif 1, shown in **B**, shows that the sequence is dominated with guanines suggesting that the secondary structure may contain a G-quadruplex. Sequence alignment of the families found in the 9th round of selection using ClustalW, shown in **C**, shows a heavy presence of the MEME motif across all families with a few conserved sequences upstream and downstream from the core motif sequence.

3.2.2 Secondary Structure Analysis

Since the binding motif discovered by MEME has high G-content, the presence of a G-quadruplex is likely and the candidates and families from the 9th round of selection are analyzed using the software Quadruplex forming G-Rich Sequences (QGRS) mapper (226). Using the QGRS mapper, information about the composition and distribution of sequences that may have the capacity to form quadruplexes is generated. The resulting output contains information on the location of the start of each possible quadruplex as well as a score on the likelihood to form a stable G-quadruplex, the G-score. The default search options were used with the max length for the quadruplex run is set to 30 nucleotides with loop sizes of length 0 to 36 nucleotides.

The QGRS mapper identified that candidate 02a had the highest G-score of 40 followed by 05b and 01a with a G-score of 38 and 35 respectively as can be seen in Table 3.1. Of the 27 families in round 9, 9 families had a G-score equal to or higher than 01a, however, 02a still had the highest G-score. There appears to be a correlation between the G-score and the affinity for the tested candidates with a higher G-score resulting in a lower K_D . Notably, 02a possesses the highest affinity as well as the highest G-score; this may suggest that the formation of a G-quadruplex structure promotes the binding of the aptamer to the TO1-biotin dye. The number of possible quadruplexes along the full-length sequence does not seem to be a good predictor of affinity, however access to multiple quadruplex folds may make structural determination difficult and require sequence simplification for downstream rational design.

Table 3.1 | QGRS data

Name	Unique G4	Number of loci	Highest G-score	K_D
01a	191	5	35	711.0
02a	121	6	40	98.7
03a	94	6	21	706.8
04a	72	6	34	351.9
05b	130	8	38	667.0
06b	81	7	20	425.5
07c	2	2	18	1910.0
08c	4	3	13	721.9

The number of plausible quadruplexes, number of unique start locations and the highest scoring quadruplex obtained from the QGRS programs is listed. The K_D was measured based on the fluorescence response curve when incubated with the TO1-biotin dye.

The RNAfold tool is part of the ViennaRNA Package (214) and is used to predict the lowest energy secondary structure from the primary sequence given. Besides RNAfold, there are many alternative secondary structure prediction programs published (234–236) most notably Mfold (213) is one of the most ubiquitously used. However, of the secondary structure predicting software, RNAfold is unique in that it is able to incorporate G-quadruplex motif formations into the calculations and is able to properly treat the competition between formation of G-quadruplexes and more typical hairpin-loop secondary structures. In this work the temperature to simulate folding is set to 25°C and the inclusion of G-quadruplex formation is used in the structure prediction algorithm.

By analyzing all the aptamer candidates, there does not seem to be an obvious trend in the resulting secondary structures that can predict the role of the motif found using MEME. When just the quadruplex motif from QGRS is inputted, the most stable secondary structure is predicted to be that of a three-tiered parallel G-quadruplex for 02a and 05b shown in Figure 3.2. The 21 nucleotide MEME motif is located in the second and third propellor loop away from the 5' end for

both sequences. Of the 27 families with high G-scores, 5 families are predicted to form stable parallel G-quadruplexes (07a, 12a, 16a 22a and 27a) when analyzed with RNAfold and they follow similar patterns to the 02a and 05a candidates with the MEME motif occupying the same place in the quadruplex. The 27a family is an exception in that it does not have the predicted MEME motif and the predicted quadruplex is a two-tiered quadruplex. The convergence of the sequence space onto families that have the same predicted secondary structure gives evidence that this G-quadruplex motif is important for the binding of TO1-biotin and that the 2nd and 3rd loop nucleotides may be involved in the binding of the dye molecule. This binding mode is consistent with the crystal structure for the RNA TO1-biotin aptamer in that the dye binds to one face of the three-tiered quadruplex with two loop adenines interact with the heterocycles of the dye and a third nucleobase holds the biotin in place (83).

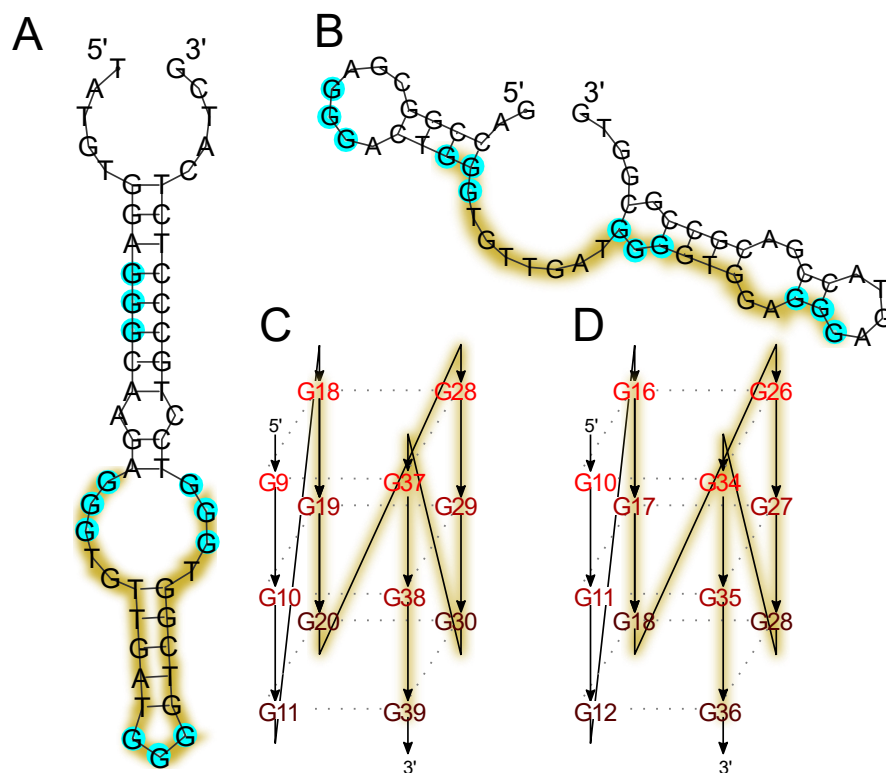


Figure 3.2 | Secondary structure

The secondary structure for the 02a and 05b aptamer candidates, **A** and **B** respectively, is predicted using the RNAfold tool resulting in a stem loop structure. The portion of the sequences that shares a similar core motif using the MEME toolkit is highlighted in orange from G18 to G38 for **A** and from G16 to G36 for **B**. The portions of the sequence that are predicted using QGRS to be involved in G-quadruplex formation is highlighted in cyan. The topology of the G-quadruplex predicted using both the QGRS and RNAfold tool is shown in **C** and **D** for 02a and 05b respectively. There is a large sequence homology for loop 2 and 3 of both quadruplexes since that is the location of the MEME motif region. Loop 1 in however is quite different between the two sequences with 02a consisting of 5 nt from G11 to G18 and 05b consisting of 3nt from G12 to G16.

3.2.3 Ion Dependency

A major factor contributing to the formation of G-quadruplexes is the stabilization of stacking interactions of the G-quartets mediated by monovalent cations, typically K^+ and to a lesser extent Na^+ or NH_4^+ (237). Due to differences in atomic radii, Na^+ complexation occurs within the plane of the G-quartet while K^+ is coordinated in the interplanar spacing between the quartets (238). Although both K^+ and Na^+ can typically stabilize the same quadruplex sequence, changes

in the loop conformation (239) or even the topology (240) can be seen in the ionic exchange. It is shown that the perturbation of the structure can be seen in the absence of such ionic species resulting loss of quadruplex stability (241–243).

In order to ascertain the effects of K^+ and Na^+ on the affinity and fluorescence enhancement, a fluorescence titration is done with the TO1-biotin dye in the presence and absence of such ions for the 02a aptamer candidate as can be seen in Figure 3.3 **A**. Both the affinity and the fluorescence enhancement are significantly increased in the presence of K^+ compared to Na^+ . The addition of Na^+ does not have a significant effect on either the affinity or fluorescence enhancement compared to the sample just in Tris-HCl buffer, however both samples have an increased affinity and fluorescence enhancement compared to the original selection library in the presence of K^+ . In the presence of 100 mM K^+ , there is a 10-fold decrease in dissociation constant from 703.09 nM to 76.77 nM and 40% increase in fluorescence enhancement from 37.63 RFU to 92.93. The sample was also tested using a Tris-HCl buffer with no added ions which provided similar results to that of the 100 mM NaCl phosphate buffered sample, however no active effort of sodium ion removal was attempted which may have allowed for trace monovalent cation stabilization of the bound complex. The results show that K^+ is not strictly required for fluorescence enhancement of the TO1-biotin dye with the 02a sequence and suggests that the K^+ plays a large role in promoting affinity to the TO1-biotin target and a much smaller role in fluorescence enhancement of the dye. The K^+ stabilized quadruplex may cause loop structure rearrangement that allows for slower off-rate binding to occur.

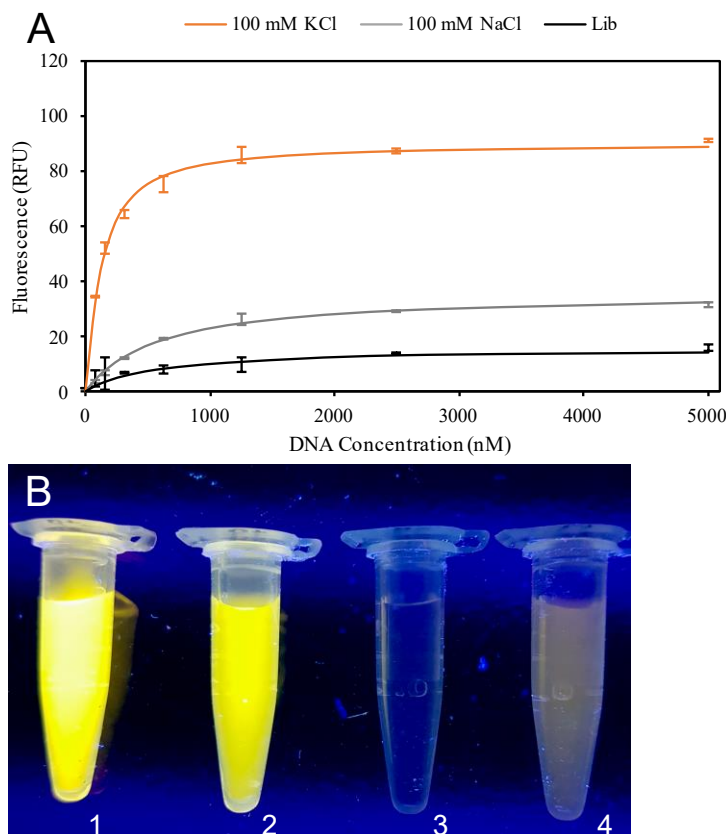


Figure 3.3 | K^+ dependency of 02a

The fluorescence titration curve, **A**, shows that the presence of K^+ ions promote the binding of TO1-biotin to the 02a sequence causing enhanced fluorescence. In the presence of 100 mM K^+ , a K_D of 84.78 nM and maximum fluorescence of 90.42 RFU was achieved. In the presence of 100 mM Na^+ , a K_D of 561.61 nM and maximum fluorescence of 35.82 RFU was achieved. Figure **B** shows the fluorescence enhancement under UV light. Sample 1 and sample 2 shows the fluorescence enhancement from the 02a aptamer in the presence and absence of K^+ , respectively. Sample 3 contains only the free TO1-biotin dye with K^+ and sample 4 shows the fluorescence enhancement of the initial selection library with K^+ .

3.2.5 Circular Dichroism Spectroscopy

Circular dichroism is a powerful tool to distinguish unique folding motifs in nucleic acid structure. The signal produced from the G-quadruplex secondary structure produces a strong and unique signature on the spectrum and is sensitive to the differences in G-quartet stacking, strand orientation and loop arrangements (232). Although each structure produces a unique signature on the

spectrum, the topology of the quadruplex can be discerned by looking at the UV region from 200 – 350 nm (244–246).

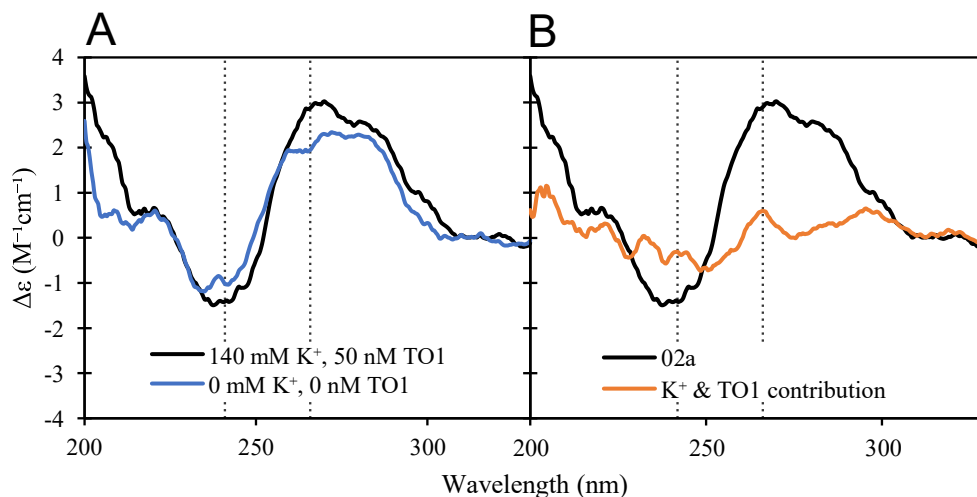


Figure 3.4 | Secondary structure analysis

CD spectroscopy was used to study the nature of the secondary structure of the 02a aptamer candidate obtained from selection. In all both figures above, the spectrum of the 02a aptamer with 140 mM K^+ and 7 μM TO1-biotin is displayed in black. In figure **A**, the blue spectrum is that of the 02a aptamer in the absence of K^+ or the dye, TO1-biotin. In figure **B**, the orange spectrum is the result of the subtraction of both spectra in **B**, thus showing the contribution of K^+ and TO1-biotin to the overall structure of the fully bound aptamer. The resulting spectrum suggests that 02a is preformed into a G-quadruplex prior to being bound to the TO1-biotin ligand and the K^+ ion plays a more functional role in aptamer-dye fluorescence enhancement.

A typical parallel G-quadruplex has a peak at 264 nm and a trough at 243 nm (247) which is consistent with what is obtained for 02a as can be seen in Figure 3.4. The spectrum suggests that the 02a aptamer is indeed a parallel G-quadruplex. Due to the fact that 02a showed some K^+ dependency for fluorescence enhancement, analysis of the CD spectrum in the absence of K^+ was performed. Doing a spectral subtraction of the resulting spectrum shows little contribution of K^+ to the overall structure of the aptamer. The addition of TO1-biotin alone also contributes minimal effects to the CD spectrum suggesting that the parallel G-quadruplex structure is preformed prior to the addition of K^+ or TO1-biotin.

3.2.4 Core Binding Regions

In order to observe the importance of the motifs obtained from MEME and QGRS for binding and fluorescence enhancement, truncations of the full length 02a sequence were tested. The motif sequences as well as extensions on the 5' and 3' ends of 5 and 6 nucleotides were custom synthesized and fluorescence titration of these sequences in the presence and absence of K^+ were performed. The fluorescent signals from the truncated sequences were compared to the full length sequence in the presence and absence of K^+ as can be seen in Figure 3.5.

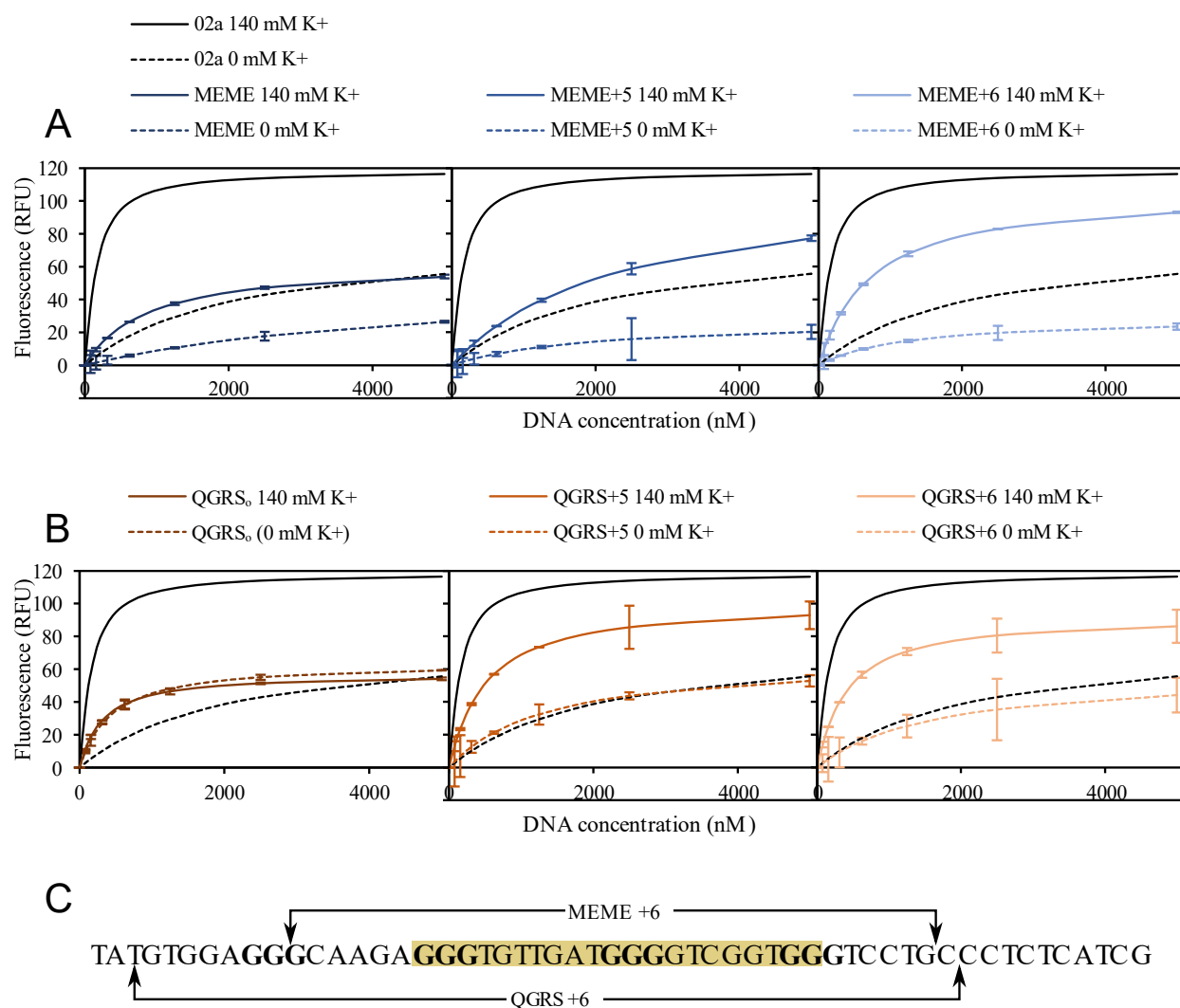


Figure 3.5 | Core binding motif elucidation

Several truncations of the 02a aptamer candidate were measured for fluorescence enhancement. All of the figures contain the full length 02a in the presence and absence of K⁺ in solid black and dash black respectively. **A** shows the fluorescence enhancement of the motif obtained from the MEME analysis of the NGS families as well as extensions of this sequence. **B** shows the fluorescence enhancement of the predicted G-quadruplex motif using QGRS as well as extensions of this sequence. **C** is a schematic of the identity of the sequences used. The highlighted areas is the motif obtained from MEME whilst the bolded Gs are involved in the quadruplex predicted from QGRS.

As expected, any truncation of the full-length sequence resulted in a drop in fluorescence enhancement as well as affinity for all sequences tested. The general trend of the MEME sequences involves a general increase in fluorescence enhancement with increasing sequence length; the

addition of two guanine from MEME+5 to MEME+6 results in both higher affinity as well as higher fluorescence enhancement. It can be seen that even though the MEME sequence is highly conserved, it is not sufficient to bind TO1-biotin by itself.

Although the affinity for TO1-biotin is hindered in the QGRS₀ sequence, the affinity is quite high, rivaling some full-length candidates, however the absolute fluorescence enhancement is significantly perturbed. Interestingly, QGRS₀ shows little change in fluorescence enhancement in the presence or absence of the K⁺ ion; this suggests the dye has some affinity to the pre-formed structure, but the loops at the 5' and 3' ends are more important for stabilizing the aptamer:target complex. The QGRS+6 sequence shows similar fluorescence enhancement character to that of the full-length sequence in the absence of K⁺ suggesting that the flanking sequences outside the quadruplex plays an important role in K⁺ mediated fluorescence enhancement. When looking at the sequence alignment of families, there are sequences outside the QGRS+6 sequence that are partially conserved that may aid in structure stability.

Table 3.2 | Binding affinity of sequence truncations

Name	Sequence	K _D (nM)	FE
02a MEME ₀	GGGTGTTGATGGGGTCGGTGG	821.9	62.8
02a MEME+5	CAAGAG GGGTGTTGATGGGGTCGGTGGGTCCT	2234.4	89.9
02a MEME+6	GCAAGAGGGTGTTGATGGGGTCGGTGGGTCCTG	654.7	107.2
02a QGRS ₀	GGGCAAGAGGGTGTTGATGGGGTCGGTGGG	282.0	61.0
02a QGRS+5	GTGGAG GGGCAAGAGGGTGTTGATGGGGTCGGTGGGTC CTG	443.1	101.9
02a QGRS+6	TGTGGAG GGGCAAGAGGGTGTTGATGGGGTCGGTGGGTCCTGC	362.1	108.7

The K_D was measured based on the fluorescence response curve when incubated with the TO1-biotin dye. Fluorescence enhancement (FE) was calculated by dividing the maximum fluorescence response by the signal obtained from the free dye.

3.3 Materials and Methods

3.3.1 Chemicals

The DNA sequences of the aptamer candidates and truncations were synthesized and purchased from Integrated DNA Technologies (Coralville, IA).

3.3.2 Fluorescence Titration

All fluorescence data was obtained using a SpectraMax M5 Microplate Reader from Molecular Devices LLC. The desalted and lyophilized DNA was resuspended in MQ to the concentration of 100 μ M. A serial dilution of the DNA from 5000 – 78.125 nM prepared in PBS-M pH 7.4 (10 mM Na_2PO_4 pH 7.4, 140 mM KCl & 1 mM MgCl_2) with 50 μ M TO1-biotin or Tris pH 7.4 (10 mM Tris-HCl pH 7.4 & 100 mM KCl or 100 mM NaCl) with 50 μ M TO1-biotin. The sample was modified with 140 mM NaCl when measurements in the absence of potassium was desired. Before adding to the 96-well plate, the DNA was heated to 90°C for 1 min then cooled to 4°C and allowed to incubate with the TO1-biotin in the well for 5 minutes before being read. The excitation wavelength of 488 nm was used and the emission of the fluorescence was measured at 535 nm with a cutoff at 530 nm. The reported value is the average of 6 readings.

3.3.3 Circular Dichroism Chromatography

All circular dichroism data was obtained from a J815 CD spectropolarimeter from JASCO. The desalted and lyophilized DNA was resuspended in MQ and prepared at 5 μ M in PBS-M pH 7.4 (10 mM Na_2PO_4 pH 7.4, 140 mM KCl & 1 mM MgCl_2) with or without TO1-biotin at 7 μ M. The buffer was modified with 140 mM NaCl when measurements in the absence of potassium was

desired. Spectra was acquired at 25°C every 1 nm from 200 to 330 nm. Each spectrum is an average of 4 scans with a 2 s integration time. Data were buffer subtracted and normalized to provide molar ellipticity values.

3.4 Conclusions

By analyzing the primary sequence of the 27 families from round 9 and the candidates selected for further testing, a consensus sequence motif was found. A 21-nucleotide motif emerged and is highly conserved across all families as can be seen with sequence alignment. The motif contains high G content which suggested the presence of a G-quadruplex structure. RNAfold and QGRS are used to find the potential location of the quadruplex in each sequence and the location of the motif sequence in relation to the predicted quadruplex is analyzed. The most stable quadruplex structure is predicted to be a three-tiered parallel G-quadruplex with the conserved motif containing the second to fourth G-triple including the 2nd and 3rd propeller loop away from the 5' end. Of the tested candidates, 2a has the highest affinity and G-score, according to QGRS. There are however several untested families that are predicted to form more stable quadruplexes at room temperature (7a, 12a, 16a, 22a and 27a) according to RNAfold.

The 2a candidate shows K⁺ dependency with a large decrease in affinity and moderate decrease in fluoresce in the absence of K⁺. The addition of Na⁺ does not recover the function of the sequence which suggests that the K⁺ ion plays an important role when forming a tightly bound complex that allows enhanced fluorescence. By using CDS, the structure of a parallel quadruplex is confirmed, however the dye does retain some affinity in the K⁺ absent structure. This is not unexpected as the RNA mango also shows a retention of quadruplex structure in the absence of K⁺ on the circular dichroism spectrogram.

Although the motif found using MEME is highly conserved across all families in the final round of selection, the sequence alone is not sufficient in eliciting a strong fluorescence enhancing effect. Since the first G-triple and 1st propeller loop is not a highly conserved area the 3' end of the sequence may be more involved in the binding of the dye molecule. A larger recovery of affinity is seen when the quartet near the 3' end is introduced in the MEME+6 sequence from the MEME+5 truncated sequence. Interestingly the fluorescence enhancement of the quadruplex sequence, QGRS₀, does not have a large difference of fluorescence signal in the presence and absence of K⁺, suggesting the 5' and/or 3' ends of the sequence are needed to hold the dye in the binding pocket and are able to access a conformation that promotes fluorescence enhancement in the presence of K⁺. Although truncation of the sequence was not achieved, it was discovered that much more than the conserved sequence and predicted quadruplex motif is needed to provide a tight binding and bright aptamer:TO1-biotin complex.

4.1 Introduction

Hypersensitivity is a set of physiological reactions caused by the immune system and there are four types of hypersensitivities, types I – IV (248). An allergic reaction falls under the type I hypersensitivity which is induced by allergens; an allergen can come from many different sources, most commonly from pollens, foods, drugs and animals. When an allergen first enters the body, the production of allergen specific IgE which induce the release of histamine which initiates downstream effectors resulting in allergic symptoms (249). Due to the elevated levels of IgE in the body, IgE is a good indicator of someone that is prone to allergies; as many as 30% of Americans suffer from seasonal allergies (250).

Traditionally, diagnosis of allergies is done in person in a clinical setting. The *in vivo* tests involve causing an allergic reaction on the body through a skin prick or intra dermal injection (251). In both cases the isolated allergen is put in contact with the patient and swelling is monitored by a physician. Alternatively *in vitro* methods are available for cases that may have an elevated risk of anaphylaxis. The gold standard for such *in vitro* testing is the radioallergosorbent test (RAST) which uses radiolabeled anti-human IgE antibodies to detect specific IgE captured by surface bound allergen (252). Since radioactive isotopes are undesirable in modern assays, the RAST method is being phased out for methods more akin to an ELISA such as Siemen's AlaTOP (253) and Thermo Fisher's ImmunoCAP (254) assays.

Recently, there has been an increased need for rapid at home testing such as blood glucose level detection, home pregnancy testing and at home COVID-19 testing. The lateral flow immunoassay (LFIA) is an emerging market for at home testing as can be seen with the increased need with the rapid antigen COVID-19 tests; however, there is currently no widely available at-

home allergy test to the same extent. Although LFIA are very useful, they are not without fault; the use case can be narrow as the results are generally qualitative, the accuracy cannot be matched by other in-solution methods such as ELISA and the accuracy can drop even more when administered by a non-expert (255).

The D17.4 aptamer is a DNA aptamer that was selected to specifically bind to human IgE and originally developed as a novel class of IgE inhibitors to fight against allergic diseases. The 37-nucleotide sequence was selected and characterized by the Tasset group (256) and has a K_D of 10 nM. It has since been used in many sensing applications using methods such as electrochemical, luminescence resonance energy transfer and quartz crystal microbalance based approaches (257–259). Due to the fact that there have been many published works on the use of this aptamer in sensing applications, the D17.4 aptamer was selected as a candidate for use in our biosensor development.

Originally, our group was approached by Kenota Health (previously ExVivo) a biotech start-up firm from the University of Waterloo-backed incubator Velocity, to help develop an aptamer-based biosensor for allergies. Herein we systemically compare several approaches used towards the application of aptamers to sense the biomarker IgE as an indicator of allergies in isolated protein samples as well as in real serum samples.

4.2 Results and Discussion

4.2.1 LSPR Approach

In order to probe the use of the D17.4 aptamer for use in our sensing application, localized surface plasmon resonance (LSPR), was used to monitor the binding of human IgE to the aptamer

on a sensor surface. The formation of the LSPR sensor was achieved using functionalized DNA aptamer sequences. The gold nanoparticle surface is prepared and 5' thiolated DNA is used to form a monolayer of the aptamer through the gold-thiol bond. Signal response and sensitivity on the gold surface was not consistent and multiple iterations of running buffer, regeneration solution and concentration ranges for the aptamer and target was tested to optimize the assay. Ultimately changing the sensor chip to be functionalized with streptavidin and the aptamer with a biotinylated 5' end resulted in more consistent signal responses. The binding affinity of the immobilized aptamer to the free IgE was found to be 3.71 nM when using a streptavidin coated sensor chip and 9.8 nM when using a bare gold sensor chip when fit to a one-site, two state binding curve.

When comparing the binding kinetics of the aptamer to an antibody, the Chen group was able to use traditional SPR to characterize the binding interaction between immobilized human IgE with the D17.4 aptamer and a goat anti-human IgE antibody (260). It was found that the affinity of the aptamer to human IgE was 74.62 nM – the affinity of the goat anti-human IgE was found to be 129.37 nM. The difference in apparent affinity can be attributed to the differences in the binding conditions and that the ligand is immobilized on the sensor in Chen's study whereas the aptamer is immobilized in this study. Because one of the species is bound to the surface, steric hinderance may reduce the degrees of freedom for the binding interaction (261). One bound species may promote higher affinity interactions in the aptamer-ligand complex formation. Although this method of detection can provide an accurate result, it would not be suitable for consumer level detection due to the low throughput and slow response output. The results show however, that the use of the D17.4 aptamer can be a great alternative to antibody-based assays as the affinity is comparable, if not better.

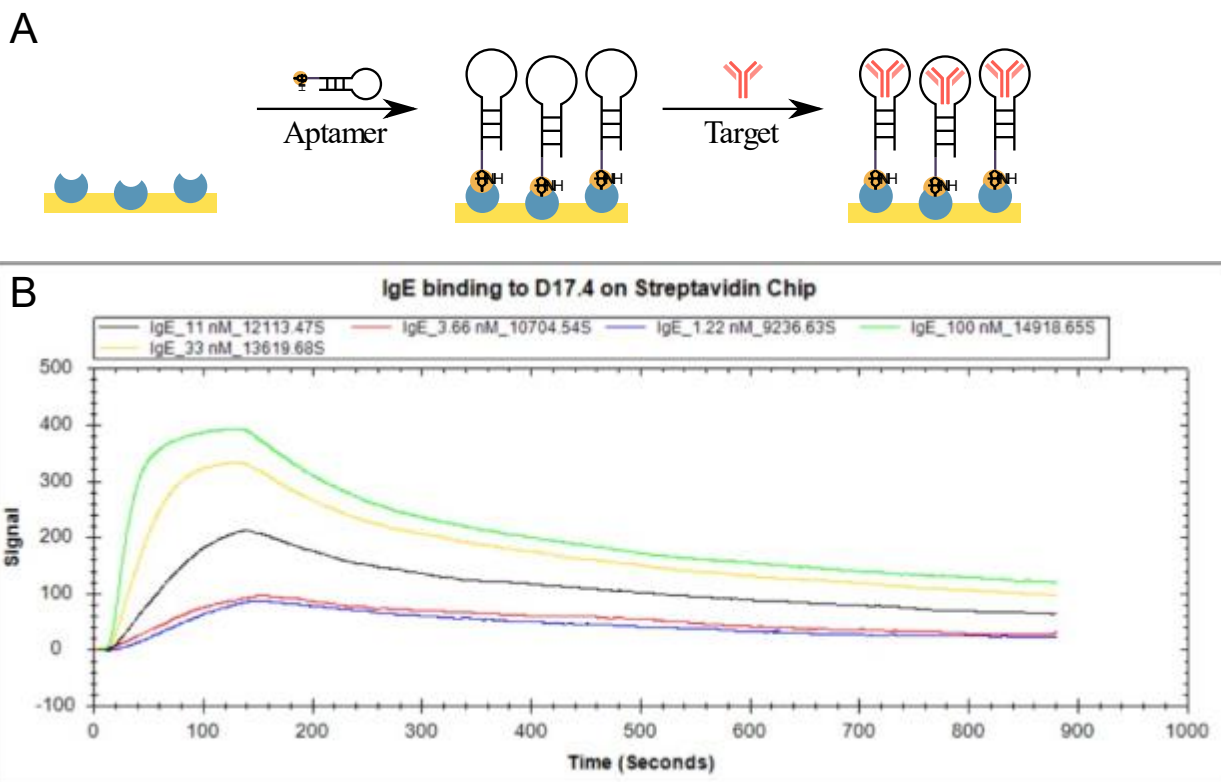


Figure 4.1 | Binding kinetics of IgE

The binding kinetics of the anti-IgE aptamer D17.4 was probed using LSPR under various conditions. A schematic of the design for the sensor used for the detection of IgE using LSPR is shown in **A**. The gold nanoparticles on the LSPR sensor chip are functionalized with streptavidin and the D17.4 anti-IgE aptamer with a biotinylated extension is added forming a monolayer on the LSPR surface. When the sample containing human IgE is injected onto the surface a signal response due to the addition of mass produces a curve on the sensogram based on the aptamer-target binding kinetics. A typical binding sensogram is shown in **B**. Varying concentrations of IgE and modeling the response curves against a one-site saturation binding curve produced a K_D value of 3.71 nM for this sensing setup.

4.2.2 Labeled Electrophoretic Approach

A challenge in the detection of IgE in human serum is that IgE is present at much smaller proportions in serum, relative to other proteins; in order to have overcome this challenge separation of proteins can be utilized to aid detection. To this end, non-denaturing PAGE can be used to separate proteins whilst maintaining the native 3D structure needed for epitope detection. Typically the method used for the detection of specific proteins in a sample via electrophoresis is

western blotting (262), however, Electrophoretic Mobility Shift Assay (EMSA) (263) can be used to detect specific protein:DNA interactions. EMSA involves monitoring the perturbation of a DNA or RNA sample on a gel due the binding of specific proteins presents in the protein-nucleic acid mixture sample and comparing with the protein free sample. When there is no protein with specific interaction with the nucleic acid present in the sample, the signal for the nucleic acid is unperturbed and the band is lower on the gel. In the case for this study, native PAGE EMSA is used for the detection of D17.4 binding to IgE in human serum samples.

A serum sample provided by Kenota Health with known elevated levels of IgE was used for the testing of the D17.4 aptamer binding in this electrophoretic approach. The amount of protein was quantified in the sample via the Bradford method (264). The D17.4 aptamer was labeled with Cy5 at the 5' end used to monitor the binding of the DNA to different protein species in the sample. The labeled aptamer was incubated with the serum sample at various concentration and then run on a native PAGE which resulted in a fluorescent signal at the location where protein interaction occurred. In order to find an optimal range for detection of IgE in the sample, several concentrations of the serum were tested. There was very weak signal for the protein of interest at the top of the gel, however, for serum samples containing 2.8 – 45.8 $\mu\text{g}/\mu\text{L}$ protein, a fluorescent signal was present near the bottom of the gel seen in Figure 4.2 **A**; that corresponding signal in the Coomassie stained gel is where human serum albumin (HSA) would be present seen in Figure 4.2 **B**. This fluorescent signal was also visible when run with BSA at concentrations as high as 10 $\mu\text{g}/\mu\text{L}$ suggesting that there may be non-specific binding to the protein in the gel.

In order to test the nature of this non-specific binding, an engineered aptamer variant, named D17.4ext, was used that has been shown to have higher specificity to the IgE target (265). The extended variant designed by the Prohaska group features a four GC base pair extension of

the stem region which resulted in an increased affinity from the unmodified aptamer in their QCM studies. The K_d of the extended aptamer was measured to be 3.6 nM whilst the original aptamer had a K_D of 8.4 nM when measured by QCM. It is thought that the increased stability of the tertiary structure via the extension of the stem results in slower dissociation which can allow for a lower detection limit.

The use of a Cy5-labeled D17.4ext aptamer in EMSA resulted in more fluorescent signal at the top of the gel, where the high molecular weight IgE is expected to be, and less signal near the bottom of the gel, where HSA is expected as seen in Figure 4.2 C. The signals in the BSA lanes are also reduced with the use of the extended aptamer. The use of the extended aptamer in solution may result in a sensor that has higher specificity for the target and higher affinity. This off-target binding may be a result of nonspecific binding as a consequence of high protein concentration, however in this work there is a detectable difference between the extended and original aptamer.

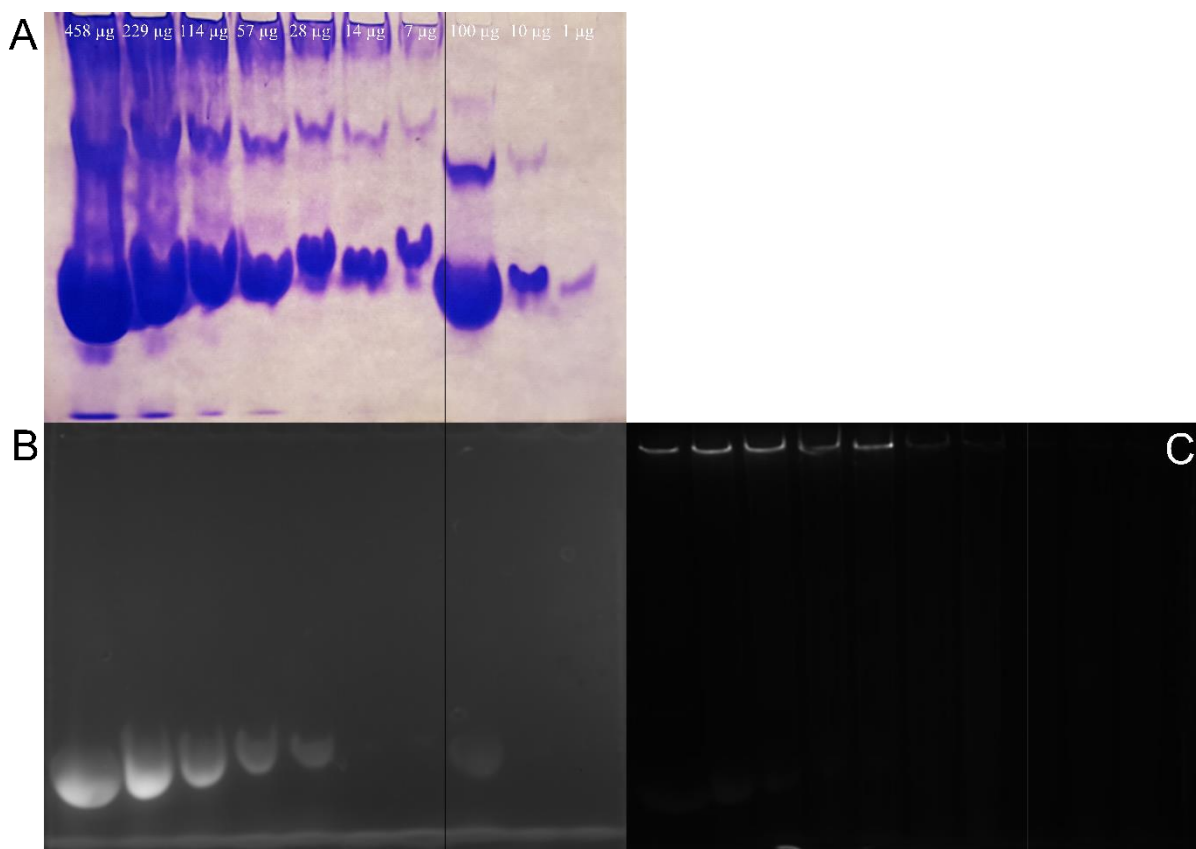


Figure 4.2 | Mobility shift with Cy5 labeled D17.4

The Cy5 labeled aptamer was tested against various concentrations of the sample in a mobility shift assay to see if IgE in the serum sample can be visualized. The Cy5 labeled D17.4 aptamer was incubated with varying concentrations of a serum sample and BSA and analyzed using native PAGE shown in **A**. The amount of protein in the serum sample was quantified using the Bradford assay and displayed at the top of the wells to the left of the black line – the BSA concentrations are on the right of the black line. When visualized using filtered light, shown in **B**, signal corresponding the Cy5 dye is shown where HSA would be present in the serum sample. When the labeled extended aptamer is used in **C**, there is a reduction of the Cy5 signal in the expected HSA location and a increased signal at the expected IgE location. There is also a reduction of signal in all BSA lanes.

4.2.3 Structure-switching Approach

In addition to the target molecule, aptamers have the ability to bind to their own complementary sequence, wherein both results in two distinct structures. When bound to the target molecule that the aptamer was selected against, a defined tertiary structure of the aptamer:target complex is formed. The canonical double stranded structure is formed when the aptamer binds to

complementary sequence via Watson-Crick base-pairing as is the nature of DNA or RNA. The interplay between the two distinct structures, target-bound and double stranded, is often exploited when designing aptamer-based biosensors (266); the binding of the target molecule displaces the complementary strand, termed structure-switching, which allows for downstream effects to occur, typically fluorescence as seen in Figure 4.3 A. To this end, the D17.4 aptamer was modified with Cy5 dye at the 3' end and a complementary oligonucleotide with Black Hole Quencher®-2 (BHQ-2) at the 5' end.

Various lengths of complementary sequences to the D17.4 sequence, ranging from 13 – 17 nucleotides in length, were synthesized with the quencher BHQ-2 functionalized at the 5' end. The labeled aptamer and quencher strands were melted and reannealed to form the sensor which was confirmed via a decrease in fluorescence of the assembly. After probing the annealed sequences for quenched fluorescence signal, it was shown that the 13-nucleotide complementary sequence showed a optimal range of signal quenching shown in Figure 4.3 B. When both strands are present at equal molar amounts, the signal is reduced by 60.6% of the unquenched signal and at 4 times molar excess of the BHQ2 modified sequence, a 98.8% signal reduction of the Cy5 signal occurred. In order to reduce the amount of background fluorescence, the 1:4 aptamer:quencher ratio construct was used for our downstream sensing applications.

When the sensing module was incubated with a serum sample that contained an elevated level of IgE, there was an apparent recovery of fluorescent signal that is indicative of a positive response due to the presence of the target molecule shown in Figure 4.3 C. When the target, IgE, is present in the sample, the double stranded structure of the aptamer:quencher complex is disrupted and the formation of the aptamer:target complex allows for the restoration of fluorescent signal. However, when a sample containing only the IgE isolate was incubated with the fluorescent

module, the signal was not recovered. The typical amount of total IgE present in serum is in the 1 ng/ μ L range (267), but signal recovery was not achieved in the IgE isolate sample even at concentrations up to 25 ng/ μ L. The fluorescent module was then incubated with serum albumin, in this case bovine serum albumin (BSA), as this is a major protein component in serum and signal recovered was significantly more than with the IgE isolate. This suggests that the high concentrations of protein itself may have been a major driver of fluorescence recovery and the aptamer:target complex is not enough to disrupt the double stranded complex. If a structure-switching module is to be used, a shorter quenching sequence or lower aptamer:quencher ratio should be used, thus sacrificing significant signal to noise ratio.

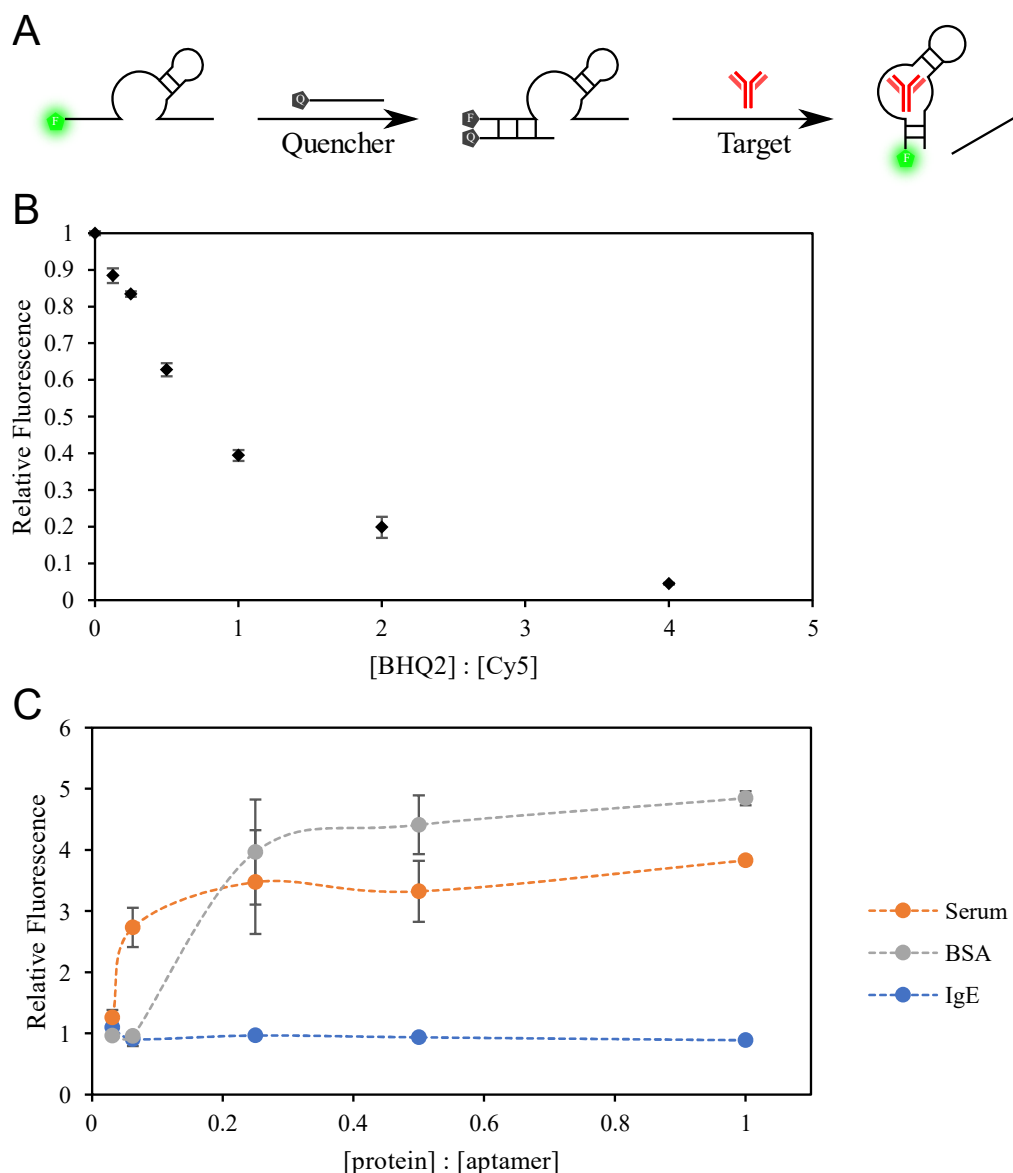


Figure 4.3 | Cy5 labeled D17.4 aptamer sensor

Testing of the molecular beacon approach for the detection of IgE using a Cy5 labeled D17.4 aptamer. A schematic of the sensor design used is shown in **A**. The fluorescence of the Cy5 labeled aptamer is quenched by the addition of a short complementary oligonucleotide labeled with a quencher, BHQ2. When the target, human IgE, is present in the sample, structure-switching of the aptamer occurs to displace the quencher strand due to target-induced conformational change. The concentration-dependent fluorescent signal reduction of the quencher strand is shown in **B**. A significant amount of fluorescence remains with an equal molar of the aptamer and quenching strand, with 39.4% of unquenched signal remaining. At 4 molar equivalence of the quenching strand to the aptamer, 0.04% of the unquenched signal remains. The concentration-dependent fluorescence rescue of various protein samples is shown in **C**. When the serum sample is incubated with the sensor, there is recovery of the fluorescent signal at as little as 6.25 μ g serum, however there is also significant signal recovery when the same amount of BSA is incubated with the sensor.

When human IgE is incubated in the concentrations that should be present in the serum sample there is not much fluorescence rescue due to target induced conformational change.

4.2.4 Lateral Flow Assay Approach

Lateral flow assays combine many beneficial aspects that make them attractive as a diagnostic tool (268); they provide rapid results, are easy to use for non-experts and are relatively cost-effective. These factors are a major driver for the development as a biosensor for the sensing of IgE. The sensing module, in this case the D17.4 aptamer, is conjugated to a fluorescent reporter, a europium doped polystyrene nanoparticle. Fluorescence is a desirable reporter due to the high sensitivity it can provide due to high signal to noise ratio. The lateral flow assay strip design was modeled after typical rapid lateral flow immunoassays (268) shown in Figure 4.4. In this study, the binding of a labeled anti-human IgE antibody is compared to that of labeled aptamer for the detection of specific IgE.

The typical lateral flow immunoassay assembly involves attaching a nitrocellulose membrane, with a test and control line, to a backing card with the sample pad and conjugation pad on one end of the test strip while the adsorbent pad is on the other side. The conjugation pad contains dried buffer, the primary antibody and the secondary antibody, and the sample is drawn from the sample pad to the adsorbent pad via capillary action. In place of a primary antibody, biotinylated allergen is used, in this case the group 5 pollen allergen for Timothy grass which is the major antigen associated with grass allergies; this provides the sensor with the specificity for the specific IgE. Biotinylating of the antigen is achieved with an NHS-ester activated biotin reagent. In place of the secondary antibody, labeled aptamer is used, in this case the aptamer is labeled with a fluorescent nanoparticle; this provides the sensitivity for the assay. In this study, to simplify assay optimization, the sample and conjugation materials are mixed in solution in a well

with one end of the strip submerged in the mixture and the sample is allowed to flow through the strip via capillary action to the absorbent pad.

If the sample contains the specific IgE for Timothy grass, both the biotinylated antigen and conjugated nanoparticle will bind to the IgE and will be captured at the test line that contains an avidin derivative, NeutrAvidin, that is able to capture the biotinylated antigen; this results in a positive fluorescent signal at the test line. The control line contains a complementary DNA strand to the aptamer for the D17.4-conjugated nanoparticle and a murine anti-goat antibody for the antibody-conjugated nanoparticle resulting in a positive fluorescent signal at the control line. Since the sensing module is in excess it will continue to the control line and be captured even with a positive test signal indicating a valid test.

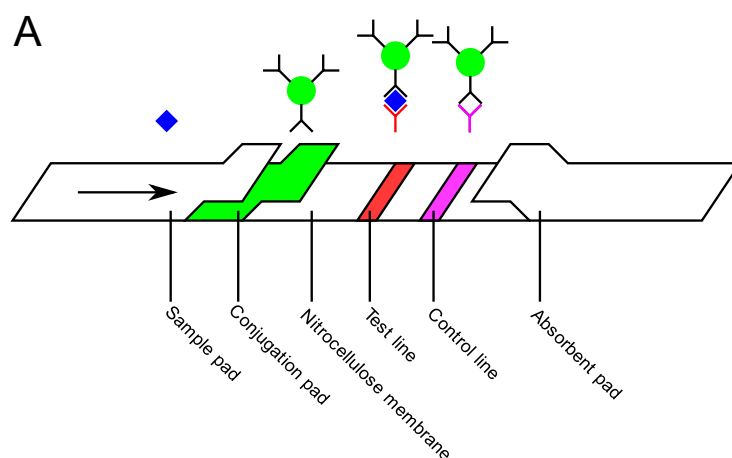


Figure 4.4 | Typical lateral flow strip setup

A schema of a typical lateral flow immune assay strip is shown in **A**. The sample is loaded onto the sample pad and the target (blue) will move towards the absorbent pad due to capillary action. The conjugate pad (green) contains dried buffering reagent and the conjugated reporter molecule. The test line (red) captures the target and reporter in a sandwich-like configuration whilst the control line (purple) is able to capture excess reporter to confirm that the movement of the sample down the test strip.

The IgE sensing aptamer was linked to a fluorescent nanoparticle in the form of a europium doped polystyrene nanoparticle. The large stokes shift of the chelated europium and the low retention of the polystyrene nanoparticles on nitrocellulose makes this nanoparticle ideal as the fluorescent module for the sensor. Standard EDC/NHS activation was used to conjugate the nanoparticles with DNA molecules show in Figure 4.5. Briefly, the carboxylate moiety of the nanoparticle is activated with sulpho-NHS to form an NHS ester via a O-acylisourea intermediate using EDC. When the DNA with a free amine at the 3' end is introduced to the activated nanoparticle, the DNA forms an amide bond with the nanoparticle via nucleophilic acyl substitution. Nanoparticles were conjugated with D17.4 or D17.4ext or goat monoclonal anti-human IgE antibody. Surprisingly, the stability of the D17.4ext nanoparticles was reduced compared to the D17.4 modified nanoparticles. Aberrant association of nanoparticles forming aggregates occurred with synthesis of D17.4ext conjugates; conjugation of the nanoparticles with a mixture of methylated PEG and the DNA helped to reduce aggregation propensity. Previous usage of D17.4ext in an electrophoretic approach showed that there was an increase in specificity, resulting in a stronger signal response on gel, however, when used in this LFIA approach, there is enhanced aggregation which lowers stability.

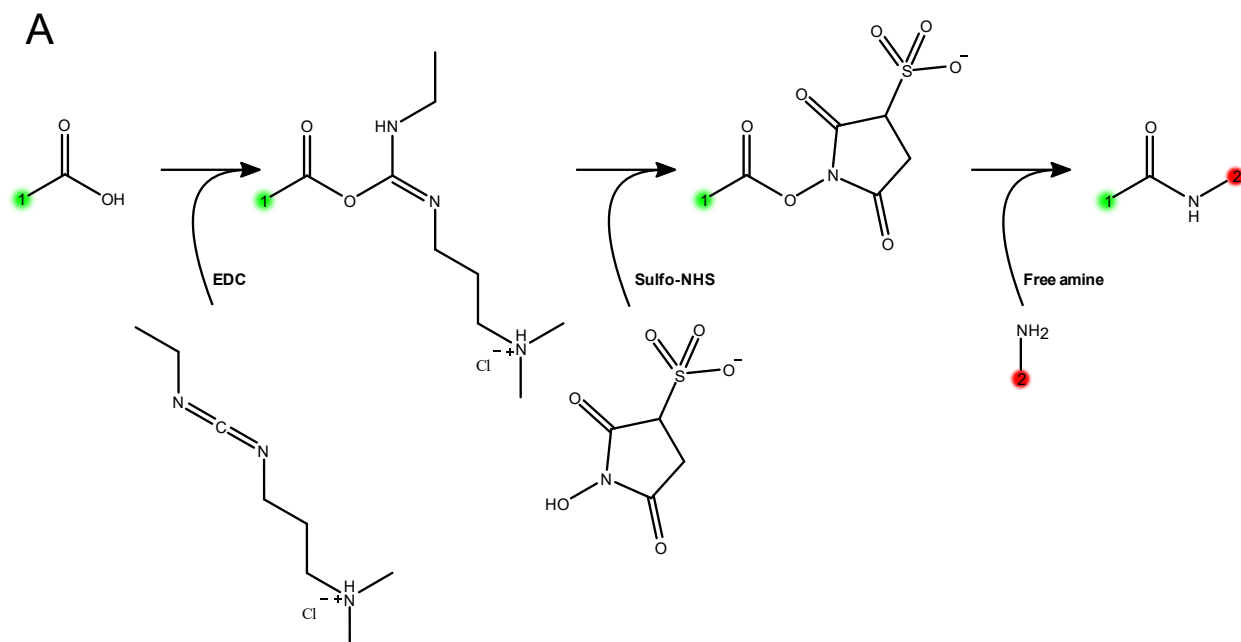


Figure 4.5 | EDC/NHS conjugation

The general reaction scheme for the coupling of carboxylate-modified nanoparticles with molecules containing free amines. The carboxylic acid group of the nanoparticle (green) reacts with the EDC to form a reactive O-acylisourea intermediate which subsequently reacts with sulfo-NHS to form the more stable NHS ester. The amine modified DNA or protein (red) reacts with the NHS-ester via nucleophilic acyl substitution to couple the two molecules through a stable amide bond.

The conjugation of the nanoparticles was confirmed on the nitrocellulose half-strip by spotting human IgE on the nitrocellulose strip and incubating one end of the test strip in the solution containing the conjugated nanoparticle. The samples that contained successfully conjugated nanoparticles with D17.4, D17.4ext and goat monoclonal antibody have a positive signal at the test spot under UV. Retention of the nanoparticles occurred with some D17.4ext conjugated nanoparticles which may be due to the aggregation of the extended aptamer conjugates as can be seen in Figure 4.6 A. Retention mitigation was attempted by reducing the density of the DNA on the nanoparticles by incorporating methylated PEG in the conjugation mixture, by blocking the nitrocellulose surface with BSA and optimizing the conjugation solution buffer.

Optimization resulted in a modest reduction in aggregation, however there is significantly less aggregation with the non-extended aptamer, therefore, the conjugated nanoparticles using the shorter sequence was used for later testing. HSA is spotted to test for off-target binding that was observed in other sensing applications and only a very slight signal is present in the HSA spot seen in Figure 4.6 **B**. Off-target binding was not as pronounced as compared to with a directly labeled structure-switching aptamer or in a mobility shift gel. When compared to the antibody-conjugated nanoparticle in Figure 4.6 **D**, the signal is much weaker at the test line for the aptamer-conjugated nanoparticle.

Although the extended aptamer resulted in a marginally brighter signal due to the higher affinity of the aptamer, the retention of the nanoparticles due to aggregation was not removed even after optimization. This may be due to the higher complementarity of the extended region of the longer aptamer resulting in cross-linking of the nanoparticles; the extension that once promoted stronger intermolecular bonding allows for intramolecular bonding. The control line was unsuccessful in capturing the aptamer-conjugate despite various optimization attempts and the signal remained weaker compared to the antibody-conjugate at the test line. Although there is excellent detection of isolated IgE that is spotted onto the nitrocellulose surface for the aptamer conjugates, detection of specific IgE in this LFIA setup leaves much to be desired. Although the antibody conjugate is able to elicit a strong fluorescent signal and the optimization of antibodies have longer history of use (269), the production of antibody-based products can be less cost-effective. The optimization for the aptamer conjugate signal will require more detailed exploration of binding conditions and sequence optimization but may result in a sensor that can be more accessible and shelf stable than an antibody-based sensor.

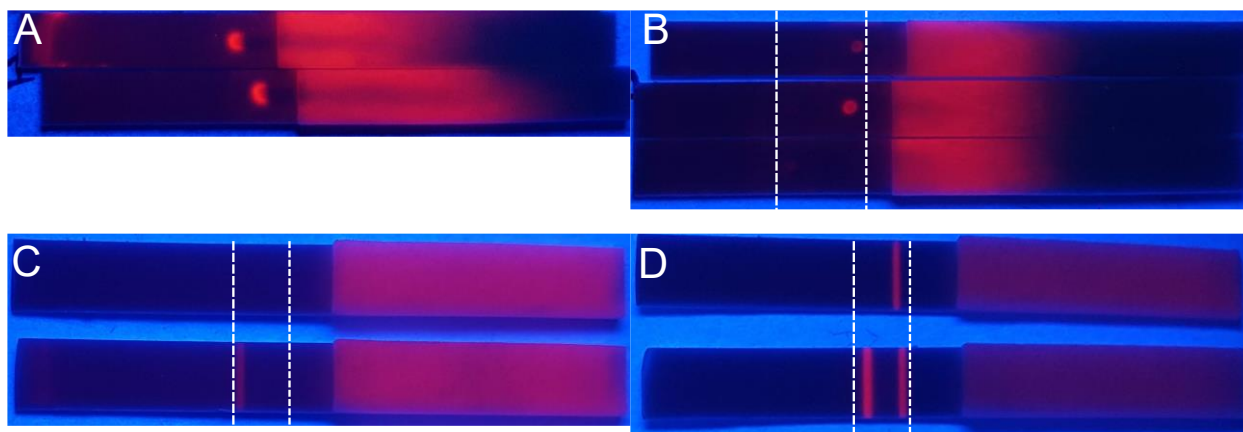


Figure 4.6 | Half strip samples

A shows D17.4 and D17.4ext modified nanoparticles that are run on test strips that have been spotted with human IgE. Both of the modified nanoparticles were successful in detecting the spotted IgE, however there is some nanoparticles that are held up in the D17.4 test strip at the application site. **B** shows three strips that have been spotted with IgE (right), IgE & HSA and HSA (left) alone since there was some concern that there might be interaction between the D17.4 aptamer and HSA in the human serum sample. There is minimal signal detected for the spot containing HSA. **C & D** show the testing of two serum samples, one that does not contain the specific antibody (top) and one that does (bottom) with the aptamer conjugated nanoparticles in **C** and an antibody conjugated nanoparticles in **D**. The test strips are printed with streptavidin (left) and a goat anti-rabbit IgG (right). Compared to the antibody conjugated nanoparticles, the aptamer signal is slightly weaker, however they function as expected with the signal only present at the test line for the sample expecting to have the specific antibody.

4.2.5 Modular Aptamer Approach

Since the aggregation of the fluorescent nanoparticles was a major driver for loss of signal in some of the test strip assays, the need for a fluorescent module that is unlikely to undergo crosslinking was desired. For these reasons, the use of an aptamer to a fluorogenic dye for use in a sensor was of interest in order to produce a turn-on fluorescent aptameric sensor. RNA mango has high affinity and has a high fluorescence enhancement to its cognate dye, TO1-biotin, therefore it was chosen in this study as the fluorescent module for the development of a modular aptamer sensor. The aptamer was synthesized by *in vitro* transcription via bacteriophage T7 polymerase.

The dye was synthesized by the Chong Lab (University of Waterloo); the product was confirmed by NMR spectroscopy and ESI-MS.

The lateral flow form factor showed promise for sensitivity and ease of use, so further development in this form factor was desired. This requires that the sensing modules are stable on a surface, typically nitrocellulose, therefore aptamers needed to be tested under these conditions to verify that the function would be acceptable under these conditions. Initial testing of the Mango aptamer on a polyvinylidene difluoride (PVDF) shows a typical response that was observed in solution with enhancement occurring when the aptamer is present in solution in Figure 4.7 **A** and **B**. However, when the TO1-biotin solution was loaded onto the nitrocellulose surface, significant fluorescence occurred in the absence of the aptamer as seen in Figure 4.7 **C**. The fluorescence enhancement in the absence of the aptamer made it clear that the aptamer-fluorophore complex would not be appropriate for sensing in this application.

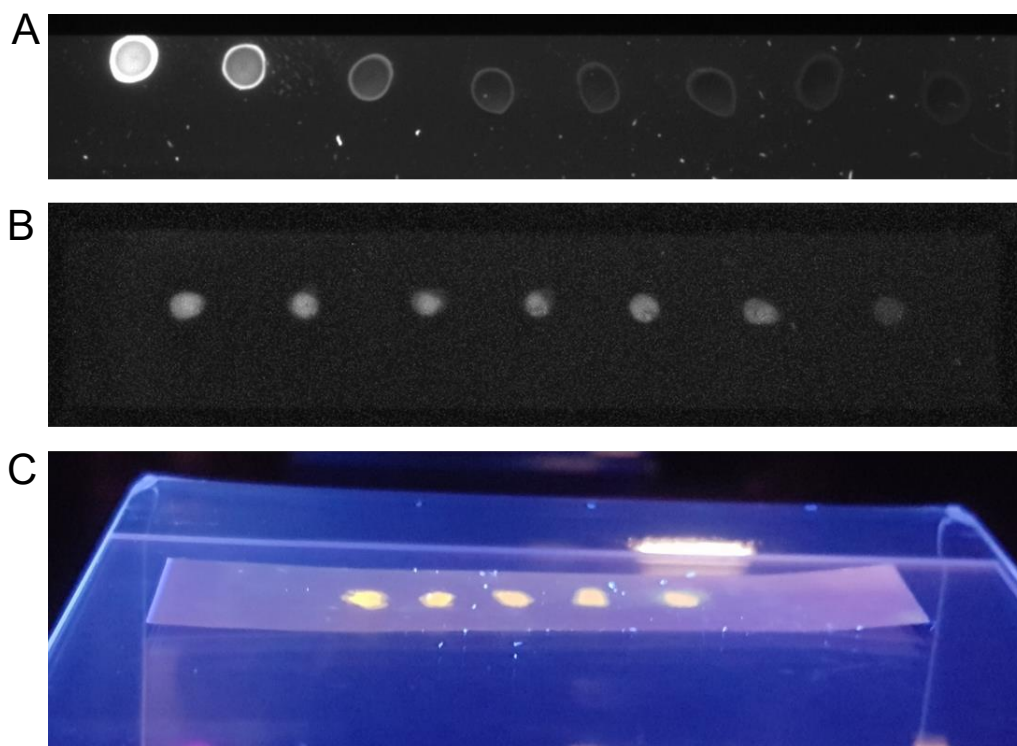


Figure 4.7 | Fluorescence enhancement on surface

After synthesizing the RNA aptamer and the dye molecule, the fluorescence enhancement needed to be confirmed in solution and on a solid surface with a fixed amount of dye and decreasing amount of aptamer from left to right. **A** shows the fluorescence of the droplets on polyvinylidene difluoride (PVDF) and when dried in **B**. However, the dye is not suitable for use on nitrocellulose due to the fact that it becomes fluorescent without being bound as can be seen in **C**.

To form a sensing module that has the uses the D17.4 aptamer as the recognition element and Mango aptamer as the signaling element, a fusion of these two aptameric modules was performed via hybridization of the aptamers through an extension of the 3' and 5' regions respectively. The D17.4 aptamer was synthesized with a 24 nucleotide 3' poly-T extension, while the Mango aptamer was synthesized with a 24 nucleotide 5' poly-A extension. The two aptamers were incubated in equal molar amounts and annealed at melted and re-annealed to form the hybridized fused sensing module. After optimization of the two sensor modules, a signal

transduction strand can be developed as a bridging strand for the modular sensor shown in Figure 4.8 A.

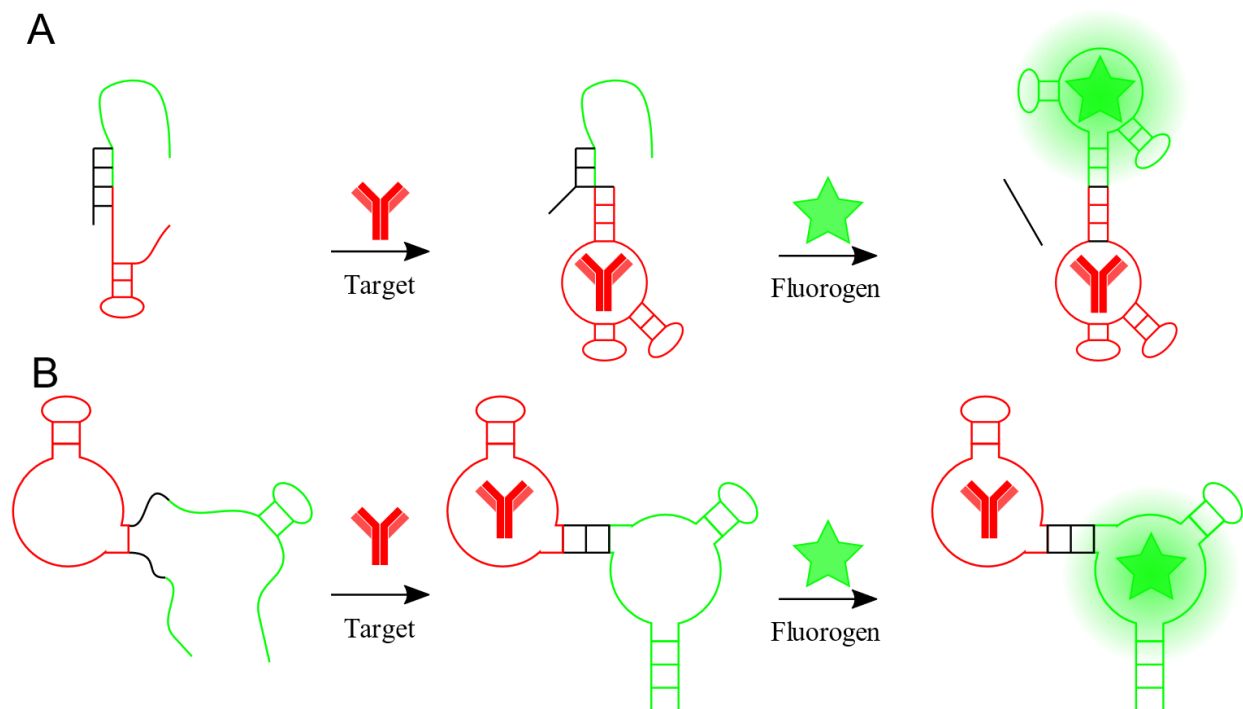


Figure 4.8 | Modular aptamer design for IgE

A schematic of a modular aptamer sensor that contains a fluorescent module and a IgE sensing domain. In **A** the sensor contains the two domains fused in tandem with a bridging strand. Two aptamers are fused through a linker region, one for the target molecule (red) and one for a fluorogenic molecule (green). The bridging strand (black) blocks the fluorogenic molecule from binding to its cognate aptamer until the target molecule binds to its cognate aptamer. After which, the bridging strand is able to be displaced by the binding of the fluorogenic molecule. Optimization of the bridging strand and linking region needs to occur in order to achieve this result. In **B** the sensor contains the two domains but with the signaling domain bifurcated and then fused via a linker region. The split fluorogenic binding domain does not form until the target binding domain is occupied. This helps stabilize the fluorogenic binding domain and allows fluorescence to occur.

The fluorescence enhancement and IgE binding activity were tested separately and in the fused sensing module. The unlinked Mango aptamer displayed a significant fluorescence enhancement over the range of concentration tested and the unlinked D17.4 aptamer displayed

negligible fluorescence enhancement seen in Figure 4.9 **A**. Once linked, there is still significant fluorescence enhancement, however, it is significantly reduced compared to the unlinked aptamer. The extent of IgE binding was tested by running different components of the fused aptamer sensor on a native polyacrylamide gel and post-staining the gel with ethidium bromide to detect the position of the nucleic acid on the gel seen in Figure 4.9 **B**. When there is IgE added to the sample containing only D17.4 aptamer and the linked aptamer sensor, ethidium bromide signal is detected at the top of the well indicating that there is aptamer binding to IgE.

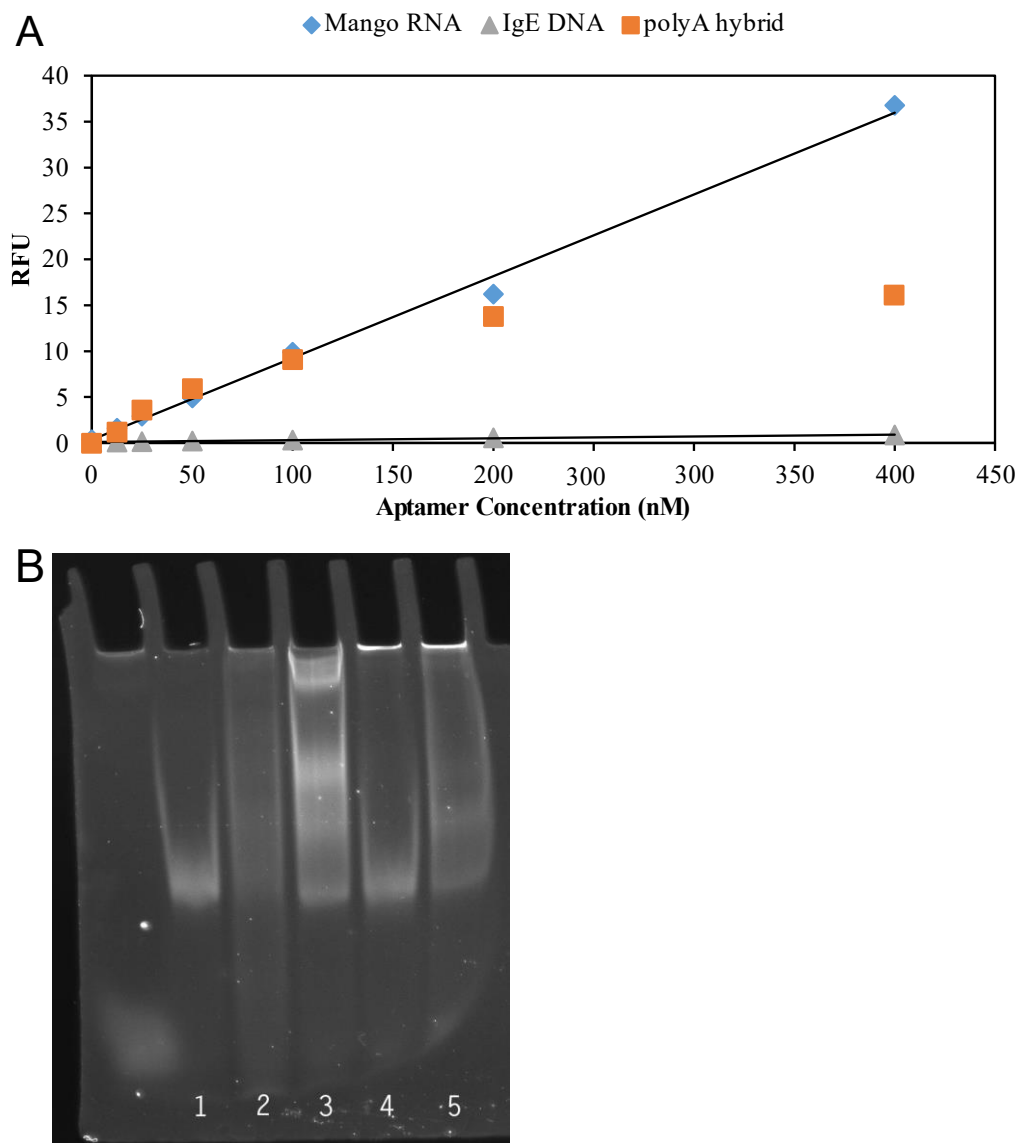


Figure 4.9 | Poly-A:poly-T linkage

The functionality of each aptamer moiety was tested after the linkage with a polyA-polyT hybrid. The fluorescence enhancement of the TO1-biotin dye molecule was measured as a function of nucleic acid concentration shown in **A**. The TO1-biotin dye was incubated with the anti-TO1-biotin aptamer (blue), anti-IgE aptamer (grey), and the linked hybrid (orange). Fluorescence enhancement was observed with the anti-TO1-biotin aptamer and minimal enhancement was observed with the the anti-IgE aptamer. The hybrid retained fluorescence enhancement activity; however maximum enhancement was reached at a lower concentration. The hybrid and their components were run on a native-PAGE mobility shift assay post-stained with ethidium bromide, shown in **B**. The samples loaded onto the gel include: 1 – anti-IgE aptamer, 2 – anti-TO1-biotin aptamer, 3 – aptamer hybrid, 4 – IgE and anti-IgE aptamer, 5 – IgE and hybrid aptamer. Although the RNA anti-TO1-biotin aptamer was not seen to form clear bands on the gel, the formation of the hybrid can be seen with the apparent band higher than that seen for the DNA anti-IgE aptamer.

When bound to the DNA aptamer, a detectable signal for the protein at the top of the gel is seen for the anti-IgE

Signal transduction can be achieved by utilizing the structure-switching phenomenon for the fluorescent module seen in Figure 4.10 **A**. Unlabeled DNA sequences complementary to various lengths, ranging from 9 – 17 nucleotides, of the RNA mango aptamer were synthesized to test the structure-switching capabilities of the aptamer. The sequences that contained 9 and 11 nucleotides of complementary sequences to the 5' end of the aptamer was able to recover over 80 percent of the fluorescence enhancement signal, suggesting that structure-switching of the double stranded sequence was successful shown in Figure 4.10 **C**. The design of a sequence that may not disrupt the IgE binding function whilst disrupting the TO1-biotin binding function and subsequently becoming unstable upon IgE binding induced structure-switch has proven to be highly challenging. Although studies have shown that this challenge can indeed be overcome (270, 271), in this work linking the RNA and DNA aptamer adds another layer of complexity.

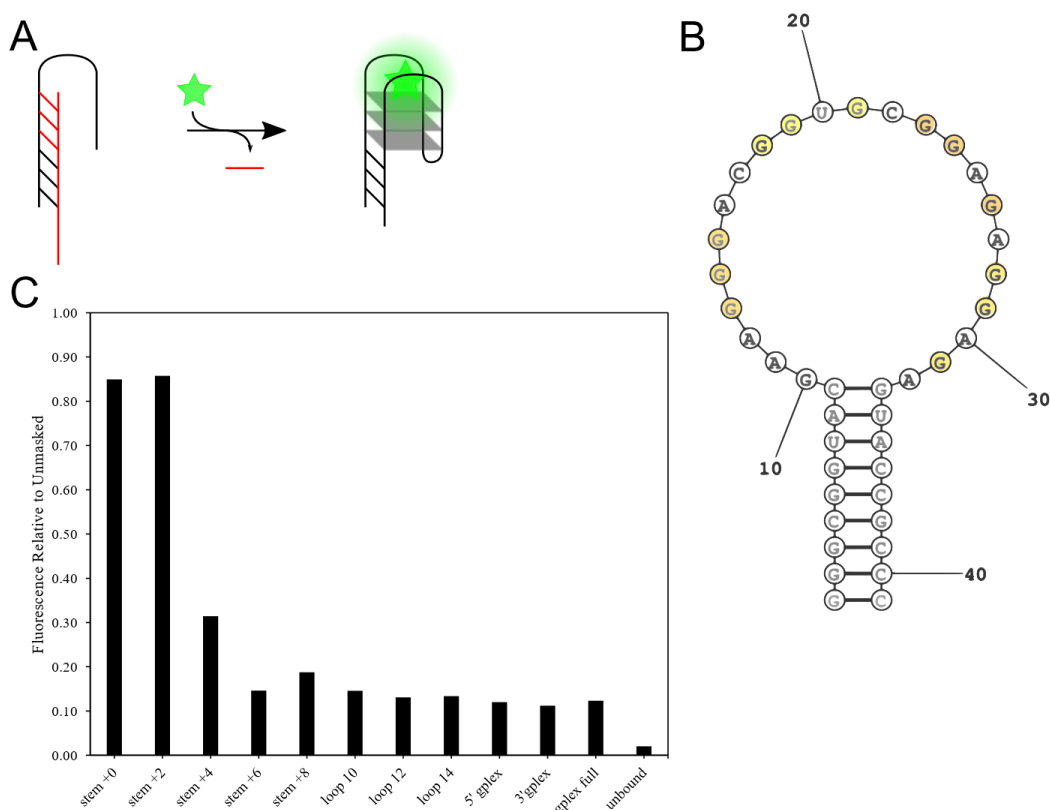


Figure 4.10 | Structure-switching RNA Mango

A schematic of the structure-switching module is shown in **A**. When a complementary oligonucleotide (red) is added to the aptamer (black), the formation of the double stranded structure occurs. When the target (green) is present in the sample, the formation of the aptamer-target complex is able to displace the oligo resulting in fluorescence enhancement. The full sequence and predicted secondary sequence are shown in **B**. The 5' and 3' ends of the aptamer come together to form a stem with the middle of the sequence forming a loop, that forms a three-tiered G-quadruplex (yellow) when bound to the TO1-biotin target. The fluorescent signal based on the sequence identity is plotted in **C**. When the masking sequence contains just sequences from the stem region of the aptamer, structure-switching of the aptamer is able to displace the sequence, resulting in fluorescence enhancement of the bound aptamer-target complex. When the masking sequence binds to any of the guanines required for the formation of the quadruplex, structure-switching of the aptamer is not allowed, resulting in a severe reduction of the fluorescence enhancement ability of the aptamer.

Ideally, the aptamers can be fused forming a single molecule as the fusion of the modules is not covalent and linkage may become disrupted, especially in the complex matrix of human serum. This can be done if both of the sensor modules are of the same chemical nature, which can be the case if the aptamer selected for TO1-biotin is used to form the modular sensor. The

formation of the modular sensor allows for flexible design, either with a bridging strand that can allow for signal transduction similar to with a linked aptamer design (80), or with the bifurcation of the signaling module that can be reconstituted once the sensing module binds to the target molecule (184).

4.3 Materials and Methods

4.3.1 Chemicals

Unless otherwise stated, all buffer material used for testing was purchased from VWR (Radnor, PA). The labeled and unlabeled DNA sequences listed in Table 4.1 was synthesized and purchased from IDT (Coralville, IA). The gold LSPR sensor chip and streptavidin coated sensor chip used for LSPR testing were purchased from from Nicoya (Kitchener, Canada). The FluoSphere carboxylate-modified microspheres 0.2 μm used for sensing the lateral flow assay was purchased from Invitrogen (Waltham, MA) NeutrAvidin protein, EZ-Link NHS-biotin, Sulpho-NHS and EDC was purchased from Thermo Fisher (Waltham, MA). *Phleum pratense* Phl p 5 was purchased from Sino Biological (Wayne, PA). The antibodies used for the lateral flow assay was purchased from Abcam (Cambridge, UK).

4.3.2 LSPR Assay

The OpenSPR system by Nicoya (Kitchener, Ontario) was used to analyze the binding chips and streptavidin-functionalized gold chips were used to analyze the binding of either thiol functionalized or biotin functionalized DNA, respectively.

Standard gold sensor chips were prepared by first rinsing with MilliQ water, ethanol, acetone then isopropanol (IPA). The sensor chip was then soaked in an IPA bath for 5 minutes and subsequently dried using filtered air. The chip was then treated with a 7:3 piranha acid solution (sulfuric acid and 30% hydrogen peroxide mixture) for 10 minutes before being quenched with MilliQ water. The chip is then rinsed with MilliQ water, followed by ethanol before being submerged in an ethanol bath for 1-2 hours.

Streptavidin sensors sensor chips were prepared by first rinsing with MilliQ water followed by IPA. The sensor chip was then soaked in an IPA bath for 5 minutes and subsequently dried using filtered air.

For both types of sensor chips, the prepared chips are mounted into the OpenSPR system using a cool light LED source, 100 μL sample loop and the running buffer, PBS-MT pH 7.4 (10 mM Na_2HPO_4 pH 7.4, 100 mM NaCl, 40 mM KCl, 1% Tween 20 and 50 μM MgCl_2). Once the prepared chip is mounted, the system is flushed with the running buffer for 10 minutes at 150 $\mu\text{L}/\text{min}$. An injection of 100 μL IPA at 150 $\mu\text{L}/\text{min}$ for 30 seconds is used to remove any bubbles in the system. Before conjugation of the sensor chip, 100 μL injections of 10 mM glycine HCl pH 2.3 at 150 $\mu\text{L}/\text{min}$ is used to achieve a stable baseline. When a stable baseline is achieved, 100 μL of 2.5 μM conjugated aptamer is injected at 40 $\mu\text{L}/\text{min}$ to form the monolayer on the surface of the sensor chip. Multiple injections are used to reach saturation on the surface monitored by the LSPR signal.

Before the injection of the sample of IgE, the conjugated sensor chip is conditioned with the regeneration buffer (10 mM glycine HCl pH 2.3) at 40 $\mu\text{L}/\text{min}$. Once stabilized, 100 μL of 1.23 – 100 nM IgE was injected, in order of increasing concentration, with an association time of

240 s and dissociation time of 150 s. Between each injection of sample 100 μL of the regeneration buffer was injected at 150 $\mu\text{L}/\text{min}$ and the baseline was allowed to restabilize.

The obtained data was analyzed using the TraceDrawer software and fit to a 1:1 Langmuir isotherm for each concentration to obtain the association (k_a), dissociation (k_d) and equilibrium dissociation (K_d) constants.

4.3.3 Electrophoretic Mobility Shift Assay

Native PAGE was used to monitor the interaction between the different aptameric species and several proteins in the sample, most notably IgE. Labeled D17.4 aptamer was used to probe the specificity of the protein-aptamer interaction in the serum sample. Unlabeled Mango and D17.47 aptamers were used to monitor the formation of the RNA-DNA hybrid and the interaction of this hybrid with free IgE and post-staining with ethidium bromide is used to visualize the nucleic acids on the gel.

When monitoring the binding of the Cy5-labeled D17.4 aptamer to the serum sample, the samples are loaded onto a 7.5% 37.5:1 acrylamide:bis-acrylamide non-denaturing polyacrylamide gel. Each well contains serum samples ranging from 7.1 - 458.4 μg protein, quantified via the Bradford method, that was incubated with 1 μM labeled aptamer. These samples were run in parallel with BSA samples ranging from 1 – 100 μg protein incubated with 1 μM labeled aptamer. The incubated samples were loaded onto the gel with a 40% glycerol loading buffer and run at 250 V using TBE buffer pH 8.0. The gel image was captured with a FluorChem FC2 Imaging System using an excitation laser of 640 nm provided by a DRC III Fiber Optic Light Source Illuminator with a 670/30 nm bandpass filter to visualize the Cy5-labeled DNA. The gel was then stained with

the Coomassie staining method to visualize the protein and the gel image was captured on a light box.

When monitoring the binding of the DNA-RNA hybrid to purified IgE, the samples are loaded onto a 7.5% 37.5:1 acrylamide:bis-acrylamide non-denaturing polyacrylamide gel. Each well contains 400 ng nucleic acid with either the D17.4 DNA aptamer, Mango RNA aptamer or a 1:1 mixture of each with some samples incubated with 5 ng IgE in 40% glycerol loading buffer. The gel was run at 250 V using TBE buffer pH 8.0 and post stained with 0.5 µg/mL ethidium bromide in TBE buffer pH 8.0 for 30 minutes, destained with MilliQ water for 10 minutes and visualized with a FluorChem FC2 Imaging System.

4.3.4 Fluorescence Assay

In order to test the structure-switching capabilities of the D17.4 DNA aptamer and Mango RNA aptamer, the fluorescence of the Cy5-labeled DNA or fluorescence enhancement of TO1-biotin was monitored using a SpectraMax M5 Microplate Reader from Molecular Devices LLC. Complementary DNA oligonucleotide sequences of the aptamers were ordered from IDT with a Black Hole Quencer® - 2 (BHQ-2) moiety attached to the 3' end of the D17.4 aptamer complement and unlabeled DNA for the Mango aptamer complement.

Table 4.1 | List of DNA sequences used in Chapter 4

Label	Sequence
D17.4	GGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCCCC
D17.4 ext	GCGCGGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCCCCGCGC
Cy5 D17.4	/5Cy5/GGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCCCC
Cy5 D17.5 ext	/5Cy5/ GCGCGGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCCCCGCGC
13Q D17.4	ATAAACGTGCCCC/3BHQ_2/
17Q D17.4	ACGGATAAACGTGCCCC/3BHQ_2/
13Q D17.4 ext	ACGTGCCCCGCG/3BHQ_2/
17Q D17.4 ext	ATAAACGTGCCCCGCG/3BHQ_2/
Am D17.4	GGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCCCC/3AmMO/
Am D17.4 ext	GCGCGGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCCCCGCGC/3AmMO/

4.3.5 Nanoparticle Synthesis

Conjugation of amine modified D17.4 DNA aptamer to carboxylate-modified nanoparticles labeled with europium is performed as followed. The 0.2 μm Fluro-Max™ Florescent Carboxylate-Modified particles is vortexed briefly (20 seconds) and a 100 μL aliquot is put into a 1.5 mL tube. The beads are then centrifuged at 14,000 rcf for 10 minutes, washed twice and resuspended with 0.1 M MES buffer pH 6.5. An EDC solution in 0.1 M MES pH 6.5 and a NHS solution in 0.1 M MES pH 6.5 are made and added to washed beads for a final concentration of 10 mg/mL EDC and 0.9 mg/mL NHS. The beads are then sonicated for 30 seconds to disburse the beads and allowed to incubate for 30 minutes. After the incubation period, the activated beads are centrifuged at 14,000 rcf for 6 minutes, washed twice and resuspended with 50 mM borate buffer pH 8.0. The activated beads are incubated with 20 μM amine modified D17.4 DNA aptamer, sonicated for 30 seconds to disburse the beads and allowed to incubate for 2 hours. The conjugated beads are then centrifuged at 10,000 rcf for 10 minutes, washed twice with PBS-M (10 mM sodium phosphate buffer pH 7.4, 140 mM potassium chloride and 1 mM magnesium

chloride) and resuspended in 1 mg/mL HSA in PBS-M. The beads are then sonicated for 30 seconds to disburse the beads and incubated overnight.

4.3.6 Lateral Flow Strip Fabrication

Half-strips of the lateral flow tests constructed from the nanoComposix (San Diego, CA) lateral flow material starter kit. The strips are composed of a backing card that has an adhesive that holds the nitrocellulose membrane and wick pads together. The sample pad and conjugation pad were excluded to allow for simpler assay optimization. Excess backing card was removed with a guillotine paper cutter. The test line and control line was printed on the nitrocellulose membrane of the half strips using an Agismart Rapid Test Printer – RP1000 from Rega Biotechnology (New Taipei City, Taiwan). The test line contains 1 µg/mL of the antigen for Timothy grass, Phleum pratense Phl p 5, and the control line contains 1 µg/mL mouse-anti-goat IgG. The assembled card was cut into 1.5 cm wide strips using a Test Strip Cutter QG001 Rapid Test Guillotine Cutter by Werfen Equipment (Haining, China).

4.3.7 TO1-biotin Synthesis

The synthesis of the TO1-biotin derivative was done following the procedure outlined in the supplementary information of the published paper for the RNA aptamer (77) and provided to us under the supervision of Dr. M. Chong. The sample was purified by silica column chromatography and confirmed by NMR spectroscopy and ESI-MS.

4.3.8 RNA Mango Synthesis

Production of the MGA RNA aptamer is done using a modified *in vitro* bacteriophage T7 RNA polymerase (T7 RNAP) protocol (272). His-tagged T7 RNAP was overexpressed in *E. coli* containing a plasmid coding for the ampicillin resistance and purified through a nickel-nitrilotriacetic acid (Ni-NTA) column. The protocol for RNA production uses double stranded DNA (dsDNA) as the template for transcription. A solution containing the DNA template, NTPs, tris-buffered saline (TBS), dithiothreitol (DTT), MgCl₂, spermidine and T7 RNAP was incubated at 37°C for 6 hours. The crude mixture is run through a urea-denatured 12% polyacrylamide gel and the bands containing the aptamer, visualized by UV shadowing, is cut out and eluted overnight using a 300 mM NaCl solution. The sample is precipitated using ethanol, lyophilized then resuspended with Milli-Q grade water. Purification is done with a weak anion-exchange fast performance liquid chromatography (FPLC) column (273) and lyophilized. The final step is to resuspend the sample and run it through a desalting size-exclusion FPLC column. Nanodrop™ analysis is done to quantitate the amount of RNA produced.

Table 4.2 | List of RNA sequences used in Chapter 4

Label	Sequence
RNA Mango	GGGCGGTACGAAGGGACGGTGCGGAGAGGAGAGTACCGCCC

4.4 Conclusions

As a sensing module for human IgE, the D17.4 aptamer has been shown to perform well, as can be seen in the multitude of publications that use this sequence as the aptamer in biosensing applications (257–259). Certainly, in our work, we show that the D17.4 aptamer is able to bind isolated human IgE with good sensitivity and selectivity, however based on the sensing platform,

these parameters can vary. When it comes to binding in a complex matrix such as human serum, the results are much more mixed with respect to the performance of the aptamer.

LSPR showed excellent selectivity, good sensitivity and was able to detect concentrations as low as 1 nM for the isolated IgE protein. Further optimization would be required to lower the detection limit to the pM range that can be present in serological samples, especially for specific IgE (274). Furthermore, the sensitivity of biosensors becomes diminished in complex media such as serum due to non-specific binding (275). The platform allows for flexible sensing with either the aptamer or the antigen bound to the surface to capture total IgE or specific IgE, respectively. A major drawback of this sensing method is that it is not accessible to many people due to cost of the instrument and the scalability of the system is limited.

Electrophoretic methods can be beneficial for detection of specific proteins in a complex matrix and have been used for the detection of allergen specific IgE in serological samples (276, 277). In this study, the gel shift assay shows a detectable amount of IgE in serum samples as low as 1.4 $\mu\text{g/mL}$ which is in the range for the average concentration of IgE in typical human serological samples. Although this test condition is a good stand-in for solution state testing, there are anomalous signals due to aberrant interaction with serum albumin, which is the most abundant component of human serum. This may be due to the nature of the test that may hinder the flow of the aptamer through the gel matrix due to the sheer amount of the protein; the fluorescent signal is not seen at the HSA location at lower serum concentrations. In this study, it was shown that an extended aptamer sequence engineered by the Prohaska group allowed for more specific interaction with the target and allowed for less off-target binding.

There are many examples of directly labeled aptamer sensors that utilize structure-switching for sensing purposes (278–280). In this work, a structure-switching sensing module was

designed that utilized Cy5 as the fluorescent module and a BHQ-2 quenching oligonucleotide strand to form the sensor. When the cognate ligand, IgE, binds to the sensor, the double strand is disrupted, and the aptamer forms the ligand bound structure, resulting in the recovery of fluorescent signal due to the separation of the quencher strand. At the concentrations tested in this work for the purified IgE protein, recovery of the fluorescent signal did not occur. However, signal recovery was seen for the human serum sample as well as with high concentration of BSA. In this case, the sensitivity of the sensor needs to be calibrated such that smaller concentrations of the specific target would need to cause structure-switching-induced fluorescence recovery. This can be achieved by modulating the length and concentration of the quenching strand.

Although lateral flow assays allow for ease of use for the end-user, the optimization of the assay can be complex. The detection of isolated IgE using both the D17.4 aptamer and its extended version proved successful. Interestingly, while the D17.4ext sequence allowed for more specific binding interactions using an electrophoretic approach, the performance in the lateral flow assay was lacking. This is due to the fact that the nanoparticle conjugates formed using the extended aptamer resulted in more aggregation that may have been due to cross-linking. However, when it came to the serum sample, the positive signal from either aptamer conjugate was much weaker than the antibody conjugate and will require more optimization to elicit a stronger response.

In order to design a more cost-effective sensing module, moving away from using antibodies and a proprietary nanoparticle is required. The RNA aptamer for TO1-biotin, Mango I, showed good fluorescence enhancement and was still functional when linked to the D17.4 aptamer. With the selection of the DNA aptamer for TO1-biotin, a functional modular aptamer for human IgE can be achieved.

Chapter 5 | Summary and Future Work

5.1 Summary

The selection of a DNA aptamer for the thiazole orange derivative, TO1-biotin, was successful with the 02a aptamer candidate showing the highest fluorescence enhancement and affinity out of the 8 families tested. From the selection pool with diversity on the order of 10^{13} sequences, 9 rounds of selection were performed using streptavidin-coated magnetic beads to capture the biotinylated dye onto the solid phase. A sample taken after the 4th and 9th rounds of selection were sequenced using NGS resulting in reads on the order of 10^5 for both samples. NGS analysis using the FASTAptamer-toolkit showed a convergence of the sequence space resulting in enrichment of sequence reads from round 4 to round 9. Although the sequence families 01a, 02a and 03a represent over 69% percent of the sequence space for the library after the 9th round of selection, the 02a candidate alone showed significantly higher fluorescence enhancement and affinity when compared to the test set.

When compared to other published DNA aptamers for fluorogenic dyes (107, 108), the 02a aptamer selected in this work performs exceptionally well with respect to fluorescence enhancement and affinity, displaying an order of magnitude better fluorescence enhancement and several orders for the dissociation constant. However, when compared to the RNA mango aptamer (77), the affinity is much lower in the selected DNA counterpart. This is not atypical, as many DNA aptamers have a reported lower affinity when compared to selected RNA aptamers to the same target (205); it is suggested that this may be due to the access to more structural modalities for RNA compared to DNA (188). However, for the use in a biosensor, DNA is the obvious choice due to the inherent stability and resistance to degradation, when compared to RNA.

Amongst the 27 clustered families from the 9th round of selection, a conserved 21-nucleotide binding motif containing high G content was discovered by primary sequence analysis via sequence alignment and motif elucidation. Using secondary structure analysis tools, RNAfold and QGRS, most sequences are predicted to form a parallel three-tiered G-quadruplex with the motif fully contained in the quadruplex. To test the predicted secondary structures for potential quadruplex formation, ion dependency titration and circular dichroism spectroscopy were used to probe the 02a aptamer sequence. The fluorescence enhancement and affinity for the TO1-biotin target is significantly enhanced in the presence of potassium ions, whereas the presence of sodium ions has a negligible effect observed via titration. The circular dichroism spectrum features a characteristic peak at 264 nm and trough at 245 nm suggesting that the overall structure of the aptamer is indeed a parallel G-quadruplex.

The discovery that the selected aptamer contains a G-quadruplex should come as no surprise as many aptamers, especially DNA aptamers, contain a quadruplex motif (281). It is suggested that this is due to the fact that the quadruplex motif is one of the most stable structures that is accessible to DNA, whereas RNA has access to alternative stable folds (222). When comparing to the classical quadruplex aptamer, thrombin binding aptamer, the RNA (28) and DNA (282) versions both form a quadruplex with the same topology, anti-parallel, and the binding modality of the aptamers is retained despite the change in chemistry. The RNA Mango aptamer is shown to adapt a parallel quadruplex motif, with the dye bound on one face of the quadruplex (83). That suggests that the interaction of the TO1-biotin dye may be favorable to any parallel quadruplex, with the loops providing specificity to the binding pocket for the dye.

Various truncations of the full-length sequence were tested in order to discern the role of the motifs in the 02a aptamer sequence. Although the 21-nucleotide motif discovered by sequence

alignment was highly conserved across all clustered families, the sequence alone was not enough to elicit a strong fluorescence enhancement signal, relative to the full-length sequence. The sequence that contained the whole predicted G-quadruplex motif had some interesting properties in that the affinity was quite high, comparable to some full-length aptamer candidates, and additionally did not display a significant difference in signal intensity in the presence or absence of K^+ . This suggests that the quadruplex sequence can be considered the core binding sequence, but the aptamer requires flanking sequences to access K^+ -mediated fluorescence enhancement conformations. This is supported by the fact that thiazole orange has an affinity for G-quadruplexes and the full-length sequence shows retention of the parallel G-quadruplex character on the CD spectrum in the absence of K^+ ; this suggests that the G-quadruplex structure may be accessed from just the motif sequence and TO1-biotin is able to bind to this K^+ -absent quadruplex to elicit a diminished fluorescence enhancement activity. It is shown that the connection between affinity and fluorescence enhancement is not always clear. This can be explained by the fact that the dye may bind in an arrangement that is not optimal for fluorescence.

In the effort to design a sensor for the detection of the biomarker related to allergies, human IgE, several sensing form factors and sample types were used to evaluate the efficacy for commercial application. LSPR, electrophoretic, structure-switching, LFIA, and modular aptameric approaches were evaluated on samples with isolated human IgE and human serum samples. The structure-switching approach aside, the other methods were able to detect isolated IgE in the sample. However, these methods may not be suitable on a commercial level due to the relative slow response and higher upfront cost. When samples of human serum were introduced, many methods fell short due to the complex mixture of proteins in the sample. In order to create a cost

effective and facile sensor, a separation step may be required, or greater optimization of the assay must be done to overcome the complex matrix of human serum.

5.2 Future Work

In order to obtain an aptamer that has a higher affinity for the target TO1-biotin, a reselection under more stringent conditions may be desired. In the case that standard reselection does not result in a tighter binding aptamer, as was the case for the Mango I (77), more advanced techniques may be needed such as microfluidic droplet-based functional selection (102), which resulted in Mango II, Mango III and Mango IV. The fluorescence enhancement can be more rigorously determined by measuring the quantum yield of the free dye and comparing it with the bound complex, as was done with the RNA Mango I aptamer. Further investigation into the structure of the aptamer sequence of 02a may provide the insight needed to guide rational sequence minimization. Structural analysis via UV-Vis absorbance at 295 nm rather than 260 nm can be used to report on the presence of a quadruplex (230). This can provide further evidentiary support to the structure of the sequence proposed in this work, however this method does not report on the topology of the quadruplex. Porphyrin binders such as 5, 10, 15, 20-Tetrakis-(N-methyl-4-pyridyl)porphine (TMPyP4) have been shown to exhibit selectivity towards parallel quadruplex (283) and can be used to probe the topology of the DNA system. Alternatively an atomistic approach may be employed; NMR spectroscopy can be used to characterize the binding face on the quadruplex (284) that was not provided with the CDS characterization of the quadruplex structure.

In order to create a modular aptamer sensor, the sequence of the D17.4 aptamer and 02a aptamer can be synthesized in tandem. A bridge design with optimized bridging sequence (285)

may be explored to create a modular sensor, however optimization of a unimolecular design may be less complex. In order to form a unimolecular modular aptameric sensor with the D17.4 aptamer, bifurcation of the 02a sequence must occur (164). There have been examples of split quadruplex sequences in literature that were able to be reconstituted in solution when both halves of the sequence were present in solution (286–288). The cleavage sites of the quadruplex must be chosen carefully to result in a stable quadruplex assembly and the typical cleavage occurs between guanine triples. The 3:9, 2:2 or 9:3 split modes, have been shown to result in successful reassembly of quadruplex (287). Interestingly, cleavage within a guanine triple, such as the 4:8 and 8:4 split modes, has been shown to result in better signal:noise ratio for analytical application when binding to a ligand (288). This may be a desirable cleavage site for the 02a aptamer.

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Appendix

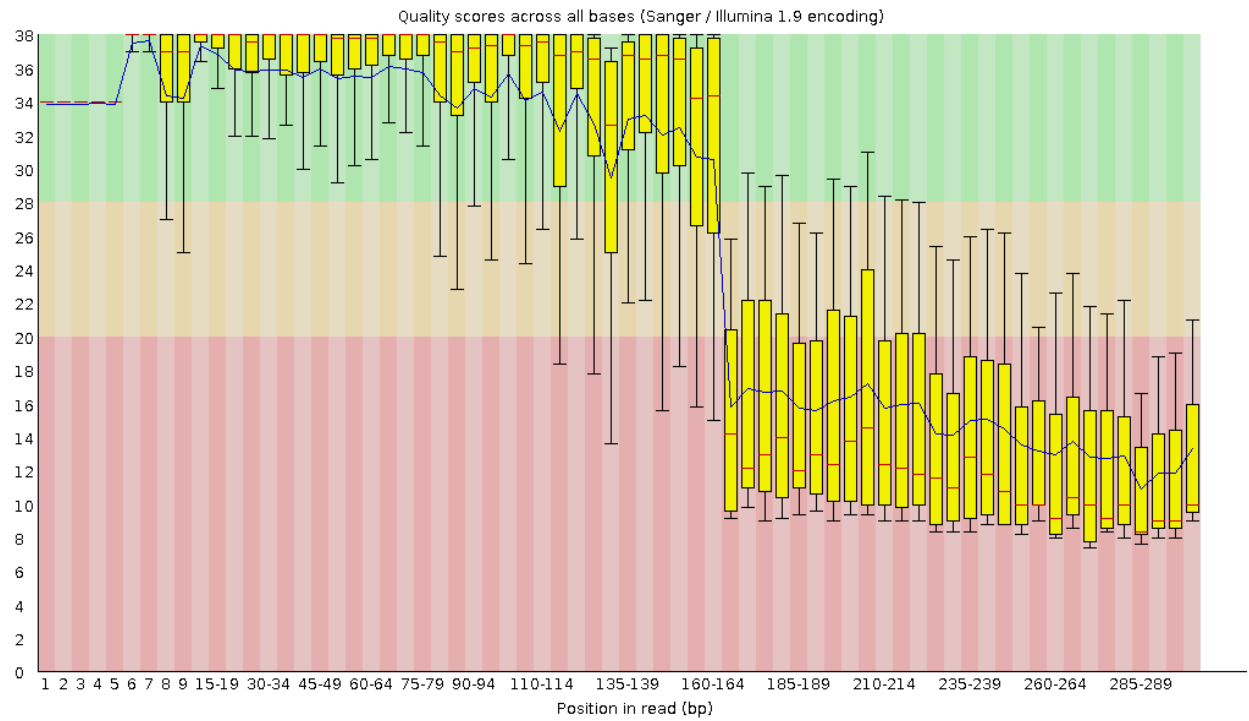


Figure 5.1 | Round 9 per base quality

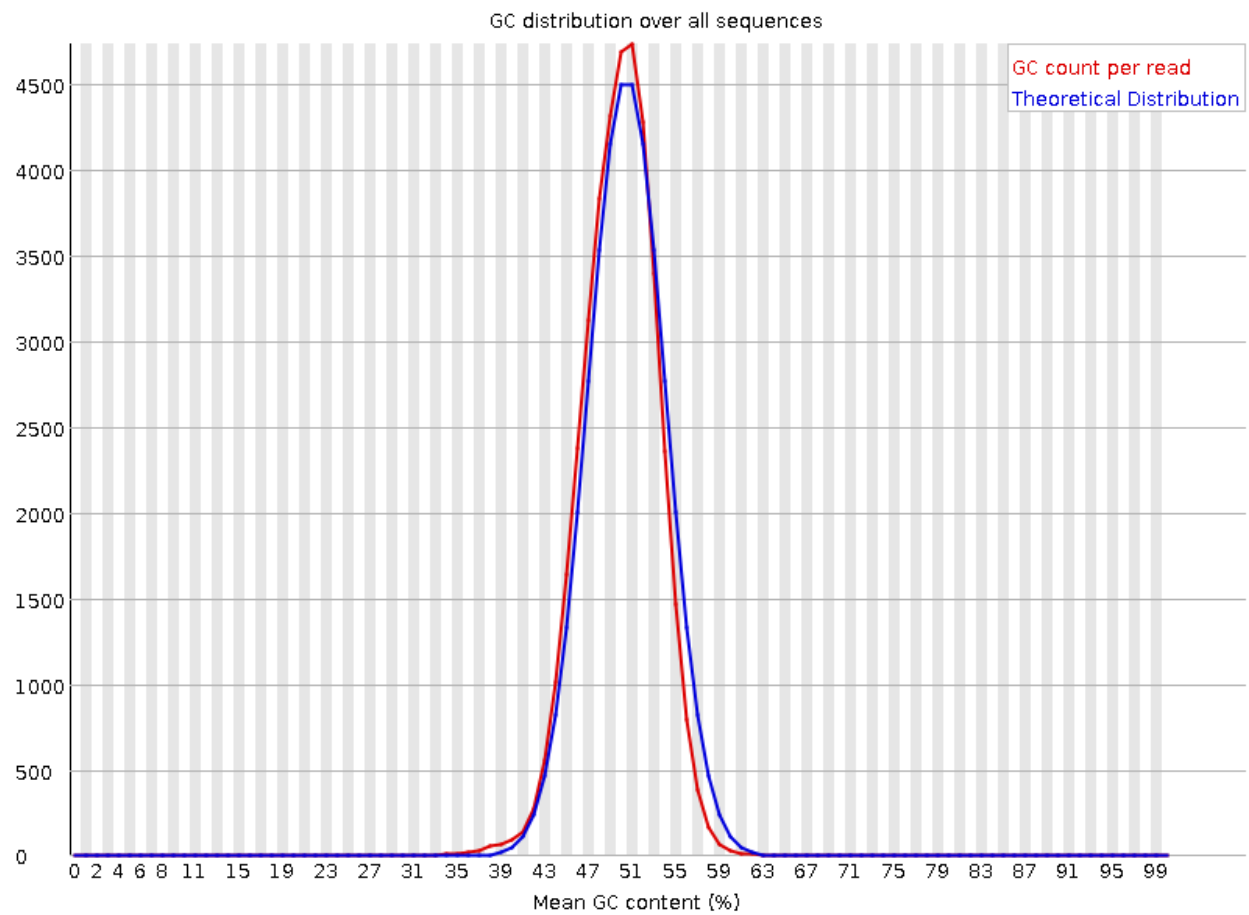


Figure 5.2 | Round 9 per sequence GC content

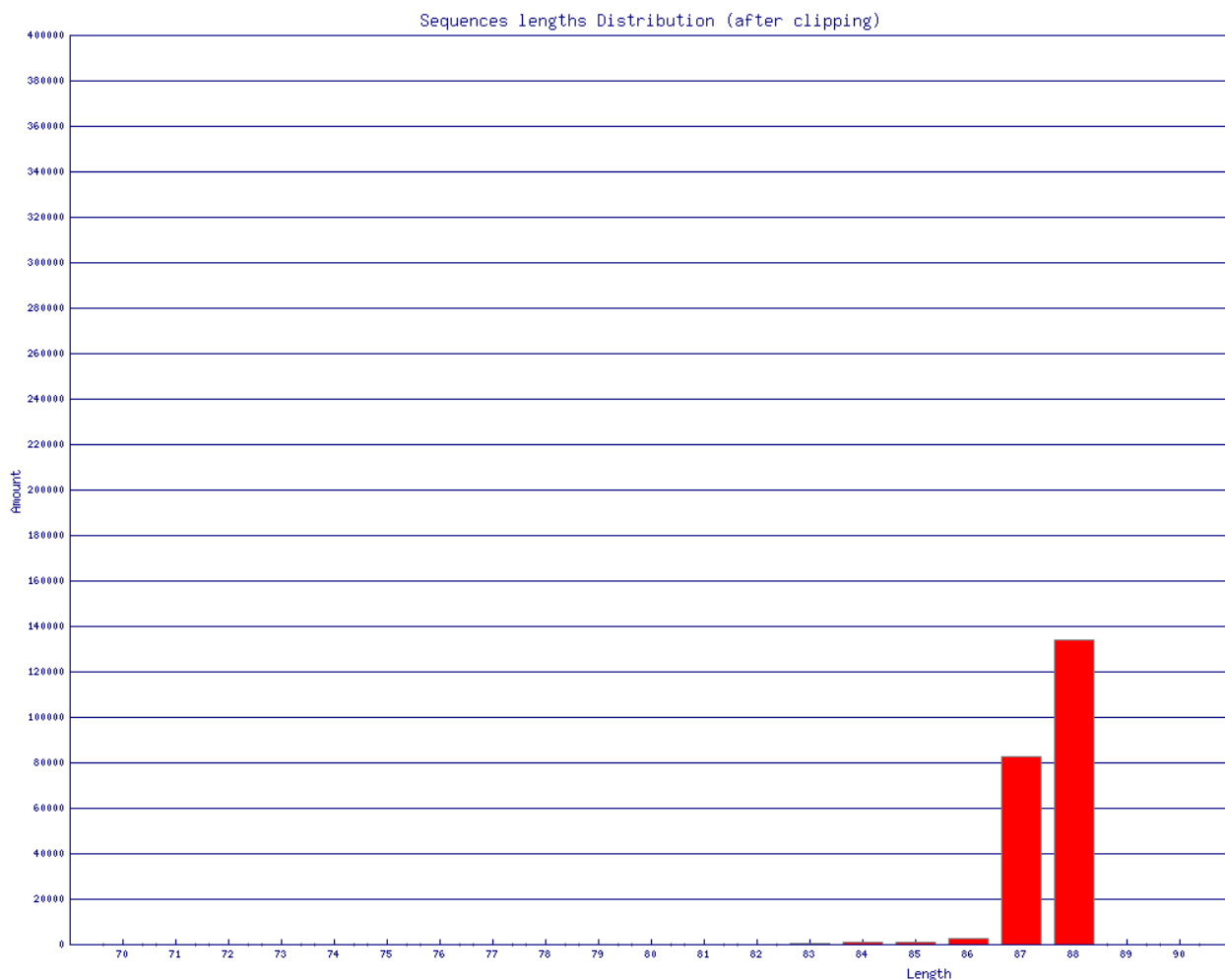


Figure 5.3 | Round 9 after clean-up

Table 5.1 | QGRS output for 02a

Position	Length	QGRS	G-Score
6	23	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GG</u>	14
6	23	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GG</u>	15
6	23	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GG</u>	15
6	23	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GG</u>	16
6	24	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GGG</u>	13
6	24	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GGG</u>	14
6	24	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GGG</u>	14
6	24	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GGG</u>	15
6	25	<u>GGAGGG</u> CAAGAG <u>GGT</u> GTTGAT <u>GGGG</u>	12

6	25	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGG	13
6	25	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGG	5
6	25	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGG	13
6	25	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGG	14
6	25	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGG	6
6	25	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGG	12
6	25	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGG	11
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	8
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	9
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	6
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	5
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	4
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	9
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	10
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	8
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	7
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	6
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	16
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	15
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	13
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	15
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	14
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	13
6	29	<u>GGAGGGCAAGAGGGTGT</u> TGATGGGGTCGG	2
9	22	<u>GGGCAAGAGGGTGT</u> TGATGGGG	13
9	22	<u>GGGCAAGAGGGTGT</u> TGATGGGG	14
9	26	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGG	17
9	26	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGG	15
9	26	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGG	13
9	26	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGG	18
9	26	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGG	16
9	26	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGG	14
9	26	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGG	5
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	19
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	18
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	16
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	8
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	21
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	19
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	17
9	29	<u>GGGCAAGAGGGTGT</u> TGATGGGGTCGGTGG	9

9	29	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	5
9	29	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	6
9	29	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	5
9	29	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	4
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	19
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	18
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	17
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	9
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	20
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	20
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	18
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	10
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	5
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	7
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	6
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	5
9	30	<u>GGGCAAGAGGGTGT</u>TGAT<u>GGGGTCGGTGGG</u>	40
9	30	<u>GGGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	39
10	21	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGG</u>	13
10	21	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGG</u>	14
10	25	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGG</u>	17
10	25	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGG</u>	15
10	25	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGG</u>	13
10	25	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGG</u>	18
10	25	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGG</u>	16
10	25	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGG</u>	14
10	25	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGG</u>	6
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	18
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	17
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	16
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	8
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	20
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	19
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	17
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	9
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	6
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	7
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	6
10	28	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGG</u>	5
10	29	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	18
10	29	<u>GGCAAGAGGGTGT</u> TGAT <u>GGGGTCGGTGGG</u>	17

10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	16
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	9
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	19
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	19
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	18
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	10
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	6
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	8
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	7
10	29	<u>GGCAAGAGGGT</u> GTTGATGGGGTCGGTGGG	6
17	18	<u>GGGTGTTGAT</u> GGGGTCGG	13
17	21	<u>GGGTGTTGAT</u> GGGGTCGGTGG	13
17	21	<u>GGGTGTTGAT</u> GGGGTCGGTGG	14
17	21	<u>GGGTGTTGAT</u> GGGGTCGGTGG	13
17	21	<u>GGGTGTTGAT</u> GGGGTCGGTGG	12
17	22	<u>GGGTGTTGAT</u> GGGGTCGGTGGG	13
17	22	<u>GGGTGTTGAT</u> GGGGTCGGTGGG	15
17	22	<u>GGGTGTTGAT</u> GGGGTCGGTGGG	14
17	22	<u>GGGTGTTGAT</u> GGGGTCGGTGGG	13
18	17	<u>GGTGTTGAT</u> GGGGTCGG	14
18	20	<u>GGTGTTGAT</u> GGGGTCGGTGG	14
18	20	<u>GGTGTTGAT</u> GGGGTCGGTGG	15
18	20	<u>GGTGTTGAT</u> GGGGTCGGTGG	14
18	20	<u>GGTGTTGAT</u> GGGGTCGGTGG	13
18	21	<u>GGTGTTGAT</u> GGGGTCGGTGGG	14
18	21	<u>GGTGTTGAT</u> GGGGTCGGTGGG	16
18	21	<u>GGTGTTGAT</u> GGGGTCGGTGGG	15
18	21	<u>GGTGTTGAT</u> GGGGTCGGTGGG	14
27	11	<u>GGGGTCGGTGG</u>	19
27	12	<u>GGGGTCGGTGGG</u>	19

Table 5.2 | S9R1 Clusters

ID	Sequence	RPM	Percent
01a	GGACTGGGGTGTTGATGGGGTGGGGGGCAGTCTGCTGTTTGTATCAGCCTCTG	337035.4	33.70%
02a	TATGTGGAGGGCAAGAGGGTGTTGATGGGGTCGGTGGGTCCTGCCCTCTCATCG	282115.3	28.21%
03a	TGATGACTTATCCGTGCGGGGTGTTGATGGGGTGGATGGGCGCGTAGGGAAGTC	70650.0	7.07%
04a	TGAGTGCGTTCTTATGGGGGTGTTGATGGGGCTGGTGGGCCACAAGGTACGCCC	26081.9	2.61%
05a	GAGTTGAATTTAGTGCCGGGGTGTTGATGGGGAGGCGGGCGGCCGATAAGATAC	24133.6	2.41%
06a	TCCCTGGAGAAGTTCGTGGGGTGTTGATAGGGTGGGGGGCAGAACTTCCCGGCT	23065.2	2.31%
07a	GACCGCGAGGGACTGGGTGTTGATGGGGTGAGGGAGTACCGACGCCGCGGTG	14437.1	1.44%
08a	ATGTCCAGACGAGGATCCCGGGGTGTTGATAGGGGGTGGGCGTTTCCTCGATG	12336.1	1.23%
09a	CTCTCGCTAGCGTATCCGGGGTGTGATGGGGTGTTGGGCGAGAGCGCTTAC	9050.1	0.91%
10a	CGGCTGGTTAAAGGGTGTGATAGGGTGGCGGGTTAACTGGGCGGCTTGTTGG	8744.8	0.87%

11a	GGATTGTAGCGGGGCATACTGGGTGTTGATGGGGTGGAGGGGTATGCCCCCTCA	8735.9	0.87%
12a	AACGGGGGCTTAGGGTGTGATAGGGTGGTGGGACTGCGCTCGGTGGTAGGTGA	6994.1	0.70%
13a	ACCGTTCGTGCTAGGGGTGTTGATGGGGCGGCGGGCAGTCCACGAACTGTTTCAG	6123.2	0.61%
14a	TCCAATAAGGTTTTGCTCGGGTGTGATGGGGCGGAGGGGATGCACCTTAATCG	5261.3	0.53%
15a	AGACTAGAGTGGCGACGTGGGTGTTGATGGTGGTGGTGGGTGGAGCCTACTCA	4552.0	0.46%
16a	GATCGCACCCCTTGGGCGGGGGGTGTTGATGGGGAGGCGGGTTCGCACGGGTCTA	4237.7	0.42%
17a	TTGTAACGTATCCGAGGTGGGTGTTGATGGGGCGGAGGGACCCGGGTTCGTTAT	4659.7	0.47%
18a	CTAGTGTCATCTCCTGAGTGGGGGTGTTGATGGGGTGGCGGGAATACCGATGGA	2765.3	0.28%
19a	AACGTTAATTGCGGCCTGGTGGGTGTTAGGGTGTGATGGGGTAGTGCCGTAAC	2567.8	0.26%
20a	TATTCCAGGTCGGGCGATTGGGTGTTGATAGTGGTGGCGGGAAAACGCCGATG	2091.9	0.21%
21a	TAAAGCAACGGTCTGGGGTGTGATGGGGTGGCGTGGCAGTCCCGGTGCTGATC	1903.4	0.19%
22a	TAGAATTGTCCCGAGCGGGTGTGATGGGGTGGTGGGGTTCGGGTCTATTCA	942.7	0.09%
23a	TCGACTGGGAATTTACAGGGTGTGATGGGGTGGCGGGTGCAAATTGCTGGTG	682.3	0.07%
24a	ATTGATGTAGGGTCTTGCAGAGGGTGTGATGGGGCGGCGGGTTTGTCTCATC	583.6	0.06%
25a	ATCTCTGTCGGACAACCTGGGTGTTGATAGCGGTGGTGGGAGGTGACAGTGGG	511.8	0.05%
26a	GATTTAGGGTCAATTAGTGGGTGTTGATAGGGTGGCGGGGCTAATTGAATCTTT	493.8	0.05%
27a	AGCCCTCGTAGCATGTAGGGGCGGGGGTGTGGGTGGCGGGAGGGCTAACTTGG	404.0	0.04%

Table 5.3 | S4R1 Clusters

ID	Sequence	RPM	Percent
07c	CACCGTCGAGGACCGTATAACCCCCACAGACGCGAGGGAGGGGTGAGGTGCTTG	1106.2	0.93%
02a	TATGTGGAGGGCAAGAGGGTGTGATGGGGTCGGTGGGTCTGCCCCTCTCATCG	536.3	0.45%
03a	TGATGACTTATCCGTGCGGGGTGTTGATGGGGTGGATGGGCGCGTAGGGAAGTC	452.5	0.38%
06a	TCCCTGGAGAAGTTTCGTGGGGTGTGATAGGGTGGGGGGCAGAACTTCCCGGCT	368.7	0.31%
01a	GGACTGGGGTGTGATGGGGTGGGGGGCAGTCTGCTGTTTGTATCAGCCTCTG	352.0	0.29%