GENERATION OF MODIFIED DNA APTAMERS TOWARD A COMPLEX WHOLE CELL TARGET AND INVESTIGATIONS INTO IMPROVING SELECTION METHODOLOGY WITH MODIFIED DNA

by

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Abstract

Since the discovery was made that DNA is capable of functions beyond genetic storage and propagation, much work has been done to explore the non-canonical abilities of this molecule. Two functionalities of DNA are of particular interest. The first is the ability of DNA to act as a catalyst, and the second is the ability of the molecule to bind with high affinity to various biological and non-biological surfaces. Although numerous DNA catalysts and binders (termed "aptamers") have been identified using the five nucleoside triphosphates found in nature, the functionality of the molecule can be expanded with the addition of protein-like functional groups to the base moiety of the nucleotide. Several modified nucleotides have been developed previously and used to discover novel catalysts and aptamers. However, the successful development of modified-DNA aptamers has thus far been limited, particularly with respect to biological targets that might be of use in a clinical or commercial context. Moreover, technical challenges in the field exist that have not yet been adequately addressed. Most significantly, the employment of the modified molecules in the discovery process can be problematic as they are not ideal substrates for many basic molecular biology procedures, notably polymerase chain reaction. New techniques to overcome these difficulties are needed and few have been developed.

This thesis will focus on two aspects of modified DNA research. The first is the application of modified DNA in aptamer discovery. Starting from the hypothesis that additional functional groups will confer chemical advantages in traditionally challenging aptamer selections, the objective was to identify a modified DNA aptamer for whole bacterial cells *in vitro*. Several promising phenol-dUTP-modified aptamer sequences were identified using a

modified SELEX procedure. The specificity and affinity of these sequences for the target bacterial strain were investigated.

The second aim of this work is to address technical problems encountered when working with modified DNA in order to develop catalysts or aptamers. Specifically, a novel selection scheme was designed that eliminated the need to amplify modified DNA. Research was conducted to develop and validate this selection system as a viable route to discovery of DNA catalysts.

Preface

This thesis is submitted as part of the requirements for the Master of Science degree at the University of British Columbia. All research was performed under the supervision of Dr. David Perrin in the Department of Chemistry at the Vancouver campus. The research included in this thesis is original and all experiments, analyses and interpretations were performed by myself. The contributions of any collaborators are listed below.

Chapter 2: The design of the initial selection scheme was aided by Dr. Marleen Renders. All bacterial strains were provided by Jessie Chen and Dr. Elena Polishchuk at the Department of Chemistry's Biological Services Laboratory. The yeast strain *Saccharomyces cerevisiae* was a gift from the Loewen laboratory at the Life Sciences Institute, University of British Columbia. The phenol-modified dUTP utilized for cell-SELEX was previously synthesized in-house by Dr. Curtis Lam.

Chapter 3: The new *in vitro* selection scheme was designed in collaboration with Dr. Marleen Renders; however, all experimental work and analyses included in the thesis was performed by myself. The modified dUTPs utilized were previously synthesized in-house by Dr. Chris Hipolito and Dr. Curtis Lam. The work described in this chapter was included in a manuscript: Renders, M.‡, Miller, E.‡, Hollenstein, M., & Perrin, D. (2015). A method for selecting modified DNAzymes without the use of modified DNA as a template in PCR. Chemical Communications, 51(7), 1360-1362. (‡ contributed equally to this work)

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A	adenosine
BSA	bovine serum albumin
C	cytidine
cDNA	complementary DNA
CFU	colony forming unit
CPM	counts per minute
dA	2'-deoxyadenosine
dATP	2'-deoxyadenosine triphosphate
dC	2'-deoxycytidine
dCTP	2'-deoxycytidine triphosphate
dG	2'-deoxyguanosine
dGTP	2'-deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNAzyme	DNA enzyme
dNTPs	2'-deoxynucleoside triphosphates
DPBS	Dulbecco's Phosphate Buffered Saline
dT	2'-deoxythymidine
dTTP	2'-deoxythymidine triphosphate
dU	2'-deoxyuridine
dUTP	2'deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
G	guanosine

IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kd	dissociation constant
kDa	kiloDalton
kobs	observed rate constant
LB	lysogeny broth
LNA	locked nucleic acid
mDNA	modified DNA
N40	random 40 nucleobase DNA
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PNA	peptide nucleic acid
Ppi	pyrophosphate
rC	cytiditine
RNA	ribonucleic acid
SELEX	systematic evolution of ligands by exponential enrichment
Sequenase	engineered Family A polymerase lacking 3'-5' exonuclease activity
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
T4 PNK	T4 polynucleotide kinase
Taq polymerase	Family A DNA polymerase from thermus aquaticus
TEMED	Tetramethylethylenediamine
TEN	tris, EDTA, NaCl buffer
TNA	threose nucleic acid

Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U	uridine
VEGF	vascular endothelial growth factor
Vent (exo-)	Family B DNA polymerase derived from Thermococcus litoralis with 3'-5' proofreading ability removed
XNA	xeno-nucleic acid
λ exonuclease	5'-3' exodeoxyribonuclease derived from Escherichia coli

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I am deeply grateful to my amazing network of friends, both old and new, for the support, encouragement and entertainment they have given me throughout my degree.

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Chapter 1: Introduction

1.1 Nucleic Acids

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are two of the fundamental biopolymers of life. The respective roles of these molecules in the storage (DNA) and propagation (RNA) of genetic information are well established, but in the past few decades, non-genetic biological as well as abiological functions of DNA and RNA molecules have emerged. Discoveries such as siRNA, miRNA and ribozymes have expanded our view of nucleic acids *in vivo*, while research conducted in the early 1990s introduced these entities as synthetic molecules that could be exploited for their chemical properties. Concurrent work in the Gold and Szostak labs established that short, single-stranded synthetic RNA oligonucleotides could bind with high affinity to a selected ligand in a process termed Systematic Evolution of Ligands by EXponential enrichment (SELEX), while the group of Joyce discovered that DNA was capable of cleaving RNA *in vitro* [1, 2, 3]. Collectively, this groundbreaking work created new fields of research invested in investigating the catalytic and binding potential of synthetic nucleic acids.

1.1.1 Biological Structure of DNA and RNA

The nucleotide is the basic subunit of DNA and RNA, and consists of three components; one or more phosphate groups, a ribose or deoxyribose sugar, and a nitrogenous base. The base moiety distinguishes the five different nucleosides that make up the DNA or RNA 'alphabet'. The purines adenine (A) and guanine (G) are heterocyclic molecules with fused 5- and 6-membered rings found in both RNA and DNA. In contrast, the pyrimidines thymine (T), uracil (U) and cytosine (C) are 6-membered heterocycles. Thymine (T) is found in DNA, while uridine (U) is most often found in place of T in RNA. A single strand of nucleic acid links the 3′-hydroxyl group of the (deoxy)ribose to the 5′-hydroxyl group of the neighbouring nucleotide

through a phosphate linker, forming a series of phosphodiester bonds between adjacent sugar rings. The order in which the nucleobases are covalently attached, denoted 5′-3′, represents the primary structure of a DNA or RNA sequence.

The secondary structure of DNA is typically composed of two polynucleotide strands that associate with each other in an anti-parallel manner, forming a duplex with 3′-5′ directionality in one strand and 5′-3′ directionality in the other. The phosphodiester backbone that is formed faces outward into the cellular environment while the nucleobases face inward and associate with nucleobases on the opposite strand. In traditional Watson-Crick base pairing, the purine adenine associates with the pyrimidine thymine and forms two hydrogen bonds, while the purine guanine associates with the pyrimidine cytosine and forms three hydrogen bonds. Most often, the orientation and association of the two strands forms an asymmetric, right-handed helical structure with major and minor grooves, termed "B" DNA, although other helical structures are also found in nature. The biopolymer is largely stabilized by the hydrogen bonds between bases, base-stacking interactions, and interactions between metal cations and the negatively charged phosphate backbone. While DNA in a biological context is most often found in this double-stranded helical form, RNA is most often found in single-stranded form, although it too can adopt a double-stranded helical form similar to "A" form DNA. However, there are many examples of non-canonical nucleic acid structures in nature, including G-quadruplexes, circular DNA, Holliday junctions, triplexes, stem-loop and pseudoknot formations. These higher order structures encompass the tertiary structure of DNA, as they represent the specific three-dimensional shape of the duplex.

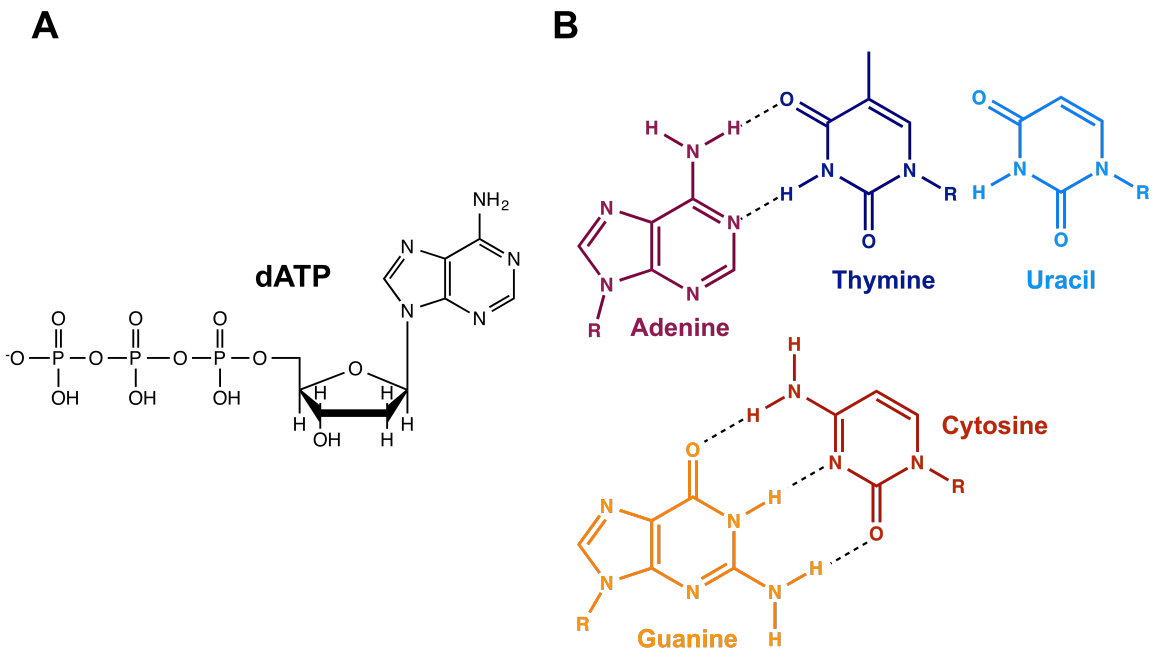


Figure 1.1 A) Nucleotide triphosphate structure B) The five naturally-occurring nucleobases shown in Watson-Crick base pairs. Uracil (light blue) replaces thymine (dark blue) in RNA.

RNAs can also act as enzymes in nature and are termed ribozymes in this capacity. Ribozymes can be divided into three categories: small self-cleaving RNAs, self-splicing introns and ribonucleoprotein enzymes. Small self-cleaving RNAs are 50-150 nucleotides in length and catalyze self-cleavage in a manner analogous to the RNA cleavage reaction performed by the protein enzyme RNAse A. An internal ribose 2′-OH group takes on the role of nucleophile and the molecule catalyzes phosphodiester bond cleavage in an S_N2-type reaction, producing 2′, 3′-cyclic phosphate and 5′-hydroxyl termini [4]. Conversely, self-splicing introns are much longer (in the range of hundreds of base pairs) and more structurally complex. They are found in many organisms and catalyze a site-specific two-step transesterification reaction reminiscent of the action of the spliceosome complex: the junction-attacking nucleophile is either an internal 2′-OH group or a free guanine nucleotide [5]. Ribonucleotide protein enzymes, of which RNAse P is the

most well-studied member, are hybrid RNA-protein enzymes in which RNA makes up the catalytic core. Ribonucleoproteins have been found to specifically cleave a variety of RNAs. In the case of RNAse P, the primary substrate is tRNA precursers [6]. Catalytic cleavage generates 5′-monophosphate and 3′-hydroxyl ends, in contrast to the cyclic moiety produced by small self-cleaving RNAs [6]. The peptidyl transferase center of the ribosome is also a ribonucleoprotein enzyme, and is somewhat unusual in that it does not catalyze RNA cleavage but promotes the formation of peptide bonds between amino acids [7]. It is also worth noting that the ubiquity of these enzymes in archaea, prokaryotes and eukaryotes constitutes one of the strongest arguments for the existence of a prebiotic RNA world.

The reaction mechanisms for some of the ribozymes described above are well understood, while others remain to be fully elucidated. The role of divalent metal ions in particular is an area of considerable interest, and has been shown to be critical for RNA catalysis in some cases but non-essential in others [5]. To date, no natural DNA equivalent of a ribozyme has been discovered.

1.1.2 Enzymatic Synthesis of Nucleic Acids

DNA is replicated in cells in a complicated process involving multiple enzymes and regulatory factors; the synthesis of RNA, termed transcription, is similarly complex. However, researchers have been able to distill the key components that make replication and transcription possible and use these simplified methodologies to enzymatically synthesize DNA and RNA strands *in vitro*. The core components consist of polymerases, many of which are now commercially available, dNTPs, a divalent metal, and two oligonucleotide strands. The first oligonucleotide strand provides the template for the desired sequence. The second strand is called the primer and is much shorter than the template strand. It is designed to be

complementary to a short segment of the template strand at the 3′-end. Once these strands have been thermally annealed, the polymerase is directed to work in a 5′-3′ direction, adding the complementary dNTP as it moves along the template strand and producing pyrophosphate (PPi) as a byproduct (Figure 1.2). Polymerase Chain Reaction (PCR) extends this basic process to obtain thousands or millions of copies of a desired sequence by repeatedly heating the double-stranded, newly-synthesized DNA in order to separate the strands, re-annealing the primer, and carrying out another round of polymerase synthesis using the thermostable *taq* polymerase [8].

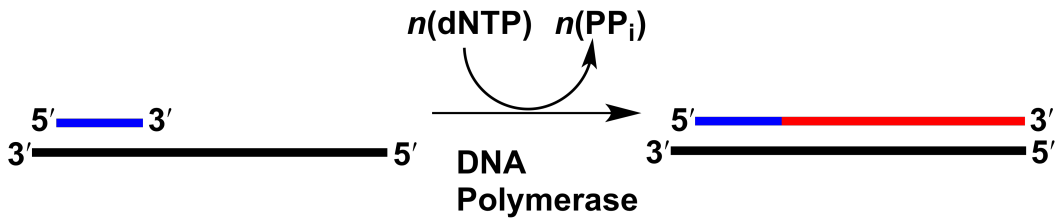


Figure 1.2 Enzymatic synthesis of DNA.

The template strand is shown in black, the primer in blue, and the newly elongated sequence is shown in red.

1.1.3 Solid-Phase Synthesis of Nucleic Acids

DNA and RNA can also be chemically synthesized via a solid-phase process developed in the 1980s from earlier work accomplished in the Khorana laboratory [9, 10]. In this process outlined in Figure 1.3, the base unit is a synthetically obtained nucleoside phosphoramidite monomer instead of a nucleoside triphosphate. The nucleoside phosphoramidite is protected at the 5′-position of the sugar, while the 3′-position is covalently linked to a solid support or modified with a phosphoramidite. The nucleobases' functional groups must also be suitably protected. Once the protective group at the 5' has been removed, a hydroxyl group is exposed that reacts with an incoming phosphoramidite under acidic conditions. A phosphite triester

linkage is formed, which is oxidized to a phosphate triester. Any unreacted 5′-hydroxyl groups on the solid-supported oligonucleotide are capped with acetyl groups, thus preventing any free 5′- hydroxyl groups from reacting with the next phosphoramidite. Successive rounds of de-protection, coupling, and capping are performed that elongate the oligonucleotide in a 3′-5′ direction. The final step is cleavage from the solid support and deprotection of the functional groups followed by purification, typically by HPLC. Although this process has grown markedly more efficient in the past decades, solid synthesis is generally suitable only for short oligonucleotide sequences. Synthesis of ribonucleotides is also performed, but remains more problematic due to the reactivity of the 2'-hydroxyl group.

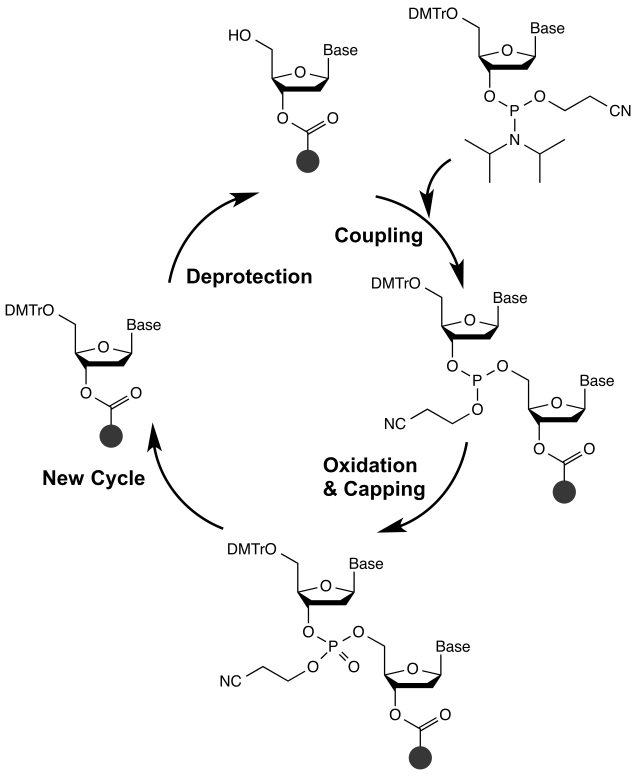


Figure 1.3 Solid phase synthesis cycle. DMTr refers to a 4,4′-dimethoxytrityl group.

1.2 Aptamers and Catalytic Nucleic Acids

1.2.1 Introduction to SELEX and *in-vitro* selection

Within the same month in 1990, the Szostak and Gold laboratories published groundbreaking research showing that short RNA sequences could selectively bind to various organic dye molecules and T4 DNA polymerase, respectively [2, 1]. Gold termed his process Systematic Evolution of Ligands by EXponential enrichment (SELEX), while Szostak called his process *in vitro* selection and anointed the selected RNA species "aptamers", from the Latin *aptos* ("to fit") and the Greek *meros* ("part"). Two years later, Szostak and co-workers were successful in generating single-stranded DNA aptamers to the same organic dye molecules that had been used in the RNA aptamer selection, and Toole *et al.* selected single-stranded DNA aptamers that were found to bind and inhibit human thrombin [11, 12]. Szostak posited that the positive results attained in generating DNA aptamers suggested that DNA might also be capable of catalysis. Catalytic RNA had been found to exist in nature, and in 1993 first artificial ribozyme was selected [13, 14, 15]. Szostak's hypothesis was borne out in 1994 with work published by Ronald Breaker and Gerald Joyce, in which they reported the selection of the first DNA enzyme[3]. This artificial, single-stranded DNA species was capable of Pb^{2+}-dependent cleavage of a single embedded ribose linkage at a turnover rate of approximately one per minute. Critically, all three pioneering works described above shared a selection method in which a ligand of interest is isolated from a large library of nucleic acids containing a random region from iterative rounds of selection, which remains the technique employed today.

Broadly, a starting pool of 10^{12}-10^{17} single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA) containing a random nucleotide region N (where N typically equals 20-60 positions) is incubated with the molecule of interest in an aptamer selection, or in appropriate

catalytic conditions in a nucleic acid enzyme selection (Figure 1.4, I). Sequences that bind to the target moiety or that display the desired catalytic function are isolated (1.4 II), PCR amplified (1.4 III), and the process is repeated with successive rounds becoming increasingly stringent. If an RNA library is employed, reverse transcriptase is used to copy the strands into DNA before the PCR amplification step, followed by *in vitro* transcription to obtain RNA sequences for the next selection round. At the end of selection, a small pool of oligonucleotides displaying either high affinity for the target molecule or having robust catalytic activity is obtained (1.4 IV). The structures generated adopt a variety of secondary motifs and fold into more complex tertiary structures: it is both the three dimensional shape and the binding or catalytic ability of key residues that create the desired properties of the aptamer or catalyst. Once the selection process is terminated, the sequences are cloned into bacteria such that there is one sequence per colony. Individual clones are then sequenced to provide information as to an oligonucleotide's sequence identity and *inter alia* its putative structure. Numerous modifications can be made to the procedure in order to suit the selection requirements of the researcher and expand the scope of targets amenable for selection.

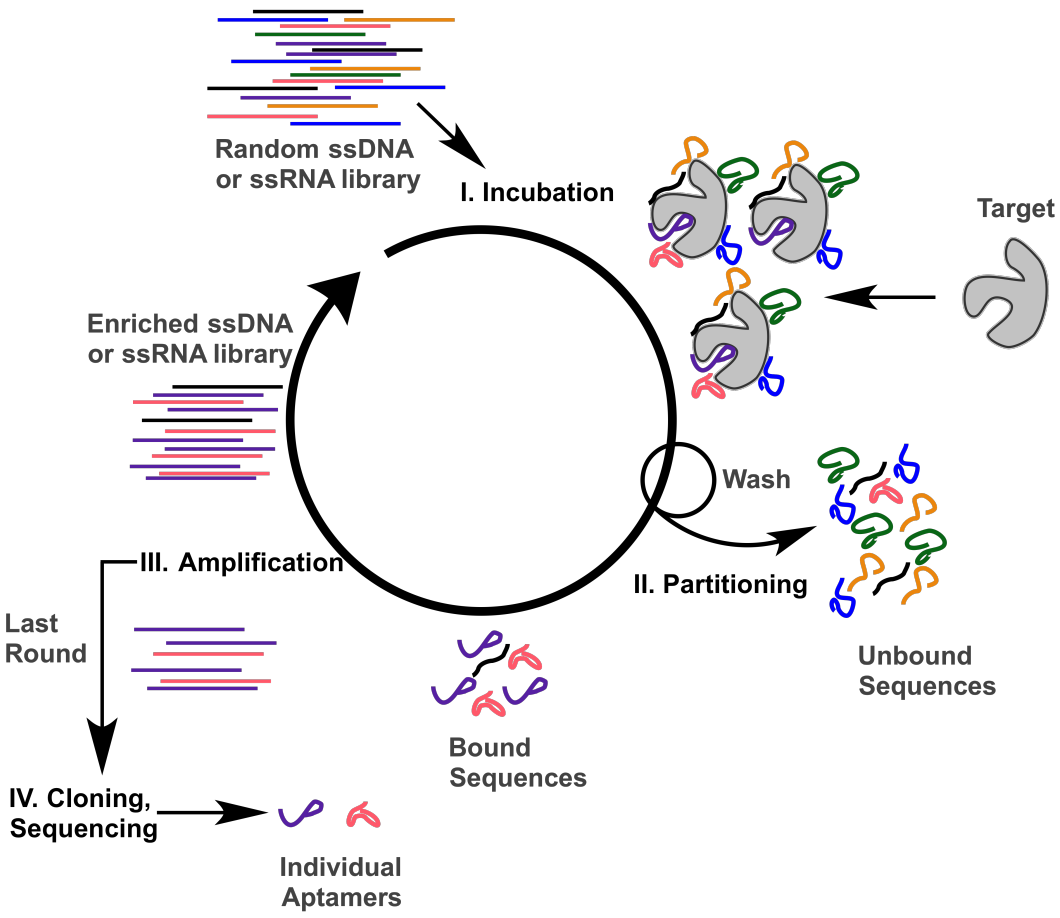


Figure 1.4 Selection process for aptamer or catalytic nucleic acid discovery.

1.2.2 Aptamers

Aptamers have become an area of intense research focus due to their antibody-like properties and because they offer many advantages over antibodies in both production capacity and potential applications. Like antibodies, aptamers are able to selectively bind a target with high affinity (the K_d is often in the nanomolar to picomolar range); however, these targets are not restricted to biological epitopes to which an immune response can be generated. Aptamers are considerably smaller than antibodies (in the range of 10-25 kDa vs 150 kDa) and are non-immunogenic. Moreover, aptamers can be easily and cheaply synthesized and are thermally

stable. Aptamers have been selected for a wide variety of targets including proteins, whole cells, viruses, metals, and toxins (see Table 1.1 for a representative list).

Table 1.1 Examples of selected aptamers in the literature.

Aptamer	Dissociation Constant	Aptamer Type
Cyanocobalamin (B12) [16]	88 nM	RNA
ATP [17]	5 μM	RNA
Thrombin (human and porcine) [18]	0.05 nM	RNA
HIV-1 Tat [19]	0.1 nM	RNA
(R)-thalidomide [20]	1000 nM	mDNA
Cocaine [21]	400 nM	DNA
Immunoglobulin IgE [22]	10 nM	DNA
Ochratoxin A [23]	50 nM	DNA
Hg^{2+} [24]	NR	DNA
Acute lymphoblastic leukemia cells (CCRF-CEM cell line) [25]	0.8 nM	DNA

Both DNA and RNA aptamers are able to adopt a wide range of complex tertiary structures, and there does not appear to be any bias towards RNA or DNA in the variety of targets that can be selected. However, DNA aptamers have the advantage of being the more stable species, as the phosphodiester bond in DNA is extremely resistant to hydrolysis and the lack of a 2'-OH group makes DNA less inherently reactive to strand scission [26, 27, 28]. Most importantly for physiological applications, DNA is resistant to ribonucleases, a widely secreted class of enzymes that specifically or non-specifically degrades RNA sequences. Chemical modifications have been introduced to RNA aptamers in order to address these deficiencies, such

as conversion of the hydroxyl to a 2'-flouro, -amino or -methoxy group [29, 30, 31]. Most of these modifications are introduced post-selection, and have been found to significantly increase the half-life of RNA aptamers *in vivo*. Because many of the properties of aptamers are ideal for physiological applications, significant interest has arisen in developing aptamers for therapeutic applications. The anti-VEGF aptamer macugen gained FDA approval for treatment of wet age-related macular degeneration and several other aptamer-based drugs are currently undergoing clinical trial (see Table 1.2).

Table 1.2 Examples of aptamer drugs. Aptamer target, targeted pathology and clinical status are listed.

Aptamer Drug	**Target**	**Application**	**Status**
Pegatnib [32, 33] (RNA, modified post-selection)	Vascular Endothelial Growth Factor isoform 165	Age-related macular degeneration	FDA approved
AS1411 [34, 35, 36] (G-rich DNA)	Nuclear factor-κB	Leukemia, Myeloid Metastatic Renal Cell Carcinoma	Phase 2
NoxE36 [26, 37, 38] (L-RNA)	Monocyte chemoattractant protein-1 (MCP-1/CCL2)	Diabetic nephropathy	Phase 2

Aptamers are also being investigated for use as drug conjugates in order to deliver cargos to targeted cells [39, 40, 41]. Still other research aims to develop precise and fast diagnostic tests for a wide variety of pathologies, such as cancers or viral infections [42, 43, 25]. The diagnostic possibilities, and to a lesser extent applications in drugs and drug delivery systems, are theoretically limited only by the ability to generate an aptamer for a desired target. Such limitations can be encountered as a consequence of the basic process of SELEX, such as the

difficulty in replicating the physiological environment during the experimental procedure. The potential result is the generation of aptamers that bind to the isolated target in a laboratory setting but do not bind to the same target *in vivo*. A SELEX variant developed by Weihong Tan, termed cell-SELEX, has aimed to address this problem by conducting the selection procedure with whole cells in media rather than isolated proteins [25]. Using this method, aptamers may be generated to complex cell surface moieties and the native conformations of proteins are not compromised [44, 45]. Moreover, an adaptation of cell-SELEX has led to the successful selection of aptamers that are internalized by prostate cancer cells but do not bind to the cell surface [46]. One notable limitation of this strategy is that the precise identity of the aptamer targets remains unknown unless further characterization work is done. Cell-SELEX remains an emerging, but challenging, field of aptamer discovery.

1.2.3 Nucleic Acid Enzymes

The range of catalytic functionalities explored in nucleic acid enzyme chemistry has heavily favoured the use of RNA as the starting material, although DNA is employed as well. The imbalance is likely due to the precedence of naturally occurring ribozymes and the widespread but unsupported belief that the 2'-OH group makes RNA a catalytically superior molecule. Reactions catalyzed by DNAzymes and artificial ribozymes include Diels-Alder [47], sulfur alkylation [48], porphyrin metallation [49], DNA adenylation [50], tyrosine phosphorylation [51], and RNA cleavage or ligation [52, 53, 54], to name a few. Although a wide variety of chemical reactions have been artificially selected for, the vast majority of reported nucleic acid catalysts catalyze the ligation or cleavage of RNA. This may be due in part to the framework of RNA-cleaving selection methods that have been developed, making selections of this type more easily accomplished, but it is certainly also due to the wide interest in these molecules for therapeutic

applications. Due to the superior *in vivo* stability of DNA discussed above, DNA enzyme development has been preferred by many researchers for this purpose despite its purported catalytic inferiority.

In fact, the majority of RNA-cleaving ribozymes and DNAzymes achieve catalytic rates far lower than the protein enzyme equivalent ribonucleose A, particularly under physiological conditions. Additionally, the limited chemical functionality of the nucleic acids in general poses limitations to the scope of reactions that can be catalyzed. Similarly, aptamer selections are limited by the chemical constraints of DNA and RNA, as the negatively charged backbone creates challenges for anionic, polar, and highly hydrophobic targets. Moreover, the lack of hydrophobic groups largely eliminates the possibility of hydrophobic contact points, which are an entropically stabilizing feature of many protein-protein interactions. Such limitations pose particular setbacks in selections with a complex target environment, such as cell-SELEX. Hence, there has been considerable effort to expand the catalytic and binding abilities of DNA and RNA molecules for use in SELEX.

1.3 Modified Nucleotides

1.3.1 Background and Development

Beginning in the 1980s, researchers have made modifications to the backbone, sugar and base components of nucleotide monomers. The interest originally lay in developing tools for molecular biology, such as fluorescent and biotinylated probes, cross-linking agents and sequence-specific nucleases [55, 56, 57, 58, 59, 60]. The modified sites on these conjugated nucleotides commonly included the C5 position of pyrimidines and the C8 position of purines, the 5' end of the phosphate backbone, and the hydroxyl groups on the sugar [61]. Modifications to the

phosphodiester backbone that increased nuclease resistance *in vivo* were also of interest early on [62]. As discussed above, groups working with RNA molecules modified the 2'-OH group of the ribose of pyrimidines with amino or fluoro groups for the same reason [29, 30, 31].

Numerous more recent reports of phosphodiester modifications can be found in the literature. For example, Caruthers *et al.* synthesized phosphonoformate oligodeoxyribonucleotides that were found to be nuclease resistant and hybridized with RNA to stimulate RNAse H1 activity (Figure 1.5) [63] . Vigroux and coworkers developed a group of constrained deoxuribonucleic acids that contain a dioxaphosphorinane ring, constraining the backbone torsion angles into canonical or non-canonical conformations depending upon the modification [64].

The sugar has also been modified extensively beyond changes to the 2'OH ribose site. One of the most well known examples is the Locked Nucleic Acid (LNA), which was developed by both the Wengel and Imanishi labs independently (Figure 1.5) [65, 66] . The introduction of a 2'-O, 4'C-methylene bridge across the ribose molecule was found to increase the thermal stability of both LNA/DNA and LNA/RNA duplexes considerably, and LNAs have since been developed for a variety of analytical and therapeutic purposes. Replacement of the sugar moiety has also been carried out; for instance, Chaput *et al.* synthesized threose nucleic acid and showed that oligonucleotides made up of threose nucleotides were capable of *in vitro* evolution (Figure 1.5) [67]. Similarly, the Holliger laboratory created nucleic acid polymers using six different non-ribose and non-deoxyribose sugars (termed "XNAs"), and demonstrated that enzymatic replication and aptamer selection was possible with the new genetic systems [68]. Modified catalysts using a subset of these XNAs were subsequently developed that exhibited *trans* RNA endonuclease activity and ligase activity [69].

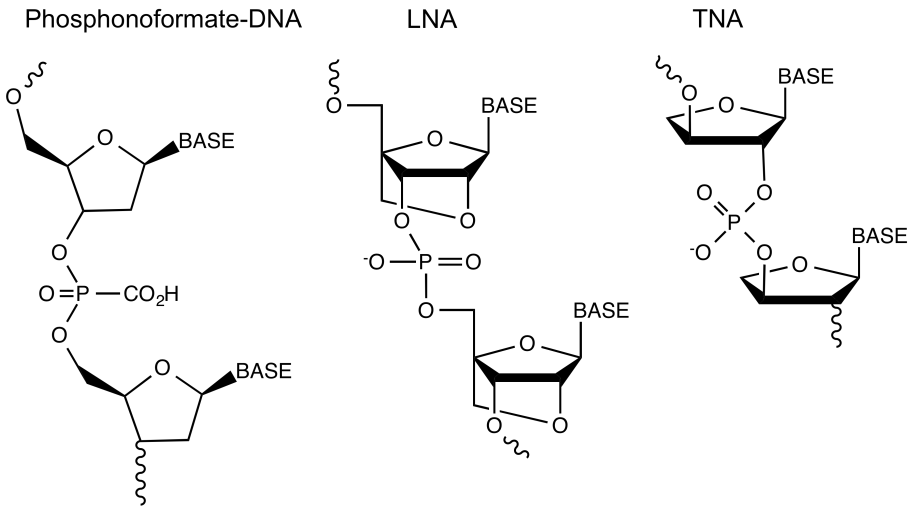


Figure 1.5 Examples of backbone- and sugar-modified nucleic acids.

From the left: phosphonoformate-DNA[63], LNA[65, 66] and TNA[67].

Notwithstanding the recent publication of an XNAzyme, in catalyst development the principal interest lies in modifications to the base of the nucleotide in order to expand the functionality of nucleic acid polymers, most often with amino acid-inspired moieties. As alluded to above, the C5 position of pyrimidines and C8 position of purines is often the chosen modification site so as not to disrupt Watson-Crick base pairing. Early examples of modified bases for use in SELEX include an imidazole-modified UTP used to select an RNA amide synthase and a pyridyl methyl modified UTP used to select an RNA Diels-Alder catalyst by the Eaton group [70, 71]. Barbas and coworkers synthesized an imidazole-modified dUTP, which was linked to the C5 position of the pyrimidine via a 3-aminopropenyl group [72]. The modified dUTP was employed to successfully select for an RNA-cleaving DNAzyme, in which it was shown that three of the imidazoles were critical for catalytic competence. The catalyst also required Zn^{2+} (10-30 μM) and 1mM Mg^{2+} for activity. Perrin *et al.* focused on the development of metal-independent DNAzymes that would be ideal for *in vivo* applications by utilizing multiple protein-like functional groups in a single selection process. A histaminyl-modified dATP was

used in conjunction with an aminoallyl-dUTP to select for Mg^{2+}-independent RNA-cleaving DNAzyme $Dz9_{25}$-11 [53]. Reasoning that increasing the chemical functionality would increase the efficiency of catalysis, the same group later selected for Mg^{2+}-independent RNA-cleaving DNAzymes Dz9-86 and Dz10-66 that rely upon guanidiniumallyl-dUTP ($dU^{ga}TP$), imidazole-dATP ($dA^{im}TP$) and aminoallyl-dCTP ($dC^{aa}TP$) modified bases (Figure 1.6 A) [73, 74].

Benner *et al.* sought to completely rearrange the hydrogen bonding patterns between bases and create new ones by introducing non-standard purines and pyridines [75]. The result was two additional nucleobases (2-amino-8- (1′-β-D-2′-deoxyribofuranosyl)imidazo[1,2-a]-1,3,5-triazin- 4(8H)-one, termed "P", and 6-amino-5-nitro-3-(1′-β-D- 2′-deoxyribofuranosyl)-2(1H)-pyridone, termed "Z") that could be incorporated into the canonical genetic alphabet to create six different hydrogen bonding patterns (Figure 1.6 B). Similarly, Kool and colleagues altered all four nucleobases by increasing the size of the pi system, converting monocyclic pyrimidines into bicyclic structures, and bicyclic purines into tricyclic structures [76, 77]. The corresponding nucleotides could be incorporated with the four canonical nucleotides to create an eight-letter genetic system that resulted in an extended but stable duplex.

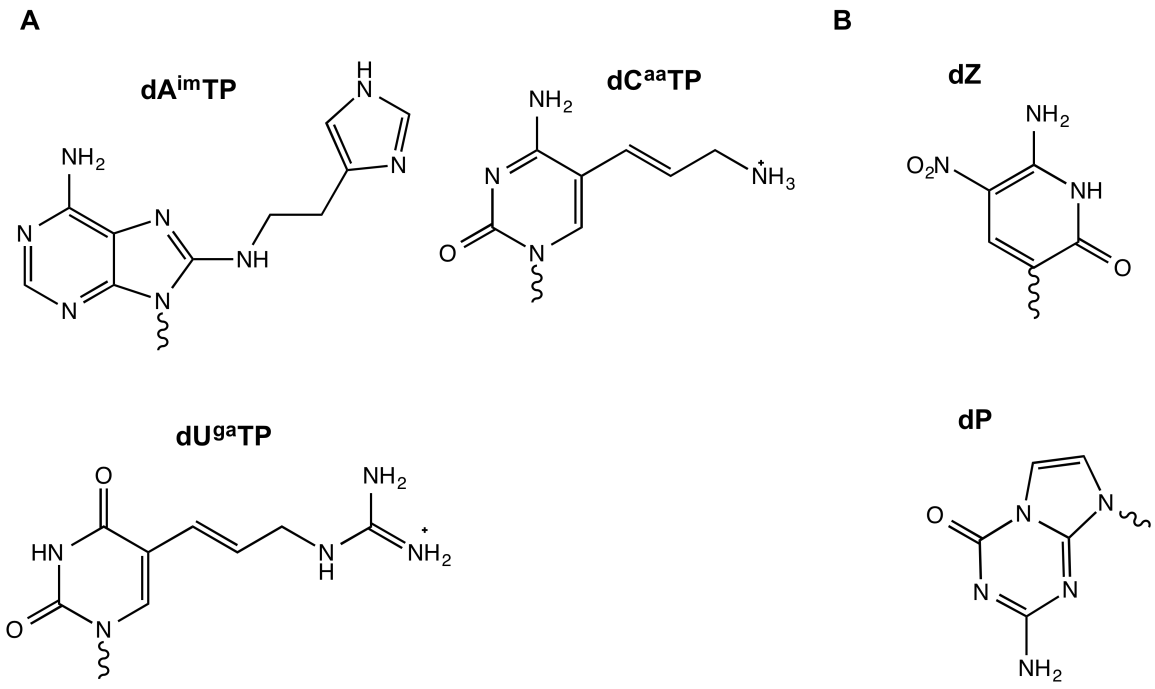


Figure 1.6 Examples of base modifications. A: dA^{im}TP, dC^{aa}TP and dU^{ga}TP nucleobases [73]. B: dZ and dP [75].

Base modifications have also been explored for aptamer selections, of which a few representative examples will be described. SomaLogic, a company started by University of Colorado researcher Larry Gold, has developed a number of modified dUTPs that append protein-like functional groups to the base of the nucleotide [78]. Selections carried out with benzyl-, naphthyl- tryptamino- or isobutyl- dUTP have resulted in high affinity aptamers for hundreds of protein targets that had previously resulted in failed or low-affinity selections using unmodified libraries. Sawai *et al.* synthesized a modified dUTP bearing a cationic ammonium group and obtained a modified DNA aptamer that was able to discriminate the (R) form of thalidomide with high enantioselectivity [20]. The Benner group, highlighted above, employed their expanded genetic alphabet to select for aptamers against breast cancer cells, and obtained one candidate with a K_d of 30 nM [45]. The modified nucleotides were found to be crucial for high-affinity binding.

1.3.2 Modified Nucleotide Incorporation

Despite the considerable interest and benefits in modifying the nucleoside structure, the number of modified aptamers and catalysts reported is far smaller than the number of unmodified examples in the literature. The imbalance is likely partially due to the fact that conducting an unmodified selection is a relatively cheap and accessible process; conversely, synthesizing and incorporating modifications is much more costly and laborious. However, the lack of modified nucleotides in most selections restricts the choice of aptamer targets and the efficiency of catalysis, and therefore conducting novel selections will become increasingly difficult with unmodified libraries. Another significant barrier to the introduction of new modifications into SELEX libraries is the poor or negligible incorporation of modified nucleotides into oligonucleotide strands by available polymerases, either in PCR or primer extension reactions. Moreover, even when incorporation demonstrates at least partial success, inefficient incorporation can cause certain library sequences to be favoured for replication, reducing the sequence space coverage of the selection and thus theoretically reducing the likelihood of a successful selection.

A few methodologies are being developed in order to address this problem. One approach is to design modifications that are well suited to incorporation and amplify with a similar efficiency to unmodified DNA, such has been done in the Williams and Sawai groups [79, 80, 81]. While this method might facilitate the eventual modified selection, the process of designing and testing modified substrates for suitability in PCR can be tedious and unpredictable.

It is also possible to re-engineer polymerases to be better acceptors of modified nucleic acids. This route was effectively employed by the Holliger and Pinheiro groups in order to

accommodate nucleic acids with alternative sugar moieties developed in the Holliger laboratory [82, 68]. The approach uses a strategy termed compartmentalized self-tagging in which a repertoire of polymerases is suspended in a water-oil emulsification and each individual polymerase is challenged to replicate a plasmid encoding for itself. Replicated plasmids are amplified and used for the next round of selection, culminating in the screening of winning enzymes. This strategy was also employed by Benner *et al.* to select polymerases suited to the group's "P" and "Z" nucleobases [83]. Conversely, Leconte and coworkers utilized an activity-based phage display method to select a *taq* polymerase variant capable of highly processive incorporation of fluorophore-tagged dNTPs [84]. Evidently, the chief limitation of such strategies is the need to develop a new polymerase for each set of non-canonical nucleotides.

This problem can also be circumvented with the design of a method in which the modified material is linked to its cDNA counterpart. Such a strategy avoids the need for direct amplification of the modified nucleotides whilst providing the template for the next round of modified nucleotide synthesis. An early progenitor of such an approach fused a peptide to its encoding mRNA by co-opting the ribosome to translate the protein moiety and linking the mRNA portion to the peptidyl acceptor antibiotic puromycin, thus producing a stable linkage between newly-synthesized peptide and mRNA. The resulting library could be manipulated to selectively enrich a desired protein-mRNA sequence via anti-sense binding to a chosen template followed by immunoprecipitation [85]. More recently, several proof-of-concept systems have been published that link modified phenotype to DNA genotype. Such studies have shown that XNAs can be successfully transcribed within DNA hairpin structures and can lead to the selection of aptamers via strand displacement of the XNA sequence from the DNA template [86, 67]. A similar strategy has been used whereby peptide nucleic acid (PNA)/DNA heteroduplex sequences are

separated via displacement of the cDNA strand, allowing PNA to adopt conformations suitable for aptamer selections [87]. The general approach has also been employed for the selection of potential glycocluster immunogens by synthesizing a library of alkyne-modified DNA that is subsequently linked to glycoclusters using "click" chemistry [88]. Nevertheless, no such strategy exists for the selection of modified RNA or DNA catalysts despite the inherent advantages of a linked phenotype-genotype system.

1.4 Aims of this Thesis

The aims of this thesis are two-fold. Firstly, there is considerable interest in developing modified aptamers to challenging targets, such as whole cells, that may not be chemically well suited to interacting with unmodified DNA. Gram-negative bacteria, such as enterogenic *Escherichia coli* (*E. coli*) are of particular relevance due to the potential for diagnostic and food safety applications. Gram-negative species are particularly good candidates for a modified selection, as the negative charge density on the outer cell surface of the bacteria is an inherent deterrent to interactions with negatively-charged DNA strands. Therefore, the aim is to develop an aptamer selection strategy to *E. coli* cells using the modified DNA previously developed in the Perrin lab and carry out a selection for high affinity aptamers towards *E. coli* cells. The methodology can subsequently be adapted for other modified DNA aptamer selections.

Secondly, this thesis aims to address the difficulty in working with modified DNA, specifically in the area of polymerase-based amplification. The work will focus on building upon incomplete research conducted in this laboratory to develop a linked genotype-phenotype system as outlined in Section 1.3.2. The novel selection strategy will circumvent the requirement to PCR-amplify modified DNA, thus decreasing amplification-based selection bias and increasing

the likelihood of a successful selection outcome. The work will aim to prove the concept by using a known DNA catalyst (the “phenotype”) and covalently linking it to its unmodified DNA counterpart (the “genotype”). Upon catalytic activity, the genotypic strand will be released and captured for amplification. The model system can then be extended to a variety of novel modified aptamer or nucleic acid catalyst selections in the future.

Chapter 2: Selection of Modified DNA Aptamers to *Escherichia coli* using cell-SELEX

This chapter focuses on the development and implementation of a selection strategy to generate modified DNA aptamers towards *E. coli* cells.

2.1 Background

2.1.1 Target Selection

Escherichia coli was a compelling choice as a selection target for cell-SELEX because there is significant interest in developing aptamers towards bacterial species for use in diagnostic tests as well as analytical assays to assess food or environmental safety [89]. Furthermore, the ability to conduct such assays with whole cells is of increasing importance in order to avoid laborious sample preparation processes and develop fast, low-cost detection methods requiring little on-site expertise [89-90]. Moreover, there is scarce precedent for the use of modified DNA in whole cell aptamer selections and therefore there is much left to exploit in terms of applying modified oligonucleotides to SELEX processes. In order to minimize working safety concerns and facilitate the establishment of new selection protocols, the DH5α cloning strain was utilized as the *E. coli* target for the experiments described in this chapter. However, the protocol developed can easily be modified toward other bacterial targets.

It should be noted that aptamers towards *E. coli* cells have been successfully selected in other groups. In 2008, So *et al.* reportedly developed an RNA aptamer to DH5α cells for use in an aptamer-based carbon nanotube field effect transistor for the detection of bacterial cells [91]. However, the procedure, selectivity and K_d of the aptamer were not reported in the paper and

therefore basic properties of the selected sequence remain unknown. Li *et al.* (2011) selected an ssDNA aptamer against K88 fimbrae protein expressed on *E. coli* K88 cell surfaces [92]. The reported dissociation constants for the aptamers obtained were approximately 30 nM. The aptamers also showed binding capacities toward whole K88 cells (K_d not reported) as well as some degree of selectivity towards the K88 strain compared to other strains of *E. coli* and *Staphyloccus aureus.* Lee and coworkers developed a 2'-deoxy-2'-fluoro-modified CTP and UTP RNA aptamer to whole O157:57 *E. coli* cells using subtractive SELEX, with K12 employed as the negative strain [93]. The minimized aptamer was found to have a K_d of 110 nM but was not tested against other bacterial strains besides K12. Importantly, the modifications to the RNA utilized in this selection serve to increase nuclease resistance in mammalian cells and not to increase chemical functionality, which is the focus of the modifications employed in the research reported herein. More recently, Kim *et al.* generated an ssDNA aptamer for *E. coli* strain K2571 using whole-cell SELEX, with reported dissociation constants of the selected sequences ranging from 12.5 nM – 25 nM [94]. They reported no significant binding activity towards non-*E. coli* bacteria; however, there was significant binding activity to other *E. coli* strains. Aptamers have also been developed for many non - *E. coli* bacterial species, including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella typhimurium* [95, 96, 97].

There has been recent interest in bacterial detection using catalytic DNA as well. In 2011 Yingfu Li and colleagues developed a fluorogenic DNAzyme to the K12 strain of *E. coli*. The DNAzyme is coupled to a fluorogenic probe that fluoresces upon cleavage, and cleavage was evolved to be dependent upon exposure to *E. coli* crude extracellular mixture [98]. Selectivity of the probe was determined via incubation with nine other gram-positive and gram-negative bacterial species; negligible fluorescence was generated in each case. The probe was shown to be

sensitive enough to detect a single live cell, although the practical limit of detection was determined to be 500 cells.

Although whole-cell SELEX has yet to employed for the discovery of modified DNA aptamers towards whole bacterial cells, Ochsner *et al.* in conjunction with SomaLogic developed a suite of modified aptamers towards cell-surface associated *Staphylococcus aureus* proteins [96]. Additionally, the Benner and Tan laboratories have recently reported the generation of modified aptamers towards the breast cancer cell line MDA MB-231 using the expanded genetic library previously developed in the Benner laboratory [99]. However, as noted above, the use of modified DNA in whole cell selections remains an extremely nascent area of aptamer discovery.

The growing number of publications in this field emphasizes the increasing interest in bacterial detection and the need for better detection strategies. Moreover, despite some precedence for aptamer selections against *E. coli*, there is nonetheless considerable merit in pursuing the selection of new aptamers to the same or similar targets. Due to the fact that there are a broad number of variables in a given selection and there are virtually no rules in determining optimal selection conditions, conducting new selections with previously selected targets can result in improved aptamers or aptamers with novel capabilities. Such has been the case with thrombin protein, which was the subject of one of the first selections in the early 1990s [100]. The selection yielded an aptamer with a Kd of 400 nM, and over two decades many groups have discovered aptamers with up to a thousand-fold improvement in affinity towards the protein, using a variety of selection strategies [101, 102]. The advantages of selecting new aptamers to identical or similar targets are even more apparent in whole cell bacteria applications. There are many strains of a given species of bacteria, and as is the case with *E. coli*, there are notable differences between them. Some are of interest from a molecular biology standpoint, others are

harmful and are of interest from a food safety and diagnostic perspective, and still others are beneficial to humans and are of increasing significance in the study of the human gut microbiome. Therefore, the development of aptamers to a wide variety of bacterial strains can have important applications in many fields. Furthermore, the aptamers must have excellent selectivity in order to discriminate a particular strain, a feature that is often lacking amongst those currently reported in the literature. For instance, in a report published by Ferreira and colleagues in 2014, an aptamer was discovered that bound to the peptidoglycan component on the outer bacterial cell wall; however, the aptamer could not discriminate between gram-positive and gram negative-bacteria [103]. Similarly, the study from Kim *et al.* discussed above found that the aptamer selected exhibited binding activity to several different *E. coli* strains [94]. This underscores the potential value in the re-selection process. Finally, aptamers that respond to the same complex cellular environment differently may be discovered by varying the many conditions that make up a SELEX experiment. For instance, aptamers that bind to different proteins or other cellular components in the same species may be uncovered. Moreover, different aptamers might permeate different compartments of the cell. This is especially pertinent in the case of gram-negative bacteria, as they have an outer and inner cell wall: aptamers might be selected that bind to outer cell wall components, inner cell wall components, or that penetrate past the inner cell wall to the cytoplasm, a capability that is so far unreported for nucleic acid aptamers. Depending upon the desired post-selection application, one of these hypothetical aptamers might be a more appropriate choice. Expanding the chemical functionality of aptamer sequences is another critical aspect in developing an array of functionalities and binding capacities towards bacterial targets and provided another impetus for the selection described in this chapter.

2.1.2 Cell-SELEX

Explicit knowledge of the target moieties is unknown in cell-SELEX, which is both advantageous and disadvantageous. In terms of advantages, the potential aptamer sequences are exposed to the complex cell surface in its entirety, which means that the target motifs are not restricted in any way. This allows for potential aptamers to be generated against protein targets that are often very difficult to express *in vitro*, such as trans-membrane proteins. Moreover, the native conformation of proteins is far less likely to be compromised in cell-SELEX compared to a selection conducted with an isolated purified protein [104]. This is particularly relevant to the development of applications with the aptamer post-selection, where the differences between native and *in vitro* expressed protein might be sufficient to severely compromise binding affinity. An additional advantage of the post-SELEX applicability is the reduction in the degree of sample preparation required for testing and detection. Conversely, the complexity of the environment can also be a disadvantage, because if a suitable target is not present at a sufficient concentration the selection will likely fail. The danger of generating and carrying non-specific sequences through a selection process is also increased due to the variety of non-specific binding surfaces available. Moreover, identification of the target in the event of a successful selection involves extensive characterization assays and might not result in a positive identification. Therefore, researchers will often utilize cell lines that naturally over-express one or more proteins. The cell lines are often derived from cancers and the protein over-expression is associated with the development of the particular pathology [25, 44]. The hypothesis is that aptamers will preferentially be developed toward the over-expressed protein, which will both increase the specificity of the aptamer towards the specific cell type and facilitate identification of the target.

Initially, the strategy for the DH5α selection was to induce the cells with Isopropyl β-D-1-thiogalactopyranoside (IPTG), an allolactose mimic that induces transcription of the *lac* operon present in the DH5α strain. Induction promotes the expression of *lac* genes, such as the cell-membrane protein lactose permease [105, 106]. It was hypothesized that aptamer generation would be favourable towards lactose permease and perhaps other *lac*-related proteins. However, it was noted that *E. coli* cells contain both an outer and inner cell membrane, in contrast to mammalian cells and gram-positive bacterial cells. Lactose permease is expressed on the inner cell membrane, and thus, aptamer sequences would have to first permeate the outer cell membrane in order to access lactose permease as a target [105]. Since the likelihood of this occurring was unknown, the hypothesis was discarded although the selection was run with IPTG-induced cells.

2.1.3 Starting Library

The initial library used in most SELEX experiments is made up of hundreds of millions of unique sequences. Each sequence is comprised of a contiguous random region "N" of nucleotides flanked by primer regions. The length of the random region is not prescribed but typically ranges from 20-80 positions, and choosing the sequence length involves the consideration of a number of factors [107]. It is simpler and more cost-effective to synthesize a shorter library, and the sequences are easier to manipulate following SELEX for the desired application, be it for sensors, diagnostic tests, or cell penetration for example. Often, researchers will try to determine the minimal sequence that provides optimal activity and continue to work with the truncated sequence for these reasons. Moreover, when working with modified DNA, longer random regions will increase the likelihood of incorporation difficulties leading to truncated sequences and a corresponding reduction in sequence space coverage. However, a longer random region will result in more folding possibilities and a greater degree of structural

complexity, which is likely to increase the probability of a successful selection [107]. A random region of 40 nucleotides was chosen for the selection and is typical of many SELEX experiments, as it is commonly thought to strike a good balance between the factors enumerated above. An N40 region represents 4^{40} (or 10^{24}) theoretical sequence possibilities, but within the context of a practical experimental volume this number is closer to 10^{12}-10^{16}, which nevertheless has often proven sufficient for a successful selection. The library used in this selection was obtained commercially via solid-phase synthesis.

2.2 Selection Design, Implementation and Results

2.2.1 Library and Target Concentration Parameters

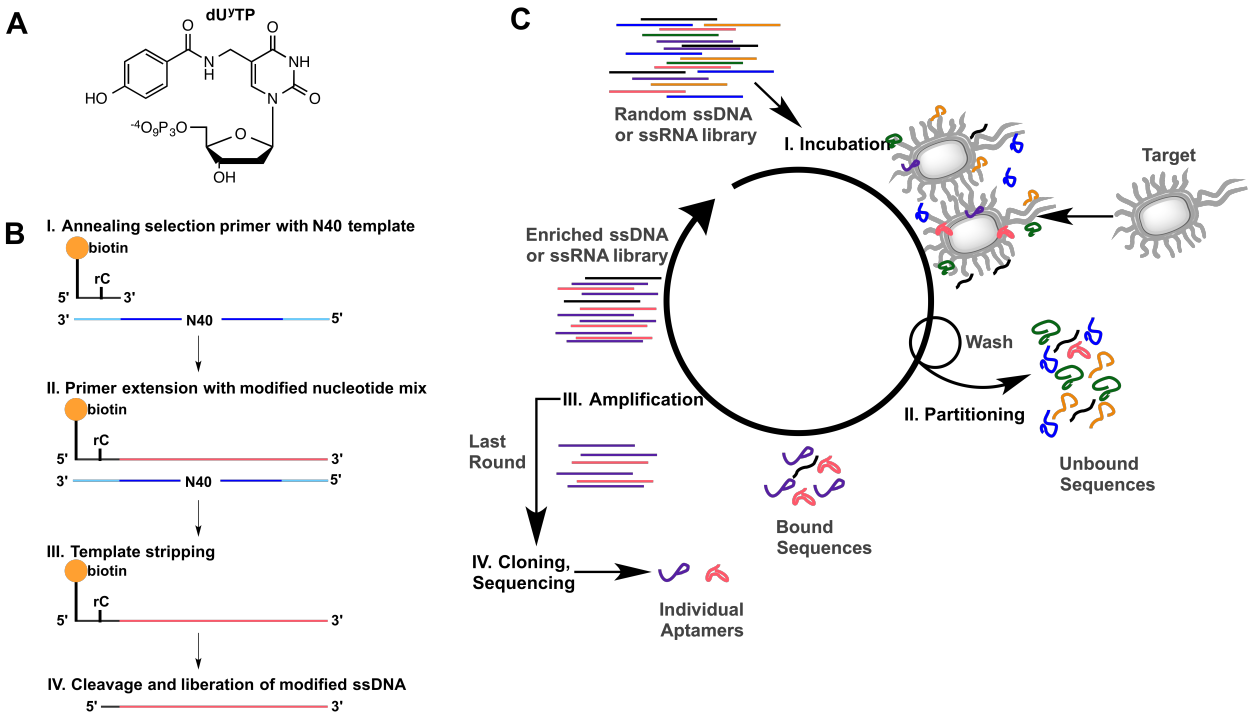
Although no rule exists as to the appropriate library and target concentrations for a SELEX experiment, generally the target concentration is kept at approximately the desired K_d value and the library concentration at least one order of magnitude more [104, 108, 102, 109]. Referring to the procedure outlined by Tan *et al.*, the target concentration was set at 20 nM for round one and 10 nM for subsequent rounds [104]. This concentration is an approximation, as explicit targets are unknown throughout a cell-SELEX procedure. Correspondingly, the protocol dictates a library concentration of 15 μM for the first round and 1 μM for round two onwards, while the incubation volume is set at 700 μL for round one and 400 μL for round two onwards. Typically, the first round contains a higher concentration of both target and ligand to allow for the maximal number of binding events with unique sequences to occur. To reach similar concentrations in the modified procedure, the incubation volume was reduced to 50 μL. This allowed for a library concentration of 400 nM from 30 pmol starting material for round one and 200 nM from 15 pmol starting material for round two onwards, assuming a 33% loss in material through preparatory

measures. While several orders of magnitude below the library concentration suggested by Tan *et al*., the library concentration was thought to be sufficiently above the target concentration to encourage competitive binding between sequences.

2.2.2 Cell-SELEX with dU^{y}TP

The selection procedure was adapted from the processes outlined by Tan *et al*. and Hollenstein *et al.*, as shown in Scheme 2.1 below [104, 73]. Sequences containing 40 degenerate positions were annealed to a primer containing a biotin tag and an internal ribose linkage. The annealed reaction was elongated in the presence of phenol-modified dUTP (dU^{y}TP) and unmodified dGTP, dCTP and dATP along with α-^{32}P-dGTP [110]. The elongated sequences were then stripped from the template stands via cleavage of the internal ribonucleotide linkage, yielding a modified library of approximately 10^{13} sequences. The sequences were dissolved in 40 μL of the appropriate buffer. DH5α cells were grown to OD_{600}= 0.6, representing approximately 10^{7} CFUs, and were induced with IPTG at OD_{600}= 0.015. A volume of 1 mL of the resulting broth culture was spun down and prepared as described in Materials and Methods. The cell pellets were re-suspended in the library solution, resulting in a total volume of 50 μL. The sequences were left to incubate with the target, and following incubation the selections were spun down and the supernatant removed ("not bound" fraction). The pellets were washed twice (rounds 1-5, 7-8) or three times (rounds 6, 9) with 100 μL SELEX Wash Buffer. Following the washes, the pellets were re-suspended in 75 μL SELEX Wash Buffer and denatured at 95° for 20 minutes, snap cooled, and spun down. The supernatant obtained contained the sequences that bound the cells, termed the "bound" fraction. Beginning in round 2 it was observed that sequences remained with the cell pellets after denaturation and centrifugation, and these sequences were labeled the "pellet" fraction. It was decided that propagating both the bound and

pellet fractions into the next round of selection was desirable. Therefore, both fractions were loaded onto a denaturing PAGE gel in order to remove any sequences of incorrect length. The purified selected sequences were pooled and amplified via PCR with unmodified DNA in two separate reactions in order to maximize the number of successful amplification rounds. The amplified double-stranded DNA was subjected to digestion by λ exonuclease, and the resulting 3' DNA strand was purified again by denaturing PAGE. The purified sequences were annealed to the selection primer for the next round of selection.



Scheme 2.1 A: Structure of phenol-dUTP. B: SELEX procedure modifications to prepare modified library. C: Adapted cell-SELEX procedure used for the selection described herein.

The selection was carried out in duplicate (notated as population "A" and population "B") starting from the 5th round and the selection was terminated after 12 rounds. Enrichment was monitored by determining the radioactivity of the library (in cpm) and measuring the amount of radioactivity in the bound and pellet fractions against this total. Initially, the sequences and the

target cells were left to incubate for one hour. The incubation period was decreased to 45 minutes in round 3, 35 minutes in round 7, and 25 minutes in round 10 in order to increase stringency. Buffer containing tRNA and bovine serum albumin (BSA) was used from rounds 4 onwards to introduce non-specific protein competition and minimize the number of non-specifically bound sequences carried forward. Lastly, the cells were washed three times in rounds 6 and 9 in order to remove more weakly bound sequences. No negative selection was conducted in order to retain as many high affinity sequences as possible. A summary of the selection round conditions are provided in Table 2.1. A summary of the not bound and total bound fractions after each round of selection is shown in Figure 2.1, and the precise values are listed in Table 2.3. Note that the values plotted for round 5 onwards in Figure 2.1 are the average values obtained from both selections as shown in Table 2.3. Also note that the "not bound" fraction represents the sequences that were removed immediately following the incubation and first centrifugation step. Therefore, the "not bound" and "bound" fractions do not add to 100% because a portion of the oligonucleotides was also removed during the wash steps. Figure 2.3 shows representative denaturing PAGE gel pictures of the "not bound", "bound" and "pellet" fractions obtained after each round of selection. The eighth round showed the highest level of enrichment (highest "bound" fraction, as shown in Figure 2.1). This generation was chosen for further characterization as it was hypothesized to have the greatest number of high-affinity sequences.

Table 2.1 Summary of selection conditions for each round.

Selection Round	Incubation Time (min)	Washes	tRNA and BSA present
1	60	2 x 100 µL	No
2	60	2 x 100 µL	No
3	45	2 x 100 µL	No
4	45	2 x 100 µL	Yes
5	45	2 x 100 µL	Yes
6	45	3 x 100 µL	Yes
7	35	2 x 100 µL	Yes
8	35	2 x 100 µL	Yes
9	35	3 x 100 µL	Yes
10	25	2 x 100 µL	Yes
11	25	2 x 100 µL	Yes
12	25	2 x 100 µL	Yes

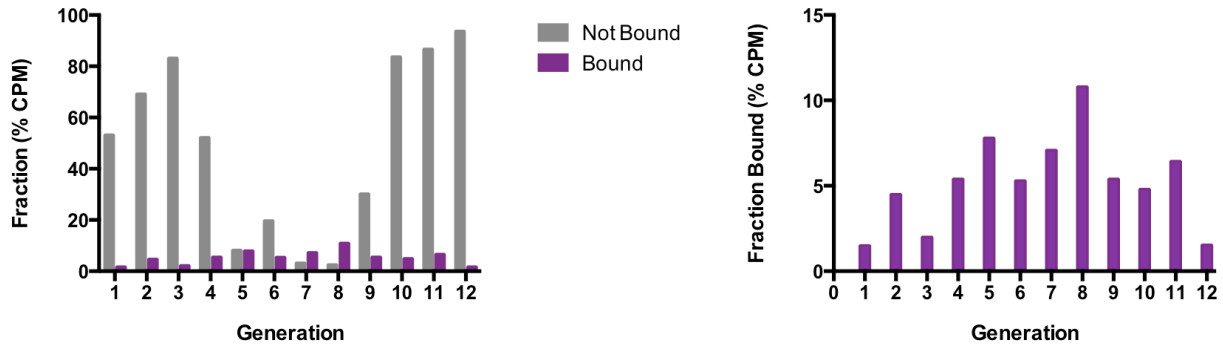


Figure 2.1 *E. coli* DH5α aptamer selection progress.

Incubation time was decreased in rounds 3, 7 and 10, and the number of washes was increased in rounds 6 and 9. The graph on the left shows the fraction of sequences that did not bind to the cells versus those that bound after each round of selection; the graph on the right is an enlarged version of the bound fraction progress shown in the left graph. The bound fraction includes both the bound and pellet fractions as explained in the text. The values from round 5 onwards are an average obtained from both selection processes.

Table 2.2 Selection progress.

Not bound and bound fractions are expressed as a percentage of the total cpm measured for a given sample. The bound fraction represents both the bound and pellet fractions as explained in the text. The selection was carried out in duplicate from round 5 onwards; the average bound and not bound values are calculated where applicable.

Generation	Pool A		Pool B		Average Not Bound	Average Bound
	Fraction Not Bound (% cpm)	Fraction Bound (% cpm)	Fraction Not Bound (% cpm)	Fraction Bound (% cpm)		
1	53	1.5	-	-	-	-
2	69	4.5	-	-	-	-
3	83	2	-	-	-	-
4	52	5.4	-	-	-	-
5	8	7.8	-	-	-	-
6	36	4.2	3.0	6.4	10	5.3
7	2.0	7.0	4.0	7.2	3.0	7.1
8	2.3	11	2.4	10.6	2.4	10.8
9	13	7.2	47	3.6	30	5.4
10	84	4.5	83	5.1	84	4.8
11	88	5.8	85	7.1	87	6.5
12	93	1.5	94	1.6	94	1.6

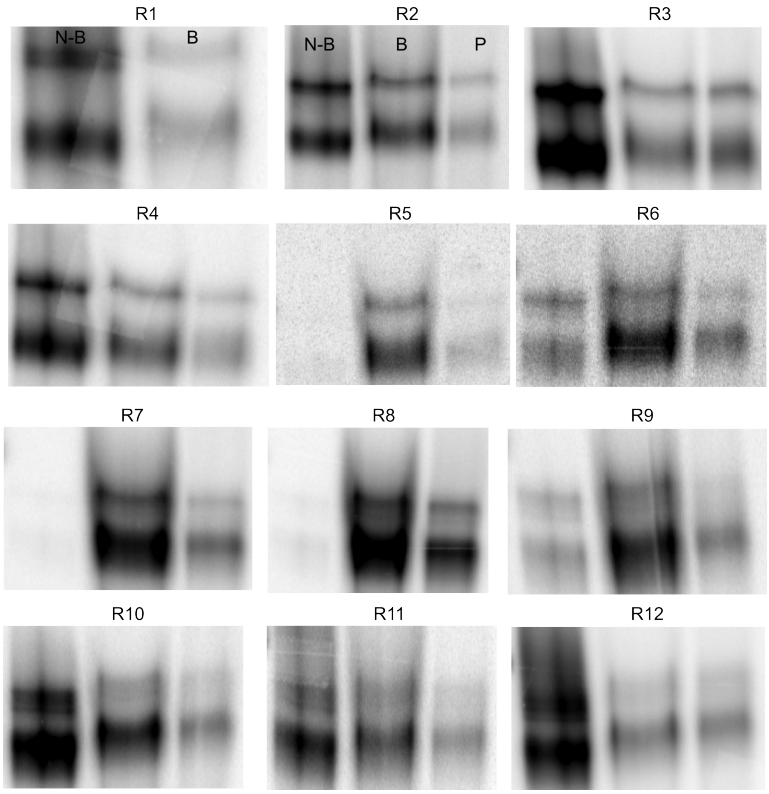


Figure 2.2 PAGE gels depicting the selection process for selection population A.

Note that quantitative information cannot be gained from the intensity of the bands; only 5% of the not bound fraction was run as a visualization aid, while 100% of the bound and pellet fractions were run on the gels in order to isolate and purify the sequences.

In order to ensure that the dU^{y}TP nucleotide was necessary for binding events, a binding assay was carried out that mimicked the conditions of the eighth selection round and that utilized the generation 7 library elongated with unmodified DNA. Radioactivity in the bound and pellet fractions was found to be negligible (less than 1.5%). With this positive result in hand, 46 clones from the eighth generation of both selections were sent for sequencing. The sequences obtained are presented in Table 2.3, along with an analysis of the modified nucleotide content of the sequences. The average number of modified nucleotides in the cloned sequences was 13.8, which is statistically greater than the average of 10 that might be expected to occur randomly. As was

discussed in the introduction, modified nucleotides suffer from lower incorporation and amplification rates by polymerases and consequently carry a negative bias for selection. Therefore, the relatively high number of modified positions was a promising indication of a successful selection that relied on the properties of the modified nucleotide.

Table 2.3 Clone sequences obtained from the 8th generation of the E. coli selection. Only the N40 region of the sequence is shown.

***Note that the phenol-dUTP (red) is read as a dTTP during the DNA sequencing process.**

Clone	N40 Region Sequence (5′-3′)	Number of phenol dUTPs*
8.1.A	ACAACAAATGTGACCATGCGATTCCCCATATCCAGGCACA	7
8.2.A	GATGCGTGTGGTGTGGAGTTGTGTGAGTGGTCTGCGTTGT	14
8.3.A	CGAGGGAATGACTTTGGTCCATGCGCTTCGGTACTCAGCT	11
8.4.A	AGAACAACACAAGCAGCATGGAACCAGTGCGCACGGTCTA	4
8.5.A	TGTCGTGCTCGCTATGGTGTATTGTGAAGAGTCCAATATT	15
8.6.A	TGTGCGTGCAGTATGGCGGTCTGTGTGTGGCATGTGCGGT	13
8.7.A	TGATAACCGCTGCCGGAACTGTGCCTGCTTCTGGTTCCCA	11
8.8.A	TGTGTGTATGCGTTCATGTGGTGAGGTCTTGCGTCTGTTT	18
8.9.A	TGGGGGGGGTGGTGGGTGGGCTTGGTTCGGTACGNCTGT	11
8.10.A	GAGTGTGTGTGGGTCCGAGTGGGTGGTCAGGGTTTGTTT	15
8.11.A	CCGCGAACCCCTAACCTATTCCTTGACTAACCTTGTTCAG	9
8.12.A	TGATAGAAATGTCGGGAGCGAACTAGTTCGAATTACGAAA	15
8.13.A	AGGGGAGTGTAAGGGGCTGATTGTTGTGTTGCGTTCTCCT	14
8.14.A	TGAAGTTCCAGCAGGCCACACCGCATGACGTTTTTCAGCA	10
8.15.A	TACGGACTCATGAAGCCAGCCATTTCTTAGTCACACACCA	12
8.16.A	GATGCGTGTGGTGTGGTGGTATGTGTGTGGCTTGTGCTGT	16
8.17.A	AGTGCATGCGTGTTTATCTGGCGTGGTTTTACGTGTGTTT	18
8.18.A	TGTGCTTTGGTGGCGTTGTGTAGTGGTTCGTCCTGTGGTA	17
8.20.A	ATGGATCGGGACGGTTGAGCAAAACTTCATGGCTTTGACA	10
8.21.A	GATGCGTGTGGTGTGGAGTTGTGTGAGTGGTCTGCGTTGT	15
8.22.A	TGTGCTTGTGGCGCGTGGGTGTGTAGGTGGTCTATGCAT	14
8.23.A	AAGTGGTGAGTGTCAGTGTGGTCTGTTGTGTTCCGTGTAT	16
8.24.A	TGTGTTTGTGGTACGAGCGTGTGTGGATGGTCCGTGTCA	14
8.26.A	ACTGTGTGCGTGCTCAAGTAGGGTGGTATTTTGTGAGCCT	14
8.28.A	TCCTCGCGTTTGGATTCATGTTGGTTTGTCGGTGTATTGT	19
8.30.A	ACAGAAAGTGTGGCCATGTGTTGTGTCCCTGGCAGGTTAGTTTA	14
8.1.B	AGGTGTGGTGCATGTGGTATGTGTTGTTGGCCTGTTAGCT	16
8.2.B	TTGAGTCAATGACACACCACGCACTAAATCTCGGCACTCT	9
8.3.B	TTGGTGGTGTGTGCCTTGGTGTTGTGTGTCGTTGGGTCA	17

Clone	N40 Region Sequence (5′-3′)	Number of phenol dUTPs*
8.4.B	TCCAATGTCTCGTACGCAACGGGCGTGGTCTAAGGATAAT	9
8.5.B	TTCATGCCCTGTGTCTTGCTCTTGTGAGTTGTTGTGTCA	18
8.6.B	ACCATCACGCACCTGCACCGTCGCCTCTATCCTTCACTT	10
8.7.B	ACCACCCCCTCGTAACACGTCTCCCGTCCTCGCCATGTTA	9
8.8.B	AGTGTGTGTGGTGTGGTGATATGCTGTTGGTCTATCTTCA	17
8.10.B	TTTGTTATGGCTGTGTTGTGTTGTGGCTAGTGTGGTGGCA	18
8.11.B	ATGGTGGTCTGTGTCTCTGTGTTGTGCGTTAGTGGGTCA	16
8.14.B	TCTGTGTGCGGTGTTATGCGGGTTGGGTTGTTTGTGTTT	19
8.15.B	TGCGTCCTGTGCTGCAGTCTTTGTGTGTCCCCTTAGTCCT	16
8.16.B	TTTGCGTCCGGGTTTATGCGGGTTGTCTCGTGTCTGTTT	18
8.17.B	TCTTGTTGGGTCGACGGTCTGTTCATGTGGGTGTTGTCCC	16
8.18.B	TTGTGTTCACGTCATTTCGCACTCCTCTCAGCTACGTTTT	17
8.19.B	TTCATGCCCTGTGTCTTGCTCTTGTGAGTTGTTGTGTCA	18
8.21.B	GTTGGAGGTGCGTGGTACGGGTGTGTGCGTGTTGTTCCA	13
8.27.B	CATGCGTGTGGGTCATGGTGAGCTGGTCCTGTCGTTGTCT	14
8.28.B	TTAGTGTGTGCCAGGGGGTTGTGTGGTTGGTTTGCGTTTG	17
8.30.B	ATGGTGGTCTGTGTCTCTGTGTTGTGCGTTAGTGGGTCAG	16

DNA templates of sequences 8.1A, 8.2A, 8.5B, 8.8A, 8.10A, 8.11B, 8.14B, 8.16B, 8.17B, 8.18B, 8.24A, 8.28A and 8.30A were ordered for further characterization. The sequences were chosen with diversity in mind, so that the largest number of divergent sequences would be assayed. Following a template purification step and elongation, all sequences were subjected to a binding experiment in which the conditions of the eighth round of selection were replicated in order to assess the ability of the individual sequence to bind to DH5α cells. Subsequently, a subset of the most promising sequences was chosen for a second preliminary assay. The second assay replicated round 8 but with unmodified DNA in order to determine if the sequence required dU^{y}TP for binding (as described previously). The results of these experiments are shown in Table 2.4 (note that the radioactive counts from the wash steps were not included, so the bound and not bound fraction do not add to 100%). From these two assays, clones 8.10A, 8.14B, 8.18B and 8.28A were selected for further characterization based on their high individual

binding ability (total fraction bound of "Modified DNA" in Table 2.4) and negligible binding ability when replaced with unmodified dUTP (total fraction bound of "Unmodified DNA" in Table 2.4).

Table 2.4 Preliminary binding assays to assess binding ability of individual clone sequences.

The column on the left lists the results of the assay carried out with dU^{y}TP; the column on the right lists the results of the assay carried out with unmodified DNA. Results are expressed as a fraction of the total radioactive counts. ND = not determined. Clones that were chosen for further characterization are highlighted in red.

Clone	Binding to DH5α, modified DNA		Binding to DH5α, unmodified DNA	
	Fraction not bound (% cpm)	Total fraction bound (% cpm)	Fraction not bound (% cpm)	Total fraction bound (% cpm)
8.1A	21	3.4	ND	ND
8.2.A	15	11	ND	ND
8.5B	30	8.6	ND	ND
8.8A	24	11	90	0.7
8.10A	38	11	91	1
8.11B	23	12	63	26
8.14B	22	17	95	0.5
8.16B	23	10	94	1
8.17B	11	11	90	1
8.18B	6.8	19	94	0.5
8.24A	53	2.9	ND	ND
8.28A	34	21	95	0.5
8.30A	29	7.8	69	1.2

An important component of a successful aptamer is its ability to specifically discriminate the desired target. Therefore, it was important to determine if the clone sequences showed cross-reactivity to other bacterial strains or non-bacterial species, particularly because negative selection steps had not been conducted during the SELEX process. A series of binding experiments were conducted in which the conditions of the eighth round of selection were once

again replicated, except that a different organism was used in place of the *E. coli* DH5α strain. The following organisms were assayed: two different *E. coli* strains, O14:K7 and K12; two non-*E. coli* gram-negative species, *Agrobacterium tumefaciens* and *pseudomonas fluorescens*; a gram-positive species, *Bacillus subtilis*; and the yeast species S*accharomyces cerevisiae*. All experiments were conducted at least three times on different days. The results of the binding tests are shown in Figure 2.3 (binding data provided in Appendix A). The total bound fraction and total unbound fraction values were of particular interest; for a sequence to be selective, the former number should be low and the latter number high for all non-DH5α strains (contrary to the desirable results for DH5α). In order to assign statistical significance to the results obtained, a one-way ANOVA analysis was performed with the "total bound fraction" mean data set obtained for each sequence. Tukey's multiple comparisons test was subsequently used in order to determine if the difference in the total bound fraction means between each of the different strains was significant using a threshold value α of 0.5 (see Appendix A for full results). Using this statistical method, it was determined that the mean differences were not significant with 8.10A (ANOVA P-value = 0.144), implying that the sequence is not able to discriminate between different strains to a meaningful degree. The results with 8.14B were found to be significant only between the target strain DH5α and *A. tumefaciens* and *P. fluorescens*, again implying limited selective ability (ANOVA P-value = 0.0366). Conversely, the mean differences for 8.18B were found to be statistically significant between all other strains and DH5α (ANOVA P-value < 0.0001), and the same was true for 8.28A (ANOVA P-value < 0.0001). These results lend confidence to the hypothesis that 8.18.B and 8.28A are highly specific for the target strain.

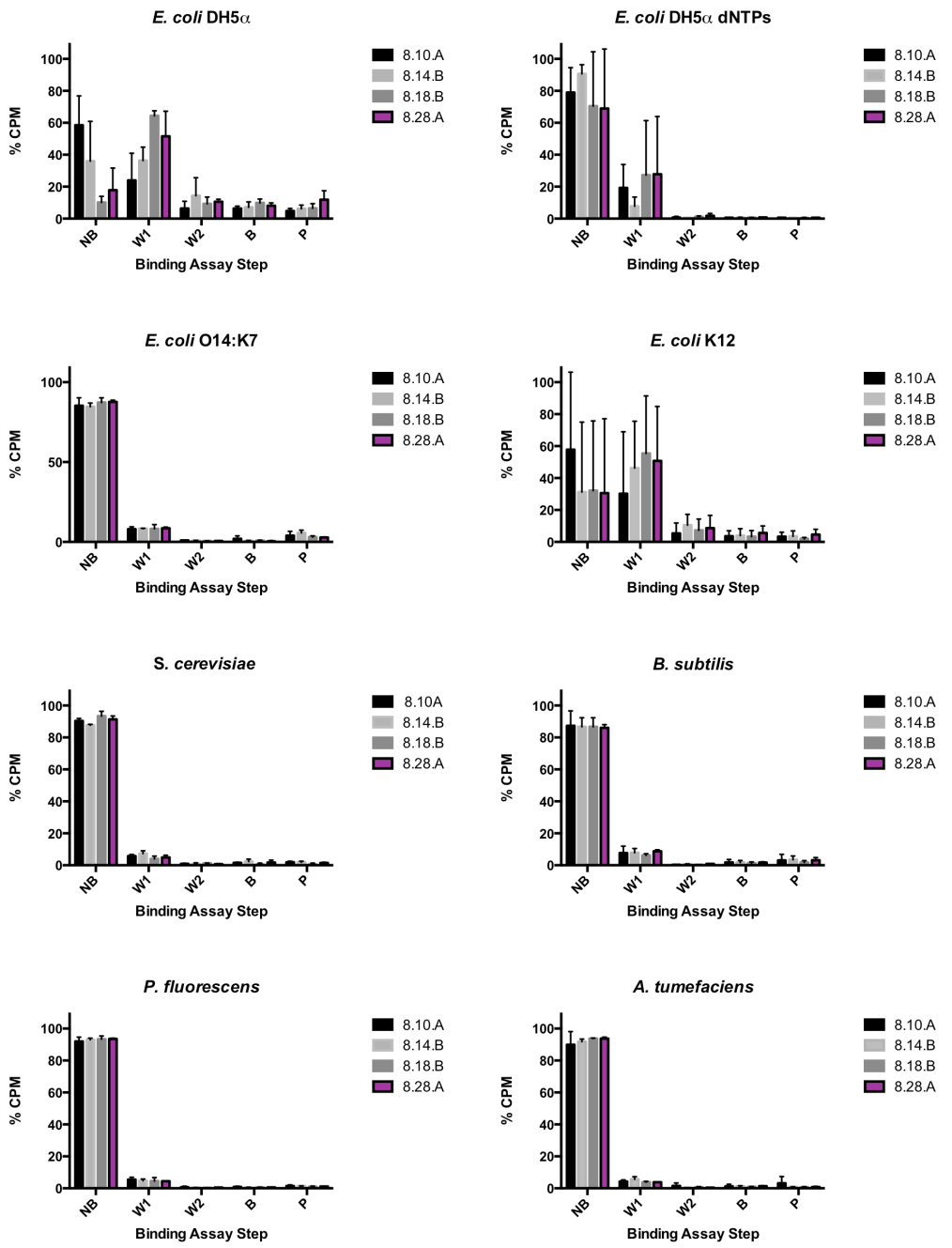


Figure 2.3 Binding assay results of cloned sequences 8.10A, 8.14B, 8.18B, 8.28A to various bacterial or yeast species as indicated.

NB - fraction not bound to cells; W1 – fraction recovered after one wash; W2 – fraction recovered after two washes; B – bound fraction recovered after denaturation; P – bound fraction remaining with cell pellet after denaturation. Experiments were performed at least 3 times independently.

Additionally, the mfold web server was used to obtain secondary structure predictions for the full-length aptamer sequences using corrected folding parameters of 138 mM NaCl, 0.5 mM $MgCl_2$, and a folding temperature of 22°C (Figure 2.4) [111, 112, 113]. While a useful tool for visualization, the software is unable to take into account any structural effects the modified nucleotide might impart. Given the high modified nucleotide content of the sequences, these effects might be significant. Furthermore, the relatively high prevalence of guanine residues in some of the sequences (notably 8.10.A and 8.28.A) is suggestive of g-quadruplex formation, which is also not predicted by mfold. The low complexity of the structures generated is consistent with other selected aptamers; however, it is unknown how the folding might change in response to target binding.

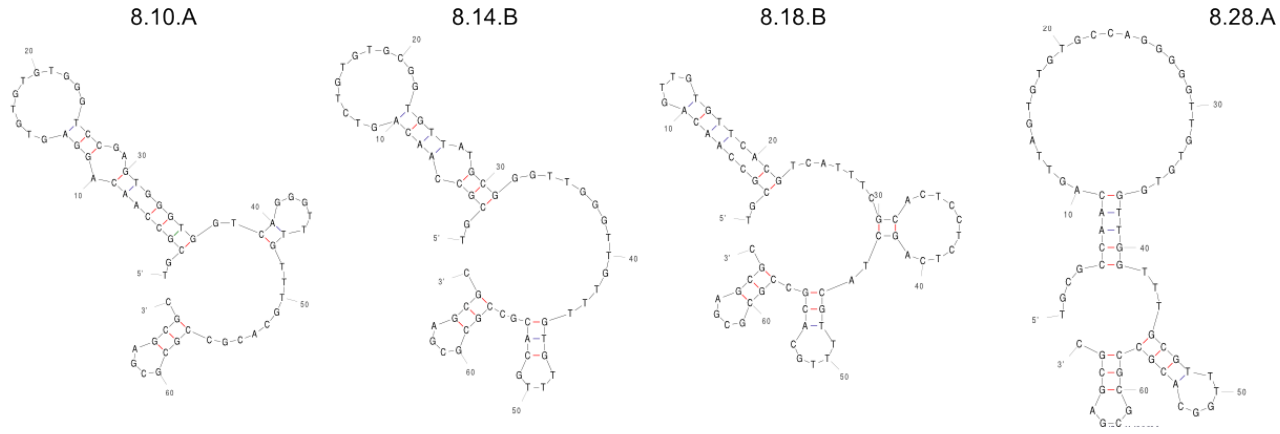


Figure 2.4 Mfold predicted secondary structure of 8.10.A, 8.14.B, 8.18.B and 8.28.A sequences.

The server does not account for the phenol modified dUTP or more complex motifs such as G quadruplexes.

The dissociation constant for aptamer candidate 8.28.A was subsequently determined by means of a saturation radioligand binding assay. Following literature guidelines, the number of cells was set at 5 nM, or about ten times lower than the predicted Kd value [114]. Seven aliquots of 10^6 DH5α cells were incubated with the following concentrations of γ-^{32}P-dATP-labeled 8.28.A in wash buffer: 10 nM, 30 nM, 60 nM, 100 nM, 300 nM, 600 nM, and 1000 nM. The samples were left to equilibrate for one hour and centrifuged at 13 000 rpm for 10 minutes. The pellets were then quickly washed with 100 μL wash buffer and centrifuged at 13 000 rpm for 5 minutes. Lastly, the radioactivity of the cells was determined using a scintillation counter (Appendix B). Since Cerenkov-type radiation was measured, no scintillation cocktail was required and the samples were simply resuspended in 1.2 mL of DEPC-treated water. The assay was repeated a total of four times. Non-specific binding was determined by incubating the same concentration range of γ-^{32}P-dATP-labeled 8.28.A sequence (10-1000 nM, with the 600 nM concentration omitted) with a saturating amount of competing ligand [115]. In this case, 1 μM of unlabelled 8.14.B and 0.3 μM of unlabelled G_o library were used as competing ligands. Linear regression analysis was performed on the data and non-specific binding at 600 nM was estimated from the y=mx+b equation. Specific binding of 8.28.A was determined by subtracting the non-specific counts from the counts obtained for total binding at the corresponding concentration according to standard protocol [115]. Data were fitted to a one-site total binding linear regression curve using GraphPad Prism v. 6.0 (Figure 2.5): $y=B_{max}(x)/(K_d+x) + NS(x)$, where NS(x) is a linear proportionality constant to account for the non-specific binding and B_{max} is the total number of receptors. The dissociation constant (K_d) of aptamer 8.28.A was determined to be 27 ± 19 nM.

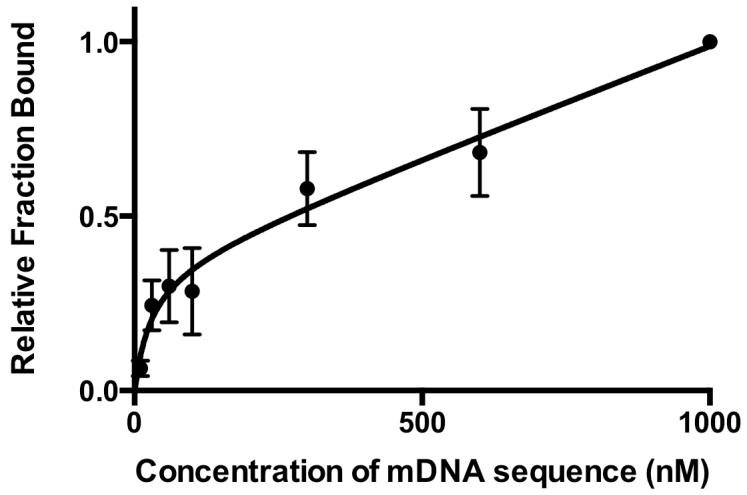


Figure 2.5 Saturation binding curve of γ-^{32}P-dATP-labeled 8.28.A (n=4) to *E. coli* DH5α cells.

Data analyzed using GraphPad Prism v. 6.0. K_d= 27 ± 19 nM.

2.3 Discussion

2.3.1 Selection Scheme Design

The additional measures undertaken in order to incorporate modified nucleotides into the cell-SELEX procedure introduced some complexities to the process. Labour and time costs were increased with the preparation of a modified library, causing the SELEX process to proceed less efficiently. Unwanted salts and truncated sequences were introduced with the extra measures described, and the steps required to remove these side products decreased the diversity and number of sequences available in the final library. Working with modified DNA also necessitated changes to the incubation step. The modified bases are not available commercially and therefore must be used sparingly, on the order of picomole amounts per experiment instead of the nanomole amounts typically used. In order for a meaningful concentration of library to be present in a selection round, the incubation volume had to be reduced. While *E. coli* can maintain viability in a small volume for the duration required in a selection, this reduction precludes the

use of mammalian cell lines as future targets using the current protocol. Indeed, in several trial experiments conducted with 4T1-hCa9 and MDA-MT1 cells, the incubation volume limitation imposed by the use of modified DNA severely compromised mammalian cell viability to such an extent that the experimental results were sabotaged by the large faction of dead cells. However, the protocol described should be suitable for most bacterial targets.

Initially, many modified nucleotides were considered as substrates for a selection. The library of base-modified nucleotides available in the Perrin laboratory provided a variety of amino acid-based functional groups to work with (see Figure 2.6), each of which might have conferred desirable binding properties to the aptamers. In order to increase the likelihood of a successful selection, different combinations of modified nucleotides were tested for the ability to elongate to full sequence length in order to mitigate excessive truncations or potential amplification issues. A subset of the combinations assayed was then chosen for a selection: $dU^{y}TP$ and $dC^{aa}TP$ with unmodified dGTP and dATP; $dU^{in}TP$ and $dA^{im}TP$ with unmodified dGTP and dCTP; $dU^{in}TP$ with unmodified dGTP, dATP and dCTP; $dU^{ga}TP$ with unmodified dGTP, dCTP and dATP; and $dU^{y}TP$ with unmodified dGTP, dATP, and dCTP. The $dU^{in}TP$ and $dA^{im}TP$ combination did not elongate well on a larger scale and was discarded prior to the selection process. Ultimately all the selections failed to show enrichment after five rounds except for the $dU^{y}TP$ selection. The high failure rate is consistent with the observation made by Larry Gold and others that fewer than 30% of protein-SELEX procedures result in aptamer discovery; it is reasonable to assume that in a more complex selection, such as cell-SELEX, the success rate would be even lower [78]. However, the failed selections provided valuable insight into optimizing the conditions for the successful $dU^{y}TP$ selection, such as the length of the incubation in each round and decision to not run negative selection rounds.

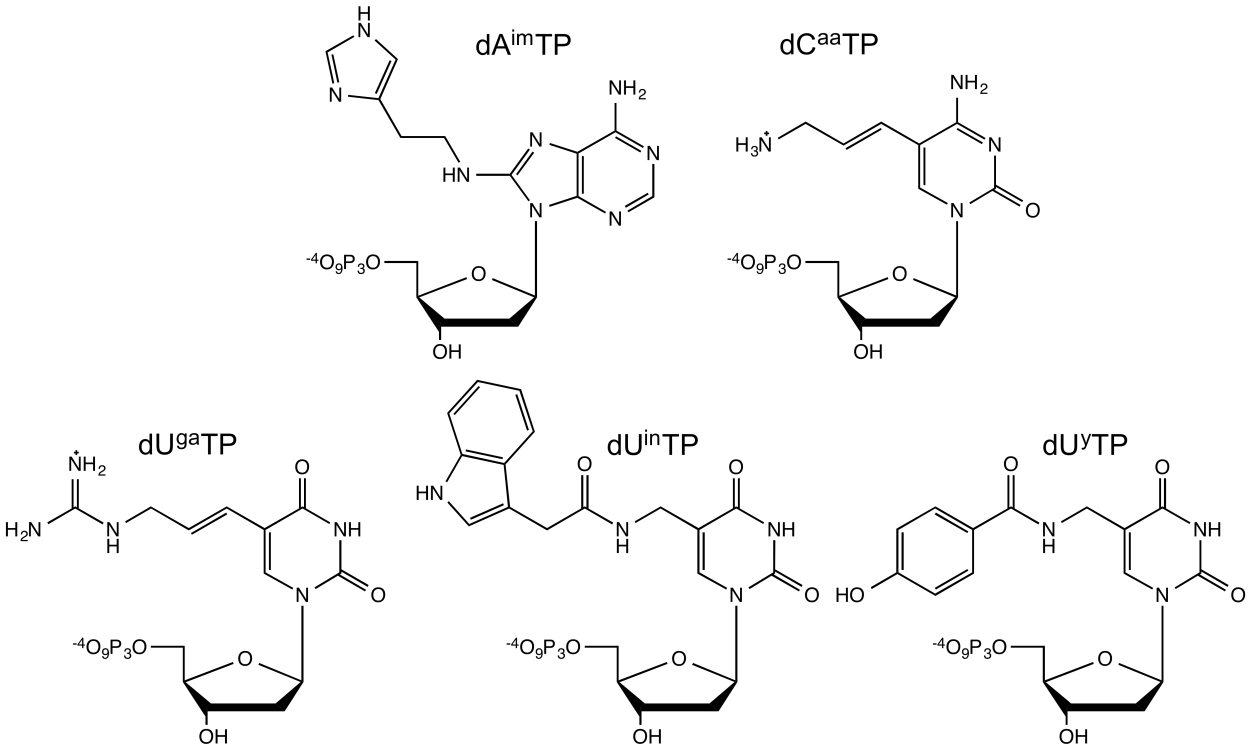


Figure 2.6 Modified dNTPs considered for the *E. coli* aptamer selection.

From clockwise from top left; imidazole-dATP, aminoallyl-dCTP, guanidinoallyl-dUTP, indole-dUTP, and phenol-dUTP.

2.3.2 Cell-SELEX with dU^{y}TP

Two interesting features emerged from the phenol-dUTP selection process. The first was the emergence of a population of sequences that were not released from the cell fraction despite multiple washes and excessive denaturation. Termed the "pellet" sequences, it is possible that these strands crossed both outer and inner cell wall and became bound to a moiety in the cytosolic compartment. If lysis was incomplete for a portion of the cells in the samples, it is reasonable to hypothesize that some of these sequences remained in the cytosolic compartment and were not released into the supernatant. Unfortunately, it was not feasible to further investigate this hypothesis. These sequences were incorporated with the bound fraction of

sequences for the subsequent selection rounds, and therefore the pellet fraction was not separated from the bound fraction for the cloning process. Some of the clones chosen for further characterization did display some indication of sticking to the cell pellet, but these results were somewhat ambiguous and no tested sequence overwhelmingly bound to the pellet. There was the possibility using confocal microscopy to visualize some of the more promising sequences with *E. coli* in order to assess their location in the cell, but time and resources did not permit for this. However, it would be a worthwhile study to conduct in the future, as it would shed insight into the nature of the aptamers that were generated and perhaps point to a unique function provided by the modified nucleotide.

The second unusual feature of the selection was the emergence of two distinct bands on the PAGE gels after each round of selection (visible in Figure 2.2). The simplest explanation for the two bands is that the upper band corresponded to full-length elongation product while the lower band corresponded to a truncated sequence. However, when the product is observed on a gel directly after elongation, only one band is visible, indicating that fully elongated sequences were almost exclusively obtained. The second possible explanation is that the upper band corresponded to sequences containing more of the modified nucleotide, while the lower band contained sequences with a lower number of modifications. Since modifications retard gel migration, this explanation could easily account for the appearance of multiple bands. However, multiple bands were also observed during the clone characterization process, when only a single sequence was being investigated at one time. Therefore, a third possibility must be introduced, which is that the aptamer sequences are able to adopt different energetically-similar conformations and are not totally denatured by the PAGE gel, causing them to migrate slightly differently from each other. It is difficult to comment with any certainty whether this is the case

without detailed structural studies that are outside the goals of this particular selection process. Additionally, there is little to no literature with which to compare this finding, as the use of PAGE gels to isolate the oligonucleotides constituted one of the changes made to accommodate the use of modified DNA. Therefore, a normal SELEX procedure does not include a step in which the isolated sequences are visualized. That said, mfold provided several predicted secondary structures for certain sequences that had similar energies of activation, which provides some credibility to this hypothesis.

Cloned Sequences

The 46 sequences obtained from the cloning process contained, on average, approximately 14 $dU^{y}TPs$ in the N40 region. This is statistically higher than would occur randomly, particularly given that the modified nucleotide is more difficult to incorporate. While certain sequences were highly similar, only two clones shared 100% sequence identity and generally the sequences were diverse and could not be grouped neatly into a small number of families. That said, certain features are seen in a majority of the sequences: a statistically low number of As, and modified Us appearing in double or triplet patterns. It should also be noted that some sequences contain only 39 bases in the N40 region, a known phenomenon in selections and a consequence of mutations, and one sequence contained an extra "tail" of 4 bases. However, the majority of the cloned sequences contained a fully conserved 40-nucleotide random region. It was only feasible to study a subset of the sequences obtained and therefore a group of 13 diverse sequences from both selection populations were chosen.

The binding assay used to determine the approximate binding ability of each individual sequence to *E. coli* cells was performed multiple times over the course of months. As in the selection process, the fractions of the sequences that were not bound to the cells, bound to the

cells and that remained in the pellet were determined using radioactivity. The results obtained for each sequence varied by as much as a factor of 2 or 3, making it difficult to separate the better clones from the poorer ones. The reasons for the inconsistent results are not completely clear but may include temperature differences (the unreliable heating/cooling system in the laboratory meant that the ambient temperature fluctuated from 16-28 °C), changes in cell morphology due to the age of the cell stock, and contaminated or spoiled reagents. However, the binding assays still allowed for a rough estimate of a clone sequence's binding ability. From the initial pool of 13, the 4 best sequences were chosen for further characterization. The 4 best sequences did not appear to share any distinct characteristics over the others, although they tended to have a cluster of $dU^{y}TPs$ at the 3' end of the sequence and a high G content.

The next phase of the project was determining the selectivity of the candidates for the target strain. In observing the results throughout the course of performing the selectivity tests, *E. coli* K12 appeared to retain the highest fraction of bound sequences. The DH5α strain is a derivative of the K12 strain, and is highly genetically similar to K12 [116]. Thus it is not surprising that some cross-reactivity towards K12 was seen, as the most of the cell surface components and overall morphology of the cell are likely shared between the two strains. It also suggests that the sequences did not bind to moieties induced by the *lac* operon because the K12 strain was not induced with IPTG. Additionally, an assay in which the candidate sequences were incubated with non-IPTG-induced DH5α cells was performed and binding did not appear to be significantly affected. However, the overall selectivity of the sequences for the target strain varied as revealed through the statistical analyses that were performed. 8.14B and 8.10A were found to have few or no statistically significant results (the null hypothesis was seldom rejected using a threshold value α of 0.05). However, the null hypothesis for each of the comparisons

between DH5α and the tested strains were rejected in the cases of 8.18B and 8.28A, implying that these aptamers are able to discriminate even between *E. coli* DH5α and K12. The excellent selectivity displayed by 8.18B and 8.28A for the DH5α strain was a highly satisfying result because no negative selection rounds had been included in the SELEX process to remove any sequences with potential cross-reactivity with other strains. Therefore the generation of poorly selective aptamer sequences was a definite risk, albeit one that did not materialize for all the sequences obtained.

Saturation Binding Assay

Determination of the dissociation constant for at least one of the aptamer sequences was a critical aspect of characterization. Sequence 8.28.A was chosen as the initial sequence for testing, and it was hypothesized that most of the sequences would have dissociation constants similar to that of 8.28.A due to the relatively similar composition and predicted secondary structures of the strands. Some constraints were faced when designing the binding assay, particularly the volume restraints posed by the use of modified material. Initially, two types of experiments were considered: a homologous competitive binding assay, where increasing amounts of cold ligand would be introduced to tubes containing a set amount of hot ligand and cell pellet; and a saturation binding assay, where increasing amounts of hot ligand would be introduced to tubes containing a set amount of cells. Since a homologous competitive binding experiment is more complex and involves more variables, a saturation binding assay was the chosen method for K_d determination. In order to obtain 5 nM of cells per tube, only 100 uL centrifuged cell stock was required, resulting in an extremely small pellet. Therefore, error due to pellet loss through steps of supernatent removal and sample transfer was a significant concern throughout the process, and likely contributed to the error reflected in the binding curve (Figure

2.5) and the corresponding dissociation constant. Seven concentrations of labeled 8.28.A were utilized that were anticipated to encompass and exceed the K_d by a factor of at least 100, a condition for obtaining useful data. Saturation binding assays also require that the system be in equilibrium for the data to be valid. The recommended incubation time to achieve equilibrium is stated as 5 x 0.693/k_{off} , which is unfortunately only useful if the k_{off} value is known prior to the equilibrium binding experiment [114]. Often this is not the case, and in practical terms the incubation time in the cell-SELEX literature is frequently set at 30-45 minutes [93, 94, 117]. The incubation time for the saturation binding experiment with 8.28.A was set at one hour to ensure that equilibrium had been reached. Accounting for non-specific binding was more challenging because the target of 8.28.A is unknown and it is even possible that the sequence is interacting with more than one cell component. In fact, this step is frequently omitted in the literature owing to the difficulty of defining non-specific binding for these systems, but remains an important component of properly assessing the binding kinetics. Utilizing unlabelled 8.28.A sequence was not a good choice because there would be direct competition for both the specific binding sites and the non-specific ones, when only competition for the former is desired. Therefore, a disproportionate mixture of aptamer sequence 8.14.B and the initial library was chosen to be the competing ligands. While not a totally ideal solution, it was expected that competition for specific binding sites and a small amount of competition for non-specific binding sites would occur, and that this would be an acceptable compromise. Non-specific binding was determined to have very little impact on the binding curve data, either in the effect on the K_d value or the standard error associated with that value. However, from the shape of the graph it appears likely that non-specific binding was under-estimated for this system, as the curve appears somewhat unsaturable at high concentrations. Another factor that might have contributed to this effect is the

presence of non-binding or non-specifically binding radioligand truncates. While the sequences used for the assay were gel purified to remove any oligonucleotides of incorrect length, it is possible that this process was not fully effective, and these radioactive truncates might have further contributed to the inflated contribution of non-specific binding. However, the consistent results that were obtained in four independent assays lends confidence to the overall analysis. The dissociation constant obtained (27 ± 19 nM) is considerably better than dissociation constants reported for most other aptamers generated against bacterial cells, which are often in the 100 -1000 nM range or higher [93, 118, 119, 120]. Nonetheless, some sub-20 nM K_d values have also been reported [94, 117]. 8.28.A can therefore be considered a robust aptamer in terms of affinity and selectivity for the target bacterial strain but does not necessarily represent a significant improvement in these areas. It would be interesting to measure the dissociation constants of the other aptamer candidates in order to determine if they are materially better, worse or comparable to the K_d of 8.28.A.

Collectively, these results show that base-modified DNA can successfully be used to select aptamers against challenging whole cell targets. These findings, in conjunction with the fact that the sequences are dependent upon the $dU^{y}TP$ nucleotide for binding, suggest that the increase in chemical diversity afforded by the modification played a beneficial role in the selection. Given that the bacterial cell membrane is negatively charged, it is an inherently difficult target for aptamer selections. The phenol modification likely facilitated binding interactions and mitigated negative charge repulsion; it is also probable that the sequences were able to bind to cell moieties not accessible to unmodified DNA. Although modified DNA can be difficult to incorporate, the high number of modifications that were present in the clone sequences points to the ease with which the modification was incorporated and propagated in the

selection process. Although the affinity and selectivity of the modified aptamer was merely comparable to the best unmodified sequences but not measurably better, these findings are a single example of the capabilities of base-modified DNA in a challenging selection. The power of mDNA is therefore still largely untested and it is too early to form any definite conclusions with regards to the improvements to cell-SELEX that could be achieved. Even if affinities and selectivity cannot be significantly improved upon, there are hundreds of bacterial strains alone for which an aptamer would be desirable, and the differences in cell surface composition between many of them are subtle. Thus, the use of modified DNA offers a powerful and perhaps necessary tool towards the generation of aptamers that can discriminate between this vast number of complex targets. The amino acid-inspired modifications explored by our group are of particular interest, as they will allow oligonucleotides to form more protein-like interactions with the cell. Future work should focus on pursuing cell-SELEX with other modified bases, including multiple modified base combinations, to other challenging targets. Doing so will provide more insight into the advantages that mDNA can provide to the cell-SELEX process and increase the number of potentially useful aptamers available. The eventual goal of this research would also include the development of a useful analytical assay or diagnostic tool that would incorporate the power of aptamer-target recognition in a commercially viable application.

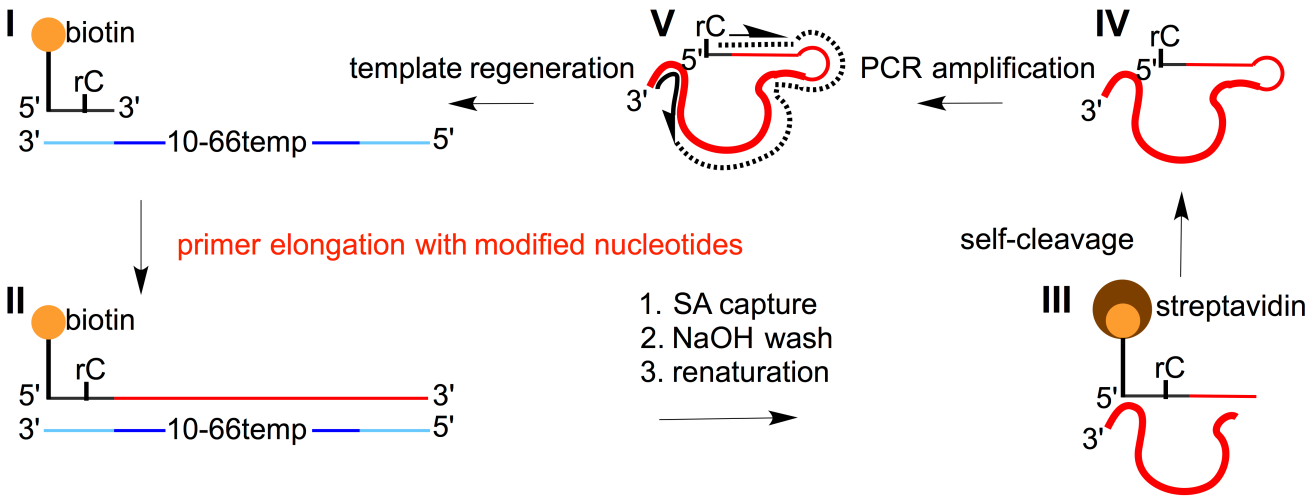
Chapter 3: A New Method for Selecting Modified DNAzymes and Aptamers without the use of Modified DNA as a Template in PCR

3.1 Method Background and Design

3.1.1 Introduction and Background

The focus of Chapter 3 is the development and validation of a novel selection scheme that facilitates the use of modified DNA for *in vitro* processes. A new selection methodology was desired that would circumvent the need to PCR amplify modified DNA (mDNA), which is often one of the most problematic selection steps encountered in the laboratory of Dr. Perrin and others working with non-canonical oligonucleotides.

Currently, most mDNAzymes are identified by '*in vitro* selection', outlined in Scheme 3.1 [72, 53]. Modified DNA is first synthesized enzymatically from an unmodified cDNA library (I-II), after which it is typically captured on streptavidin-coated magnetic beads and subjected to a number of washes with a 0.1 M NaOH solution in order to remove the template strand. The modified DNA strand is then renatured into a catalytically active structure (III) and the desired catalytic activity is selected for (IV). In the case of the work conducted in the Perrin group, the desired activity involves self-cleavage at an internal ribophosphodiester bond, as depicted in the scheme. The desired active sequences are collected and a PCR step amplifies these sequences by copying the mDNA into its cDNA (V). Subsequent enzymatic synthesis of the enriched mDNA library from the cDNA templates begins the next round of selection (I).



Scheme 3.1 The current process for selecting mDNAzymes.

Enzymatic synthesis of mDNA (I –II) is followed by template removal and renaturation (III), self-cleavage (IV), amplification (V) and regeneration of the template for the next round of selection (I). SA: streptavidin, rC: ribophosphodiester bond.

This selection scheme can be extended to the selection of many mDNA aptamers, except that during steps III and IV, the mDNA strand is cleaved from the biotin support via incubation with sodium hydroxide prior to the selection step as described in Chapter 2. The desired mDNA strands are renatured into a complex structure suitable for binding to a target and the sequences are screened based upon binding ability in place of catalytic activity. However, other strategies exist for mDNA aptamer selections. For instance, a variation to the process described above may be pursued in which the embedded ribose linkage is included in the template strand instead of the modified DNA strand [121]. The chimeric double-stranded DNA is subjected to high molar NaOH in order to hydrolyze the ribose linkage and separate the two strands. Alternatively, following the single-primer elongation step, the dsDNA can be captured on beads via biotinylation of the template strand and the mDNA eluted with NaOH [78]. Other methods have utilized direct symmetric and/or asymmetric PCR amplification followed by some method of purification;

incomplete or poor amplification of material has been observed in a number of these publications [122, 123, 124]. Therefore, regardless of the method chosen, the difficulty in amplifying the selected mDNA remains a common obstacle.

3.1.2 New Design Scheme

The idea of linking genotype to phenotype was used as the conceptual starting point for designing a new selection method. Using this strategy, the template cDNA (the "genotypic strand") remains linked to the desired mDNA (the "phenotypic" strand) in a single DNA construct throughout the activity or binding selection step. The two components are then separated and the cDNA or genotype is used in the following steps. Thus, only unmodified DNA is used as a substrate for PCR amplification. In addition to promoting a more efficient selection that reduces the loss of sequence diversity, the design allows the use of modified substrates that may not be amenable to polymerase amplification. Additionally, the requirements for PCR amplification can pose structural restrictions on catalytic and aptameric strands, as rigid structures may inhibit amplification; this selection system frees the active strands from such restrictions, thus expanding the conformational space available for selection.

The genotype-phenotype DNA construct was initially designed with the selection of RNA-cleaving mDNAzymes in mind and consists of six core DNA components (Figure 3.1). Four of these components are intrinsic to the principal, or "template" construct: the unmodified cDNA "genotypic" region (dark blue); the 5' and 3' primer regions to allow for PCR amplification of the cDNA (light blue); the hairpin loop, which provides structural flexibility (green); and the modified catalytic "phenotypic" region that is encoded by the cDNA sequence and that produces the targeted function (red). Two additional components, the selection primer

and the displacement primer, are extrinsic to the primary structure. The selection primer is 27 nucleotides in length and contains an internal biotin tag to allow for binding to a streptavidin bead support, as well as an embedded ribose to permit catalytic activity (dark grey). The 71-base displacement primer contains a region that is complementary to the hairpin region and controls the conformation of the template construct (purple). The detailed structural roles of these primers are discussed below.

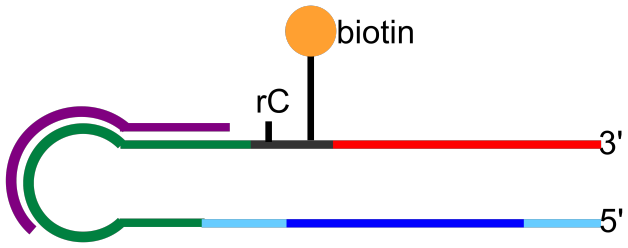
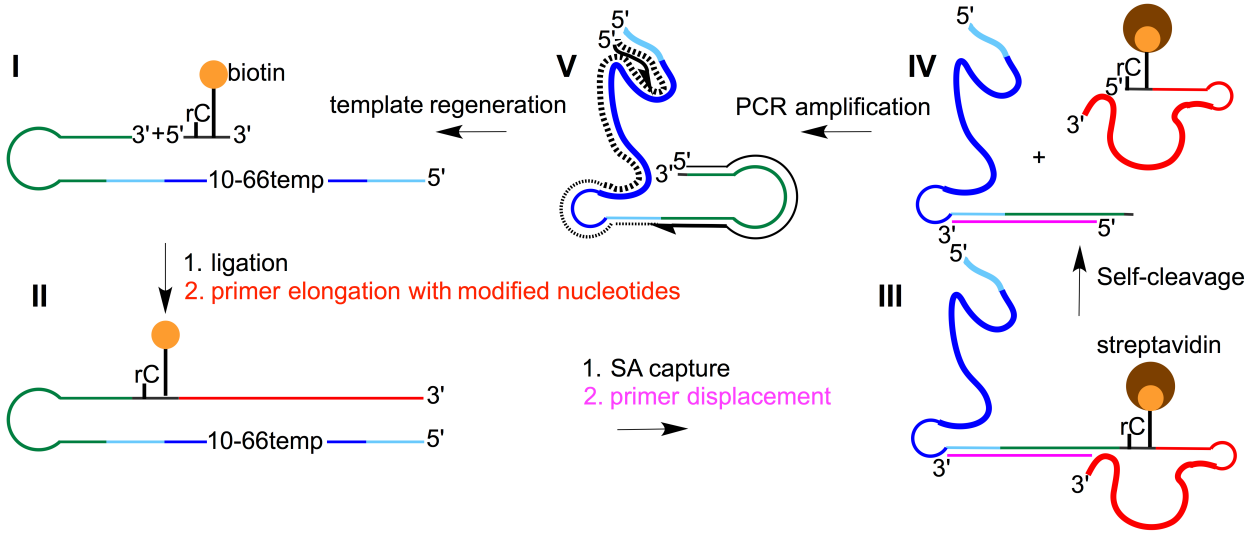


Figure 3.1 Linked phenotype-genotype construct design.

The six components are shown in different colours: cDNA genotype region (dark blue); cDNA primer-binding regions (light blue); hairpin loop (dark green); selection primer (dark grey); mDNA phenotype region (red); and displacement primer (purple). The primary construct refers to the whole cDNA region including primers and hairpin loop.

In principle, the selection process begins with the ligation of the selection primer to the 3' end of the primary construct as shown in Scheme 3.2, (I). Once ligated, the product is elongated in the presence of the requisite modified and unmodified dNTPs (II). Following enzymatic synthesis of the modified DNA (mDNA) region, the construct is captured on streptavidin beads and is subjected to a number of washes in order to remove any non-ligated templates. Subsequently, the hairpin region is denatured using NaOH and renatured rapidly in the presence of the displacement primer. The displacement primer binds to the hairpin region and prevents the construct from refolding into a long loop (III). This allows the catalytic mDNA region to adopt a conformationally competent structure and undergo catalysis (IV). Cleavage of the embedded

ribose liberates the cDNA (unmodified) region of the construct into solution and retains the catalytic (modified) region as well as any uncatalyzed constructs on the streptavidin beads. The supernatant is collected and the cDNA region directly amplified by PCR via the light blue primer-binding regions to begin the next round of selection (V). Thus, the typical need to amplify the mDNA region is circumvented. Although shown for an RNA-cleaving DNAzyme selection, the scheme could be easily modified to accommodate an aptamer selection or a DNAzyme selection for a different catalytic function.



Scheme 3.2 The new selection scheme, shown for an RNA-cleaving DNAzyme selection.

The selection primer is ligated to the template construct (I), and the mDNA sequence is synthesized (II). Addition of the displacement primer (III) allows for a catalytically competent structure to form which self-cleaves, liberating the cDNA from the mDNA (IV). PCR amplification of the selected cDNA strands generates the cDNA library for the next selection round (V-I).

3.2 Validation of New Design using Dz10-66

The methodology first required validation using a known DNAzyme sequence before a *de novo* selection could be attempted. The self-cleaving DNAzyme 10-66, previously developed

by the Perrin lab using standard protocols and depicted in Figure 3.2, was chosen for this purpose [74]. Dz10-66 was designed to operate efficiently under physiological conditions and contains three modified nucleosides, dU^{guan}TP (guanidinoallyl), dC^{aa}TP (aminoallyl) and dA^{im}TP (imidazole) along with unmodified dGTP. Self-cleavage at a single embedded ribophosphodiester bond was found to occur with an average rate constant k_{obs}=0.57±0.04 min^{-1} at room temperature and k_{obs}=0.63±0.04 min^{-1} at 37°. Accordingly, the primary construct was designed with the 40-base active sequence of Dz10-66 as the cDNA genotype region, with short 5-base flanking sequences added to the 5' and 3' ends to separate the sequence from the primer regions.

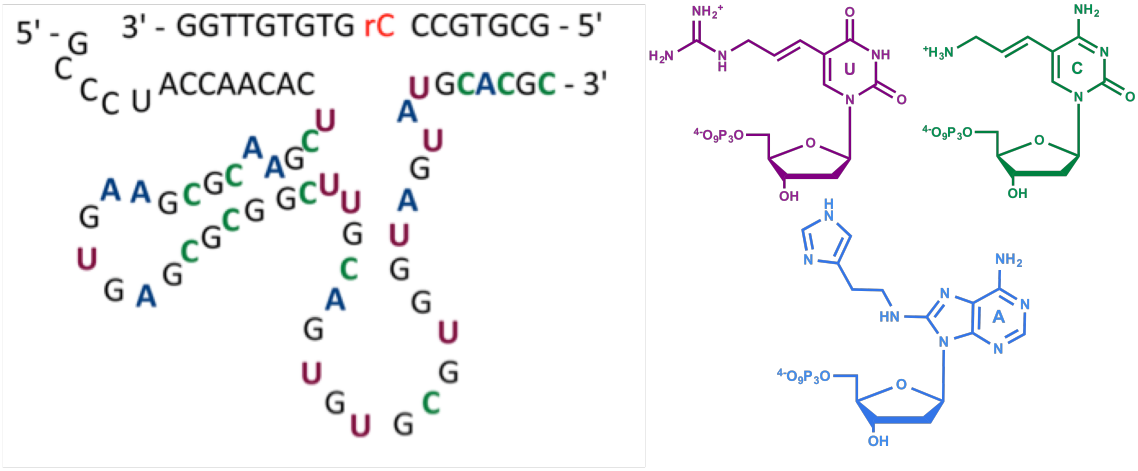


Figure 3.2 The mfold predicted 2D structure of Dz10-66 prepared from dU^{guan}TP (purple), dA^{im}TP (blue), dC^{aa} TP(green) [111]. The cleavage site is highlighted in red.

As shown in Figure 3.3 A, ligation of the template construct to the selection primer described above was achieved, followed by successful elongation of the template strand with the modified dNTPs. As a control, a 5' γ-^{32}P-dATP-labelled construct was treated with 0.8M NaOH, cleaving the embedded ribose linkage and producing a radioactive product 147 nucleotides in length as expected. It should be noted that the gel migration of the construct at different stages

does not necessarily correspond to the predicted position based on molecular weight, as the large and architecturally complex nature of the construct can cause supercoiled structures that migrate to a "lower" molecular weight position. In order to verify that elongation was taking place, un-elongated and elongated samples of the construct were run on a 1.5% agarose gel and subsequently stained with ethidium bromide (Figure 3.3 B). Ethidium bromide is a well-known DNA intercalating agent, and is widely used to detect double-stranded DNA [125, 126]. However, ethidium bromide also has a weak affinity for single-stranded DNA, largely by intercalating short helical motifs found within the single-stranded structure [126]. Therefore, by comparing the intensity of ethidium bromide staining, it is possible to qualitatively assess the degree to which a sample is double-stranded. As expected, the elongated sample fluoresced much more intensely compared to the single-stranded un-elongated sample, providing further confirmation that the template construct had been successfully elongated despite its low migration position on 8M urea PAGE gels.

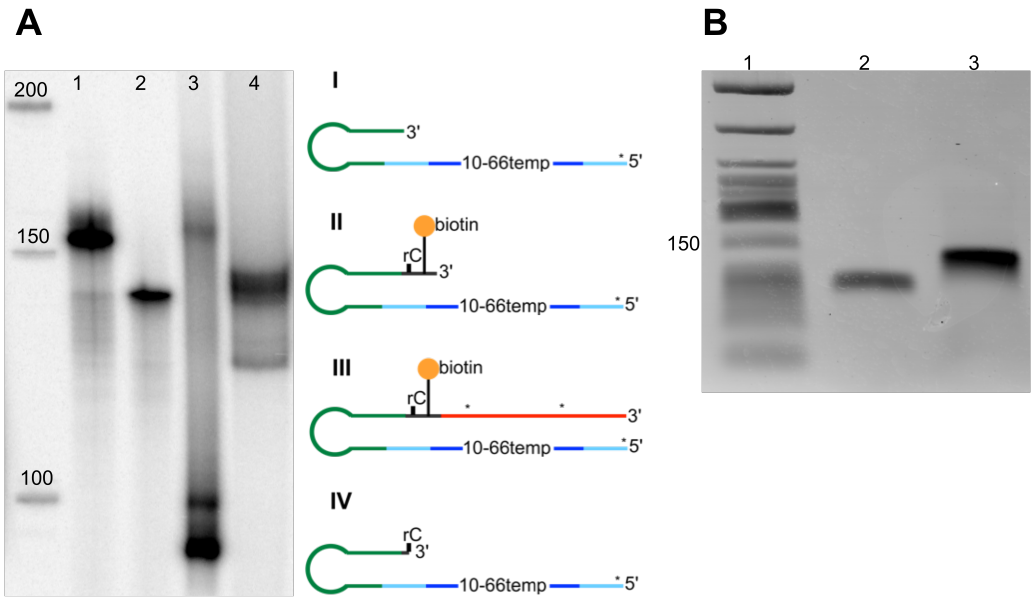


Figure 3.3 Characterization of Dz10-66 construct.

A: Denaturing PAGE gel showing 1) the ligation product of the biotinylated primer and the Dz10-66 template construct (II), 2) the 5' labeled unligated Dz10-66 template construct (I) , 3) the ligation producted elongated with dU^{guan}TP, dC^{aa}TP, dA^{im} TP and α-^{32}P-dGTP (III), 4) the supernatant of the beads after incubation with elongation product and treatment with NaOH (IV). The numbers above the bands indicate the length (in nucleotides) of the products. B: Ethidium bromide-treated agarose gel depicting 1) low molecular weight ladder, 2) unelongated ligation product (lane 1 in part A) and 3) elongated ligation product (lane 3 in part A).

In order to assay the cleavage abilities of Dz10-66, the original cleavage conditions were integrated into the selection scheme. The elongated construct was internally α-^{32}P-dGTP-labelled, allowing for the observation of product formation over time. Time points were taken after 30 seconds, 1 minute, 3 minute, 10 minutes, 30 minutes and 1 hour by removing 5 μL of the reaction slurry and mixing with 5 μL gel loading buffer. In addition, a positive cleavage control was taken by removing 4 μL of the reaction slurry after the final preparatory wash, incubating in 0.8 M NaOH, and mixing with 4 μL gel loading buffer. Negative control experiments were also conducted to confirm the necessity of the modified nucleotides and displacement primer in

forming a competent mDNA catalyst. The observed Dz10-66 self-cleavage activity showed that the construct folds into the secondary structure required for catalysis (Figure 3.4 A). However, analysis of the autoradiographic density indicated that the cleavage rate is depressed approximately six-fold (k_{obs} = 0.11 min^{-1}) compared to the rate achieved by the original Dz10-66 (Figure 3.4 B). This is hypothesized to be due to the fact that the unmodified template may be transiently hybridizing with the mDNAzyme and obstructing its ability to cleave the embedded ribose.

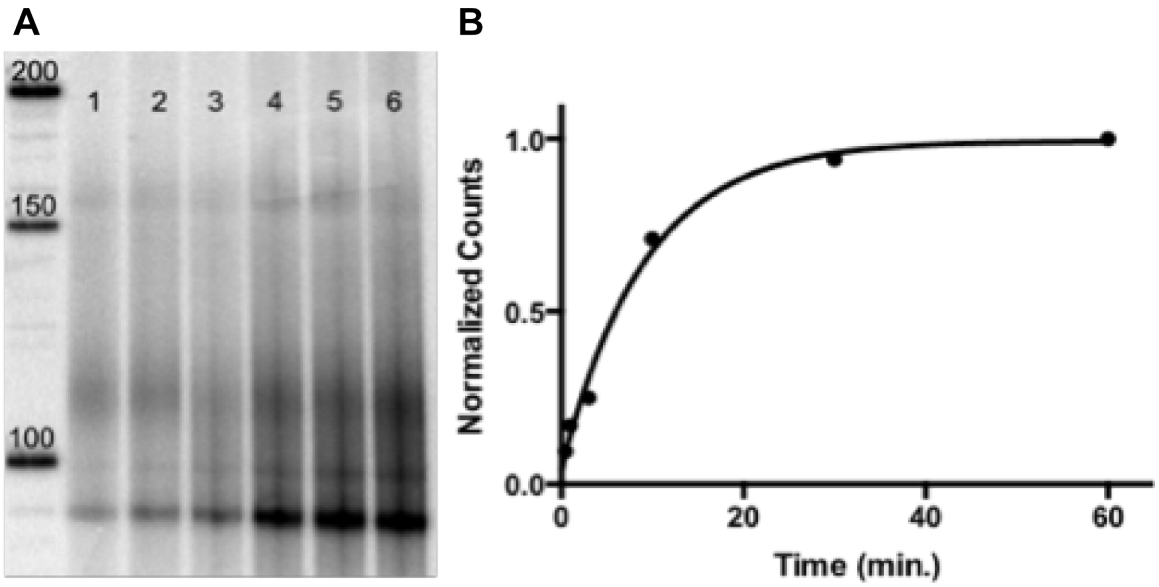


Figure 3.4 Time course of Dz10-66 cleavage when incorporated into the template construct in Figure 3.2. Lanes 1-6 correspond to the cleavage of the construct on a streptavidin bead support after 0.5, 1, 3, 10, 30 and 60 minutes. The autoradiographic density corresponding to the cleavage product (y-axis) is plotted against time (x-axis). The data were fitted to a first-order exponential decay function.

Although catalysis in the construct was achieved, the depressed rate constant raised concerns that the selection system was unreliable when dealing with a known mDNAzyme and that this could translate into significant problems in a *de novo* selection. Further experiments were proposed to characterize Dz10-66 and confirm that the behaviour in the construct is largely

consistent with that of the original catalyst. The original study conducted on Dz10-66 included a pH profile in which it was shown that the catalyst reaches an optimum cleavage rate at a pH of approximately 7.3. Activity is depressed at a pH of 8 and almost completely inhibited at a pH of 8.5 and higher [74]. Therefore, cleavage trials were undertaken with the cleavage buffer adjusted to pH 7, 8, 8.5 and 9 as indicated in the Figure 3.5 below. Qualitative inspection of the cleavage progression indicated that Dz10-66 in the construct achieved good cleavage rates at a pH of 7 and 7.5. Cleavage ability decreased at a pH of 8 and was severely compromised at a pH of 8.5 and 9 (no time-dependent cleavage is observed under these conditions). The observations are consistent with the behaviour of the original catalyst, although Dz10-66 in the construct appears to be less sensitive to pH changes compared to the original selection set-up. These experiments provided some additional evidence that the catalyst did behave in a predictable and reproducible manner within the context of the template construct.

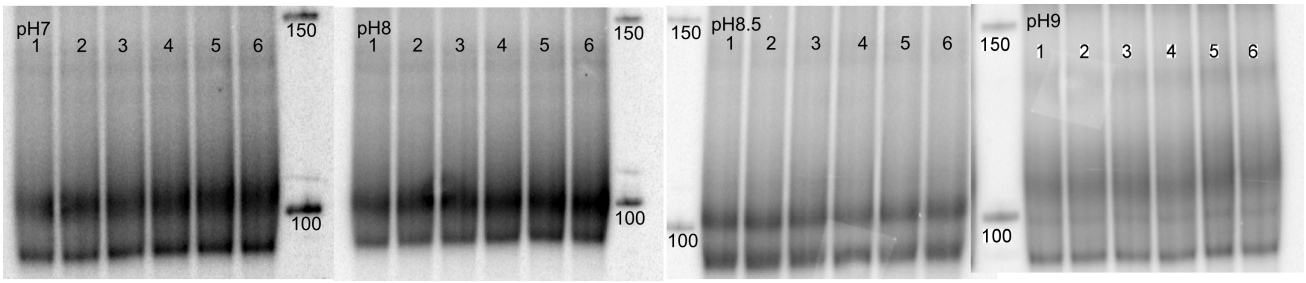


Figure 3.5 pH cleavage profile of Dz10-66 in the new selection system.

From left to right, cleavage activity was monitored at a pH of 7, 8, 8.5 and 9. Lanes 1-6 correspond to the cleavage of the construct on a streptavidin bead support after 0.5, 1, 3, 10, 30 and 60 minutes.

3.3 Discussion

Significant problems were overcome with the new selection methodology, chiefly the requirement to PCR amplify modified DNA. Moreover, by linking genotype and phenotype in one functional structure, tedious template separation and product purification steps were avoided and selection rounds proceeded faster and with less product loss compared to the current selection process. However, several weaknesses were also revealed in the course of developing and working with the system. As was mentioned above, Dz10-66 displayed a depressed rate constant in the construct. If this is in fact due to the unmodified region obstructing the active site of the catalyst, then a potentially unwanted source of selection stringency is inherently built into the methodology. Given that many traditional catalyst selections do not show any initial activity for several rounds, this effect might be exacerbated in the new system, leading to the premature abandonment of potentially promising selections. The construct itself also proved difficult to manipulate due to its large size and complex structure. Results were largely analyzed on 8M urea PAGE gels and were often ambiguous because the structure sometimes migrated to unusual positions. In a strange coincidence, the fully elongated product was found to migrate to a similar position as the cleaved product, which posed a particular problem when measuring cleavage progression. Typically, cleavage activity is ascertained by monitoring cleaved product over time versus uncleaved product over time; however, the overlap made this type of analysis unfeasible. Instead, a relative cleavage rate was determined, which is a less robust calculation. It is uncertain whether this would be a difficulty encountered in other selections or if it is a unique feature of the Dz10-66 template. Conducting a selection for a new DNAzyme would provide insight into these ambiguities and enhance our understanding of the new method. In-depth structural studies could be incorporated that would vary the length of the hairpin loop region in order to probe the

hypothesized obstructive effects of the cDNA region on the catalytic region. Additionally, some potentially beneficial techniques could be attempted that were not a part of these studies, for instance varying the denaturant in the gel solution in an effort to obtain truer gel migrations.

In summary, a novel selection system was developed to facilitate the discovery of modified DNA catalysts and was validated with the previously discovered mDNA catalyst Dz10-66. Future work should focus on the selection of new mDNAzymes using this methodology. The system is sufficiently flexible that the scheme may be adapted to select for a wide range of bond-forming and bond-breaking reactions, in addition to aptamer selections. A new selection would provide insight as to whether the new methodology yields mDNAzymes or aptamers with improved characteristics, and some of the concerns raised in the previous paragraph could be further examined.

Chapter 4: Materials and Methods

4.1 Materials

General chemical reagents and salts were obtained from commercial sources (Sigma-Aldrich, Fisher Scientific, Acros Organics). All oligonucleotides were purchased from IDT (San Diego, CA, USA). $dU^{guan}TP$, $dU^{y}TP$ and $dA^{im}TP$ were synthesized as described previously [127,128]. $dC^{aa}TP$ was purchased from Trilink Biotechnologies (San Diego, CA, USA). Ultrapure dNTPs were obtained from Fermentas. Sequenase Version 2.0 T7 DNA polymerase was acquired from Affymetrix. T4 polynucleotide kinase, T4 DNA ligase, λ exonuclease, $Vent_{R^{®}}$ (exo-) DNA polymerase and yeast inorganic pyrophosphatase were purchased from New England Biolabs. PCR products were visualized on agarose gels (agarose D, Bio Basic) using in-gel ethidium bromide staining. Sephadex G-25 fine from GE Healthcare was used to freshly prepare G-25 columns. α-^{32}P-dGTP (10 mCi/ml) and γ-^{32}P-ATP (10 mCi/ml) were obtained from Perkin Elmer. Streptavidin magnetic particles (10mg/ml) were purchased from Roche. Sheared Salmon Sperm DNA (10 mg/mL) was obtained from Eppendorf. Polyacrylamide gels were made using a 40 % 19:1 acrylamide/bisacrylamide solution (Accugel, National Diagnostics). The low molecular weight DNA ladder was purchased from New England Biolabs. Dulbecco's Phosphate Buffered Saline was obtained from Life Technologies, and bovine serum albumin and yeast tRNA were purchased from Invitrogen. All bacterial cell strains were obtained from UBC Chemistry Department Bioservices. *Saccharomyces cerevisiae* cells and culturing reagents were received from the Loewen Lab, Life Sciences Institute, UBC.

4.2 General Methods

4.2.1 5′-end ^{32}P-labeling of oligonucleotides

400 pmol of the unlabelled oligonucleotide was added to a mixture containing 4 μl of a 10 mCi/ml solution of γ-^{32}P-ATP, 45 units of T4 polynucleotide kinase and T4 polynucleotide kinase buffer (10x). The mixture was incubated at 37 °C for two hours after which the kinase was inactivated at 65 °C for 20 min and the solution was desalted over a freshly prepared G-25 column. A 5′-end ^{32}P-labeled solution of the oligonucleotide in H_2O was obtained.

4.2.2 Denaturing PAGE gels:

Polyacrylamide gels were made using a 40% 29:1 acrylamide/bisacrylamide solution with a 7M urea, 100 mM Tris-borate (pH 8.3) and 2.5 mM EDTA solution. The gel was polymerized with 10% (w/v) ammonium persulfate and 0.1% TEMED.

Oligonucleotides were purified using either a small gel (170 x 165 x 1 mm) with a final polyacrylamide concentration of 10%, or a large gel (420 x 330 x 0.4 mm) with a final polyacrylamide concentration of 7%. Samples were mixed with loading buffer and were denatured at 95° for 5 minutes and snap cooled to 0° before loading. Gels were visualized by UV shadowing or by autoradiography detection depending upon the application. Radioactivity was detected with a Typhoon 9200 phosphorimaging scanner from GE 197 healthcare and analyzed using ImageQuant software. Data analysis was carried out using Graphpad Prism Ver. 6.0c.

4.2.3 Diethylpyrocarbonate (DEPC)-treated water:

70 μL of DEPC was added to 100 mL distilled water, left at 37° for at least two hours and autoclaved before use.

4.2.4 Gel Elution:

Following visualization, the desired bands of DNA were cut out, placed in a 1.5 mL Eppendorf tube and frozen for five minutes. The gel bands were mashed with a flame-sealed 1 mL pipette tip and DNA elution buffer was added. The mixture was vortexed and the resulting suspension was heated at 65° for 15 minutes and centrifuged at 13 000 rpm for 5 minutes. The supernatant was collected, another aliquot of DNA elution buffer was added, and the elution process repeated. The supernatant fractions were dried in a vacuum centrifuge until approximately 50 µL of liquid remained. The fractions were pooled and 1.2 mL absolute ethanol was added in order to precipitate the oligonucleotides. The solution was centrifuged at 13 000 rpm for 15 minutes, the supernatant removed, and another 0.4 mL absolute ethanol added. Following centrifugation at 13 000 rpm for 10 minutes, the supernatant was removed again and the pellet dried at 65° until all ethanol was evaporated. The pellet was dissolved in 40 µL of DEPC-treated water and spun through a freshly made G25 spin column for 3 minutes. The supernatant was collected into a fresh 1.5 mL eppendorf tube and used immediately or placed in the freezer.

4.2.5 G25 Spin Columns:

Columns were prepared using 1 mL pipette tips plugged with sterile silanized glass wool. G25 Sephadex beads in a DEPC water and 1% sodium azide solution were added to the prepared pipette tips. The tips were centrifuged at 2000 rpm to remove excess liquid and were washed three times with 0.4 mL DEPC water, followed by another DEPC water wash using the volume required in the given procedure. All columns were prepared directly before use.

4.2.6 PCR Program:

OPT1ST: (1) 3 minutes at 95°; (2) 15 sec. at 95°; (3) 15 sec. at 58°; (4) 40 sec. at 75°; (5) repeat steps (2) – (4) variable number of times; (6) 5 minutes at 72°; (7) hold at 4°.

4.3 General Buffers and Solutions

Tris Buffer: 50 mM Tris pH 7, 100 mM NaCl, 1 mM EDTA

Gel Loading Solution: Formamide (27 mL), EDTA(aq) (3 mL, 0.5 M), xylene cyanol(aq) (300 μL, 0.05 %) and bromophenol blue(aq) (300 μL, 0.05 %)

DNA Elution Buffer: 1% $LiClO_4$, 10 mM Tris pH 8

4.4 Materials and Methods for Chapter 2

4.4.1 Buffers/Solutions

SELEX Wash Buffer: 50 mL DPBS, 5 mM $MgCl_2$, 4.5 mM glucose

SELEX Binding Buffer: 50 mL DPBS, 5 mM $MgCl_2$, 4.5 mM glucose, 5 mg bovine serum albumin, 0.5 mg yeast tRNA

Amplification Cocktail: PCR amplification cocktail: 15 μL 1^{st}_amp_1 primer (1mM), 15 μL 1stamp_2 primer (1 mM), 4 x 6.9 μL dNTPs (100 mM), 23 μL $MgSO_4$ (100 mM), 230 μL 10X thermopol reaction buffer, 130 μL DEPC water.

Phenol-dUTP and dNTP cocktail: 2 μL dATP (1 mM), 2 μL phenol-dUTP (1mM), 1 μL dCTP (1 mM), 1 μL dGTP (1 mM)

Modified Phenol-dUTP and dNTP cocktail: 2.5 μL dATP (1 mM), 2.5 μL phenol-dUTP (1mM), 1.5 μL dCTP (1 mM), 1.5, μL dGTP (1 mM)

4.4.2 Methods

4.4.2.1 Elongations for SELEX process

1.5 μL of 10 μM selection primer and 4 μL of 10x thermopol enzyme buffer were combined with a volume of DNA template equal to 15 pmol of material. DEPC water was added to make a total volume of 24.5 μL. The mixture was quickly heated to 100°C in a water bath and left to cool slowly at room temperature until the water bath reached 30°C. The elongations were removed from the bath and 6 uL of phenol-dUTP and dNTP cocktail was added, along with 1.5 μL (3 U) vent (exo-) polymerase and 1.5 μL (10 mCi/mL) α-^{32}P-dGTP. The mixture was covered with mineral oil and left to react for 2 hours at 72°C before being cooled down to 4°C. The elongation reactions were either kept at 4°C or stored in the freezer until use.

4.4.2.2 Elongations for clone testing phase

The elongation reactions carried out during the sequence-testing phase of the project were identical to 2.1.2 except that 2 μL of 10 μM DNA template and 2 μL of 10 μM selection primer were used.

4.4.2.3 Elongations for saturation binding assay

3.5 μL of 10 μM selection primer, 3.5 μL of 10 μM sequence template and 5 μL of 10x thermopol buffer were combined with 28 μL DEPC water. The mixture was quickly heated to 100°C in a water bath and left to cool slowly at room temperature until the water bath reached 30°C. The elongations were removed and 8 μL of modified phenol-dUTP and dNTP cocktail were added, along with 2 μL (4 U) vent (exo-) polymerase and 1 μL (10 mCi/mL) α-^{32}P-dGTP. The mixture was covered with mineral oil and left to react for 2 hours at 72°C before being cooled down to 4°C.

4.4.2.4 Template stripping and preparation of elongations:

30 μL of a streptavidin bead solution was magnetized to the magnetic bead support and the supernatant was removed. The particles were washed and magnetized three times with 100 μL of Tris buffer. The elongation reaction was added and left to incubate at room temperature for a minimum of 30 minutes. Following incubation, the solution was magnetized and the supernatant was removed. The particles were washed, magnetized and the supernatant removed with the following solutions in the order they are listed: three times with 100 μL Tris buffer, five times with 100 μL 0.1 M NaOH and 1 mM EDTA, once with 200 μL Tris buffer, once with 100 μL DEPC water. In order to cleave the embedded ribonucleotide linkage and liberate the desired modified DNA strand, the particles were incubated with 15 μL 2 M NaOH and 20 μL DEPC water at 65° for 25 minutes. The solution was neutralized with 30 μL 1 M HCl and spun through a fresh G25 spin column. The resulting solution was transferred to a fresh 1.5 mL Eppendorf tube and dried in a vacuum centrifuge.

The dried elongations were dissolved in 40 μL of SELEX wash buffer or SELEX binding buffer, denatured at 95° for 7 minutes and snap cooled for 5 minutes at 0°.

4.4.2.5 Preparation of Cells

a) *E. coli* DH5α glycerol stock (stored at -80°) was used to start a stock culture. A few microlitres of glycerol stock was grown in 10 mL of LB in a 50 mL Eppendorf tube overnight at 37° with mild shaking, and was then stored at room temperature with mild shaking. Stock cultures were kept for one month and then were replaced with a fresh culture from glycerol stock. On the day or day prior to a selection experiment, 225 uL of stock culture was pipetted into a 50 mL Eppendorf tube containing 10 mL LB broth. The culture was grown at 37° with mild shaking until an OD_{600}=0.15-0.2 was reached, at which point IPTG was added to a final

concentration of 1 mM. The culture was again left to grow at 37° with mild shaking until an OD_{600}=0.5-0.7 was reached (typically about 2 hours). Optical density was measured using a spectrophotometer. Once the bacteria reached the correct density, the cells were prepared for experiments immediately or stored at 4° for a maximum of one day. To prepare the cells, 1.2 mL of cell culture was spun down at 10 000 rpm for 5 minutes and the supernatant removed. The pellets were resuspended in 0.5 mL cold SELEX wash buffer, centrifuged at 10 000 rpm for 5 minutes and the supernatant removed a total of three times. The pellet was immediately resupended with the 40 uL SELEX library solution. Following resuspension, the cell-library mixture was transferred back to the Eppendorf tube that originally contained the SELEX library for incubation.

b) *E. coli* strain O14:K7 cells were revived from glycerol stock and plated on agar medium. A few colonies were collected with a pipette tip and ejected into 10 mL LB to start a stock culture. The stock culture was incubated with shaking overnight at 37° and then stored at room temperature with mild shaking. 0.3 mL of stock culture in 10 mL LB broth was grown at 37° until an OD_{600}=0.5-0.7 was reached. Cells were used the day they were cultured. The cells were not induced with IPTG. Otherwise, cells were prepared for binding experiments as described for E. coli DH5α cells.

c) *E. coli* strain K12 cells were revived and grown as described for E. coli strain O14:K7. Cells were prepared for binding experiments as described for E. coli DH5α cells.

d) *Bacillus subtilis* cells were revived and grown as described for E. coli strain O14:K7. Cells were prepared for binding experiments as described for E. coli DH5α cells.

e) *Pseudomonas fluorescens* cells were revived and grown as described for E. coli strain O14:K7, except cultures were incubated at 23° instead of 37°. Cells were prepared for binding experiments as described for *E. coli* DH5α cells.

f) *Agrobacterium tumefaciens* cells were revived from glycerol stock and plated on agar medium. A few colonies were collected with a pipette tip and ejected into 10 mL LB broth. The culture was grown overnight at 30° until an OD_{600}=0.5-0.7 was reached. Cells were used the day they were cultured. Cells were prepared for binding experiments as described for E. coli DH5α cells.

g) *Saccharomyces cerevisiae* cells were plated on standard agar plates with added yeast extract, peptone and dextrose (YPD medium). A few colonies were collected with a pipette tip and ejected into 10 mL YPD broth with 0.2% adenine to start a stock culture. The stock culture was incubated with shaking overnight at 30° and then stored at room temperature with mild shaking. 0.25 mL of stock culture in 10 mL YPD broth was grown at 30° until an OD_{600}=0.5-0.7 was reached. The cells were used the day they were cultured.

Determining CFUs: 100 μL of *E. coli* cells of the correct density (see **a** above) were added to a Falcon tube containing 9.9 mL distilled water. This preparation was vortexed and serially diluted twice, to obtain a 1: 10 000 dilution and a 1: 1 000 000 dilution. 50 μL of the 1: 10 000 dilution and 1 mL of the 1: 1 000 000 dilution were plated on agar plates and incubated at 37°C overnight. Colonies were counted and used to determine the concentration of the *E. coli* growth solution.

4.4.2.6 Selection

A mixture consisting of bacteria (approximately 10^7 cells) and library in 40 μL SELEX wash buffer (rounds 1-2) or SELEX binding buffer (rounds 3 onwards) was incubated at room temperature for 1 hour, 45 minutes, 35 minutes or 25 minutes (total volume of mixture was 50 μL). The mixture was dispersed periodically by gently pipetting up and down. Following incubation, the solution was centrifuged at 10 000 rpm for 5 minutes to remove all unbound sequences. The cell pellet was gently resuspended in 100 μL SELEX wash buffer and immediately centrifuged at 10 000 rpm for 5 minutes and the supernatant removed. This procedure was repeated once (rounds 1-5, 7-8, 10-12) or twice (rounds 6 and 9). The cell pellets were resuspended in 75 μL SELEX wash buffer, denatured at 95° for 20 minutes and cooled at 0° for 5 minutes before being spun down at 13 000 rpm for 6 minutes. Both the supernatant (bound fraction) and cell pellet were retained. All of the supernatant fractions were dried in a vacuum centrifuge. The radioactivity of the dried fractions and the cell pellet was measured using a Geiger counter. The bound and pellet fractions were prepared for gel purification by dissolving in 25 μL loading buffer. The unbound fraction was dissolved in 50 μL loading buffer and was used as a visualization aid to observe the enrichment of the bound and pellet fractions. All of the bound and pellet fractions were loaded on the gel for purification; however, only 2 μL of the unbound fraction was loaded.

4.4.2.7 Amplification Procedure

10 μL of gel-purified oligonucleotide from the selection (consisting of both the bound and pellet fractions) was combined with 8 μL selection amplification cocktail, 21 μL DEPC water and 1.5 μL (3 U) vent (exo-) polymerase in a PCR tube. The oligonucleotides were amplified following

the OPT1ST PCR program (30 cycles). The efficiency of the first amplification was monitored by running a 1 μL sample on a small agarose gel. In order to ensure robust amplification from modified DNA starting material, a second amplification was carried out with the product of the first. 20 μL of the 1st amplification sample was added to 40 μL of selection amplification cocktail, 130 μL DEPC water and 9 μL (18 U) vent (exo-) polymerase. The mixture was separated into 4 PCR tubes (50 μL/tube) and amplified a second time using the OPT1ST PCR program with a variable number of cycles depending upon the quality of the first amplification. Following the second amplification, the 4 samples were pooled into a 1.5 mL Eppendorf tube and 200 μL phenol chloroform/isoamyl alcohol was added. The mixture was agitated with a vortexer, centrifuged for one minute at 13 000 rpm, and the top layer was removed to a fresh 1.5 Eppendorf tube. 1.2 mL of cold absolute ethanol was added, and the sample was agitated with a vortexer. The solution was centrifuged at 13 000 rpm for 15 minutes, the supernatant removed, and another 0.4 mL absolute ethanol added. Following centrifugation at 13 000 rpm for 10 minutes, the supernatant was removed again and the pellet dried at 65° until all ethanol was evaporated. The sample was dissolved in 89 μL DEPC water to which 10 μL λ-exonuclease buffer and 1 μL (5 U) λ-exonuclease was added. The reaction was left to digest at 37° overnight. Following digestion, the sample was treated with 100 μL phenol/chloroform, and precipitated and washed with cold absolute ethanol as described above. The pellet was dissolved in 25 μL loading solution and purified on a small gel. Using UV light to detect the DNA, the desired band was cut out and gel eluted. The concentration of the eluted 40 μL of material was measured using a blank spectrophotometer. 15 pmol of the product was used as the template DNA for the next round of selection.

4.4.2.8 Cloning and Sequencing

Purified 2nd amplification material from the 8th round library was used as a template for PCR amplification with *taq* polymerase in order to add 3' dATP overhangs to the PCR products in a 4x 50 μL sample preparation using the OPT1ST PCR protocol with 25 cycles. Following amplification, the samples were pooled, phenol chloroform extracted and precipitated and washed with cold absolute ethanol as described above. The product was dissolved in 25 μL loading solution and 5 μL 6x agarose loading solution, and the entire sample volume was purified on a 2% agarose gel. The oligonucleotide band was purified according to the protocol provided in the GeneJET Agarose Gel Extraction Kit from Life Technologies.

The purified sample was cloned with the TOPO TA cloning system according to the protocol provided in the TOPO TA Kit for Sequencing from Life Technologies. The plasmids were transformed into DH5α competent cells and plated on ampicillin-selected agar plates and subjected to an IPTG/X-Gal screening. White colonies were selected and the plasmids were purified according to the protocol provided in the Miniprep Plasmid Purification Kit from Invitrogen. Purified plasmids were restriction digested with EcoRI and samples were run on a 2% agarose gel to ensure that the correct fragment length was obtained. Plasmid samples were sent to the Nucleic Acid Protein Service Unit at the University of British Columbia for sequencing.

4.4.2.9 Selectivity tests with different cell lines

Protocol for the selection procedure was followed with the given cells used in place of *E. coli* DH5α competent cells. Data supplied in Appendix A.

4.4.2.10 Saturation binding assay

Sequence 8.28.A was elongated according to **4.4.2.3** to yield 245 pmol of material (7x35 pmol elongations). Elongations were prepared according to **4.4.2.4** and gel purified on a small PAGE gel. The 8.28.A elongation was then labeled with γ-^{32}P-ATP according to **4.2.1**. Concentration was ascertained using UV spectroscopy; overall yield was generally 20%, or 49 pmol of fully purified and labelled 8.28.A sequence. Labelled sequence was aliquoted into 7 Eppendorf tubes such that a concentration of 10 nM, 30 nM, 60 nM, 100 nM, 300 nM, 600 nM and 1000 nM would be obtained in a given tube with a final volume of 20 μL. The tubes were dried down and the initial radioactivity was recorded with a Geiger counter. The sequence was dissolved in 19 μL of SELEX wash buffer, denatured at 95°C for 7 minutes and snap cooled on ice for 5 minutes.

7x120 μL of *E. coli* DH5α competent cells (representing a receptor concentration of approximately 5 nM) were spun down and prepared according to **4.4.2.5a**, with 200 μL of SELEX wash buffer employed for the washes instead of 500 μL.

Cell pellets were resuspended in one concentration of 8.28.A sequence solution and the mixture was left to equilibrate for one hour. After one hour, the solution was centrifuged at 13 000 rpm for 5 minutes. The supernatant was removed and the pellets were quickly resuspended in 100 μL SELEX wash buffer and centrifuged at 13 000 rpm for another 5 minutes. The supernatant was removed and the radioactivity of the pellets was measured directly using a Geiger counter. The pellets were then resuspended in 1 mL of water, the Eppendorf tubes were placed in scintillation counter vials and the Cerenkov radiation of the samples was measured using a PerkinElmer TriCarb Liquid Scintillation Counter (Appendix B). Data were analyzed using Graphpad Prism Ver. 6.0c and fitted to the non-linear regression curve: $y=B_{max}(x)/(K_d+x) + NS(x)$, where NS(x)

is a linear proportionality constant to estimate non-specific binding and B_{max} is the total number of receptors.

4.4.2.11 Oligonucleotide sequences for aptamer selection

Oligonucleotide name	Oligonucleotide sequence
N40 template	5′ - GCG CTC GCG CGG CGT GCN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN CTG TTG GCG CAG GCC GAC GC - 3′ (77)
Selection Primer	5′ - /5Biotin/ GCG TGC CrCrG rUCT GTT GGT TTT GCG TCG GCC TGC GCC AAC AG- 3′ (41)
PCR primer forward (1^{st}_amp_2)	5′ - /5Phos/GCG TCG GCC TGC GCC AAC AG- 3′ (20)
PCR primer back (1^{st}_amp_1)	5′ - GCG CTC GCG CGG CGT GC-3′ (17)

4.5 Materials and Method for Chapter 3

4.5.1 Buffers/Solutions

TEN buffer: 50 mM Tris HCl pH 7, 1 mM EDTA, 100 mM NaCl, containing salmon sperm DNA (100 μM final)

Modified nucleotide cocktail: Natural or modified nucleotides were added to a final concentration of 10 μM for the natural nucleotides, 20 μM for $dU^{guan}TP$, $dC^{aa}TP$ and dGTP, and 50 μM for $dA^{im}TP$.

4.5.2 Methods

4.5.2.1 Ligation reactions

The template DNA (12.5 μM) was annealed to the selection primer (10 μM) in T4 DNA ligase buffer by heating to 100 °C and slowly cooling down to room temperature. T4 DNA ligase (2,000 units) was added and the mixture was incubated at 37 °C for one hour after which it was cooled to 15 °C and left to react overnight.

4.5.2.2 Elongation with Sequenase Version 2.0

15 pmol of the 5′-labelled construct was self-hybridized in the presence of Sequenase reaction buffer by heating up the mixture to 100 °C and slowly cooling down to room temperature. To this mixture, DTT was added to a final concentration of 5 mM. α-^{32}P-dGTP was added to a final concentration of 0.375 mCi/ml for the internal labeling of the elongation product unless stated otherwise. Yeast inorganic pyrophosphatase (YIPP, 0.1 unit) was added to drive the reaction equilibrium towards the incorporation and 15 units of Sequenase Version 2.0 were added to initialize the reaction. The resulting 40 μl reaction solution was topped with mineral oil and kept at 32 °C for four hours, after which it was cooled to 4 °C.

4.5.2.3 Self-cleavage experiments

Elongation reactions were incubated for thirty minutes with 40 μl streptavidin magnetic particles prewashed three times with 100 μl TEN buffer. The first wash was left to incubate for 5 minutes, followed by two 30-second washes. The unbound fraction was removed by washing the particles with TEN buffer (3 x 100 μL). The non-ligated templates were removed and the constructs were denatured by washing the beads with NaOH 0.1 M containing 1 mM EDTA (4 x 100 μL). The samples were renatured by incubating with TEN buffer in the presence of 20 μM displacement primer for five minutes. After a final wash with 60 μl H_2O, cleavage buffer (100 μL; 50 mM sodium cacodylate pH 7.4, 200 mM NaCl, 1 mM EDTA) was added and the reaction was incubated at room temperature for one hour. After 30 seconds, 1 minute, 3 minutes, 10 minutes, 30 minutes, and 1 hour, 5 μL of the slurry was removed and mixed with 5 μL gel loading buffer containing 1 mM biotin. Cleavage control samples were prepared by removing 4 μL of the slurry

from the reaction in the final wash, adding 1 μL of a 2 M NaOH solution and incubating the sample at 65 °C for twenty minutes before mixing with an equal volume of gel loading buffer.

4.5.2.4 Agarose gel analysis

5 pmol of the ligation product of the Dz10-66 template to the selection primer and of the elongation product of the ligated Dz10-66 construct with $dU^{guan}TP$, $dC^{aa}TP$, $dA^{Im}TP$ and dGTP (containing a fraction of α-^{32}P-dGTP) were heated at 95°C for 5 minutes and snap cooled on ice for 10 minutes. The products were analysed on a 1.5% agarose D gel containing 2 μg/mL ethidium bromide ran at 100 V for 25 minutes.

4.5.2.5 Oligonucleotide sequences

Oligonucleotide name	Oligonucleotide sequence
Dz10-66 template	5′ - TGT CTA CAC GCA AGC TTA CAG CGT GC A TAC ACG CAC GCA CAC TCA TAG CGC GCC TCA CTT GCG CTG CTA G TG TTG GTA GGG CCC AAC AGA CGG GCA CGC ACT ACG TAC CCA CAA CCT CGG CCG TAC CAC GGT ACG TAG TGC - 3′ (141)
Nx template	5′ - TGT CTA CAC GCA AGCTTA CAG CGT GCNx TGT TGG TAG GGC CCA ACA GAC GGG CAC GCA CTA CGT ACC CAC AAC CTC GGC CGT ACC ACG GTA CGT AGT GC - 3′ (117 or 137)
Selection Primer	5′ - GTGCCrCGTCTGTTGGGCCCTbioACCAACA - 3′ (27)
PCR primer forward	5′ - Bio - GC ACT ACG TAC CGT GGT ACG GCC GAG GTT G - 3′ (30)
PCR primer back	5′ - TGT CTA CAC GCA AGC TTA CA GCG TGC -3′ (26)
displacement primer	5'- /5Phos/GC ACT ACG TAC CGT GGT ACG GCC GAG GTT GTG GGT ACG TAG TGC GTG CCC GTC TGT TGG GCC CTA CCA ACA -3' (71)

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Appendices

Appendix A - Chapter 2

A.1 Selectivity data for aptamer sequences

Data were measured in CPM using a Geiger counter. Values are expressed as percent CPM of the total CPM of the sequence (measured prior to the start of the assay). Data is included for all bacterial and yeast strains tested as indicated. Not bound = radioactivity of supernatant immediately following incubation and first centrifugation step. W1 = radioactivity of supernatant following one wash step. W2 = radioactivity of supernatant following second wash step. Bound = radioactivity of supernatant following denaturation and centrifugation. Pellet = radioactivity remaining with cells after denaturation and centrifugation. Total Bound = total combined radioactivity of "Bound" and "Pellet" fractions.

***E. coli* DH5α**

8.10.A *E. coli* DH5α				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	37.8	73	64.7	58.5
W1	43.2	17.5	11.2	23.9
W2	8.1	1.3	9.8	6.4
Bound	6	5.1	8	6.37
Pellet	4.9	3.2	6.3	4.80
Total Bound				11.17

8.14.B *E. coli* DH5α				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	21.5	64.8	21.7	36.0
W1	43	26.9	39.2	36.4
W2	18.5	1.6	22.9	14.3
Bound	9.2	3.2	9	7.13
Pellet	7.8	3.5	7.2	6.17
Total Bound				13.30

8.18.B *E. coli* DH5α				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	6.8	14.4	9.2	10.13
W1	60.8	65.6	66.7	64.37
W2	13.5	9.4	5	9.30
Bound	12.2	7.4	10	9.87
Pellet	6.8	3.3	9.2	6.43
Total Bound				16.30

8.28.A *E. coli* DH5α					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	33.6	12.3	8	7.5	15.35
W1	33.6	61.5	77.1	59.7	58.0
W2	12	10.8	6.2	9	9.5
Bound	6.3	9.8	6.2	8.2	7.63
Pellet	14.5	5.5	2.6	15.7	9.58
Total Bound					17.2

E. coli K12

8.10.A *E. coli* K12						
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
Not Bound	2	79.6	2.1	91.2	91	53.2
W1	59.2	8.8	74.5	6.7	7.9	31.4
W2	13.8	2.7	12.8	0.7	0.4	6.08
Bound	7.1	2.7	7.4	0.7	0.4	3.66
Pellet	4	6.2	3.2	0.7	0.4	2.9
Total Bound						6.56

8.14.B *E. coli* K12						
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
Not Bound	2	7.4	4.4	81.8	90.2	37.2
W1	57.1	59.3	66.7	12.7	7.7	40.7
W2	20.4	17	11.1	3.6	0.5	10.5
Bound	12.2	8.9	2.2	1	1	5.06
Pellet	8.2	7.4	2.2	1	0.5	3.86
Total Bound						8.92

8.18.B *E. coli* K12						
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
Not Bound	2.6	3.8	10.3	75.1	82.4	34.84
W1	68.9	70.5	80.5	10.7	14.1	48.94
W2	17.2	15.4	5.7	1.1	1.2	8.12
Bound	8.6	7.7	1.1	1.1	1.2	3.94
Pellet	2.6	2.6	2.3	1	1.1	1.92
Total Bound						5.86

8.28.A *E. coli* K12						
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
Not Bound	2.5	8	4.8	87.2	84.3	37.36
W1	62.5	53.6	76.9	10.1	12.1	43.04
W2	17.5	17.9	7.8	0.9	1.2	9.06
Bound	10	13.4	5.8	0.9	1.2	6.26
Pellet	7.5	7.1	4.8	0.9	1.2	4.3
Total Bound						10.29

E. coli O14:K7

8.10.A *E. coli* O14:K7				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	82.9	81.5	90.9	85.1
W1	8.3	9.3	6.5	8.03
W2	1.1	1.1	0.6	0.93
Bound	1.1	4.1	0.6	1.93
Pellet	6.6	4.1	1.3	4
Total Bound				5.93

8.14.B *E. coli* O14:K7				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	85.6	82.3	85.5	84.4
W1	8.1	8.4	8.5	8.33
W2	0.9	0.8	0.9	0.87
Bound	0.9	0.8	0.4	0.7
Pellet	4.5	7.6	4.7	5.6
Total Bound				6.3

8.18.B *E. coli* O14:K7				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	88.9	84.3	88.8	87.3
W1	7.4	11.2	5.9	8.17
W2	0.7	0.5	0.6	0.6
Bound	0.7	0.5	1.2	0.8
Pellet	2.2	3.4	3.6	3.07
Total Bound				3.87

8.28.A *E. coli* O14:K7				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	88.4	87.2	87.3	87.6
W1	8.3	9.4	7.9	8.53
W2	0.6	0.5	0.8	0.63
Bound	0	0.5	0.8	0.43
Pellet	2.8	2.4	3.2	2.8
Total Bound				3.23

B. subtilis

8.10.A *B. subtilis*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	95.1	72.9	90.4	86.2	86.1
W1	3.81	18.2	6.82	8.13	9.24
W2	0.211	1.22	0.34	0.65	0.61
Bound	0.422	1.51	1.02	2.44	1.35
Pellet	0.422	6.07	1.37	2.6	2.61
Total Bound					3.96

8.14.B *B. subtilis*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	93.1	77.6	84.7	89.2	86.2
W1	4.9	14.3	9.68	6.48	8.84
W2	0.245	1.22	0.806	0.541	0.70
Bound	0.49	0.82	1.61	1.35	1.07
Pellet	1.23	6.12	3.22	2.43	3.25
Total Bound					4.32

8.18.B *B. subtilis*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	93.4	77.9	90.6	87.7	87.4
W1	5.05	1.52	6.3	5.76	4.65
W2	0.253	0.866	0.394	0.524	0.51
Bound	0.505	0.866	1.18	2.88	1.36
Pellet	0.758	5.19	1.57	3.14	2.66
Total Bound					4.02

8.28.A *B. subtilis*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	86	77.5	87.5	90.1	85.3
W1	9.56	14.1	7.81	5.91	9.34
W2	0.849	1.06	0.938	0.46	0.83
Bound	1.27	1.06	1.56	0.91	1.2
Pellet	2.34	6.33	2.19	1.81	3.17
Total Bound					4.37

P. fluorescens

8.10.A *P. fluorescens*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	91	90	90	95	91.5
W1	5.6	6.8	5.6	3.9	5.48
W2	1.4	0.4	0.8	0.3	0.73
Bound	1.2	1.1	1.1	0.3	0.93
Pellet	1.8	1.9	2.5	0.5	1.68
Total Bound					2.61

8.14.B *P. fluorescens*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	92	93	61	94	85
W1	5.9	4.6	15	3.6	7.28
W2	0.4	0.5	10	0.4	2.83
Bound	0.4	0.5	5.5	0.7	1.78
Pellet	1.4	1.5	8.5	1.4	3.2
Total Bound					4.98

8.18.B *P. fluorescens*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	91	94	93	95	93.25
W1	7.1	3.4	4.3	3.1	4.48
W2	0.3	0.3	0.9	0.4	0.48
Bound	0.6	0.7	0.6	0.4	0.58
Pellet	1	1.4	0.9	0.4	0.93
Total Bound					1.51

8.28.A *P. fluorescens*					
% CPM	Trial 1	Trial 2	Trial 3	Trial 4	Average
Not Bound	93.5	90	94	93	92.625
W1	4.7	8.4	4.3	4.2	5.4
W2	0.3	0.4	0.5	0.4	0.4
Bound	0.6	0.4	0.5	0.7	0.55
Pellet	0.9	0.7	1	1.4	1
Total Bound					1.55

A. tumefaciens

8.10.A *A. tumefaciens*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	93.8	80.4	95.4	89.8
W1	3.9	5.4	3.5	4.27
W2	0.55	3.6	0.4	1.52
Bound	0.55	2.7	0.4	1.22
Pellet	1.1	8	0.3	3.13
Total Bound				4.35

8.14.B *A. tumefaciens*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	90.6	93.7	91.2	91.8
W1	7.1	3.9	6	5.67
W2	0.3	0.4	0.4	0.37
Bound	1	1.2	1.6	1.27
Pellet	1	0.8	0.8	0.87
Total Bound				2.07

8.18.B *A. tumefaciens*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	93.9	93.4	94	93.7
W1	3.9	3.3	4.3	3.83
W2	0.6	1.1	0.4	0.70
Bound	1	1.1	0.9	1.0
Pellet	0.6	1.1	0.4	0.7
Total Bound				1.7

8.28.A *A. tumefaciens*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	93	93.5	94.7	93.7
W1	4.1	3.5	3.7	3.77
W2	0.4	0.6	0.3	0.43
Bound	1.7	1.2	1	1.3
Pellet	0.8	1.2	0.3	0.77
Total Bound				2.07

S. cerevisiae

8.10.A *S. cerevisiae*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	88.5	89.8	92	90.1
W1	6.6	5.9	4.6	5.7
W2	0.9	0.4	1.2	0.83
Bound	1.8	1.6	1.1	1.5
Pellet	2.2	2.3	1.1	1.87
Total Bound				3.37

8.14.B *S. cerevisiae*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	88	87.6	86.7	87.4
W1	8.4	5.2	8.2	7.27
W2	0.85	0	1.4	0.75
Bound	1.3	4	1.8	2.37
Pellet	1.3	2.6	1.8	1.9
Total Bound				4.27

8.18.B *S. cerevisiae*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	93.4	95.7	90.2	93.1
W1	4.7	2.1	5.3	4.03
W2	1	1	1.5	1.17
Bound	0	0	1.5	0.5
Pellet	0	0	1.5	0.5
Total Bound				1.0

8.28.A *S. cerevisiae*				
% CPM	Trial 1	Trial 2	Trial 3	Average
Not Bound	88.6	93	92	91.2
W1	6.3	3.7	4.8	4.93
W2	0.4	0.9	0.8	0.7
Bound	3.4	0.9	0.8	1.7
Pellet	1.3	1.9	1.2	1.47
Total Bound				3.17

A.2 ANOVA and Tukey's Multiple Comparisons Test

Appendix Table 1. ANOVA Summary

	8.10.A	8.14.B	8.18.B	8.28.A
P-value	0.1444	0.0366	<0.0001	<0.0001
P-value signficant? (<0.05)	No	Yes	Yes	Yes
F-value	1.879	2.965	11.52	13.73
R square	0.4672	0.5805	0.8432	0.8573

Appendix Tables 2a-d. Tukey's multiple comparisons results. The means of the bound fraction of each strain (N=3), mean difference, standard error of mean difference, 95% confidence interval of mean difference, significance, and degree of significance are listed for each sequence. A larger number of asterisks indicate a more significant difference. ns = not significant. Note that only the results comparing *E. coli* DH5α to the other tested species are listed, but comparisons between the means of all species to each other (24 comparisons total) were made. All analyses conducted with Graphpad Prism v 6.0c.

8.10.A							
Compared Strains	Mean 1	Mean 2	Mean Diff.	SE of Diff.	95% CI of Diff.	Significant?	Degree of Significance
E. coli DH5α vs. E. coli O14:K7	11.13	5.933	5.200	3.131	-5.736 to 16.14	No	ns
E. coli DH5α vs. E. coli K12	11.13	6.967	4.167	3.131	-6.770 to 15.10	No	ns
E. coli DH5α vs. P. fluorescens	11.13	2.267	8.867	3.131	-2.070 to 19.80	No	ns
E. coli DH5α vs. A. tumefaciens	11.13	4.333	6.800	3.131	-4.136 to 17.74	No	ns
E. coli DH5α vs. B. subtilis	11.13	4.833	6.300	3.131	-4.636 to 17.24	No	ns
E. coli DH5α vs. S. cerevisiae	11.13	3.033	8.100	3.131	-2.836 to 19.04	No	ns
E. coli DH5α vs. E. coli DH5α dNTPs	11.13	1.050	10.08	3.500	-2.144 to 22.31	No	ns

8.14.B							
Compared Strains	Mean 1	Mean 2	Mean Diff.	SE of Diff.	95% CI of Diff.	Significant?	Degree of Significance
E. coli DH5α vs. E. coli O14:K7	13.27	6.300	6.967	3.154	-4.050 to 17.98	No	ns
E. coli DH5α vs. E. coli K12	13.27	7.567	5.700	3.154	-5.317 to 16.72	No	ns
E. coli DH5α vs. P. fluorescens	13.27	1.967	11.30	3.154	0.2830 to 22.32	Yes	*
E. coli DH5α vs. A. tumefaciens	13.27	2.133	11.13	3.154	0.1163 to 22.15	Yes	*
E. coli DH5α vs. B. subtilis	13.27	5.133	8.133	3.154	-2.884 to 19.15	No	ns
E. coli DH5α vs. S. cerevisiae	13.27	4.267	9.000	3.154	-2.017 to 20.02	No	ns
E. coli DH5α vs. E. coli DH5α dNTPs	13.27	1.150	12.12	3.526	-0.2008 to 24.43	No	ns

8.18.B							
Compared Strains	Mean 1	Mean 2	Mean Diff.	SE of Diff.	95% CI of Diff.	Significant?	Degree of Significance
E. coli DH5α vs. E. coli O14:K7	16.27	3.867	12.40	2.114	5.017 to 19.78	Yes	***
E. coli DH5α vs. E. coli K12	16.27	5.333	10.93	2.114	3.551 to 18.32	Yes	**
E. coli DH5α vs. P. fluorescens	16.27	1.500	14.77	2.114	7.384 to 22.15	Yes	****
E. coli DH5α vs. A. tumefaciens	16.27	1.700	14.57	2.114	7.184 to 21.95	Yes	***
E. coli DH5α vs. B. subtilis	16.27	3.133	13.13	2.114	5.751 to 20.52	Yes	***
E. coli DH5α vs. S. cerevisiae	16.27	1.000	15.27	2.114	7.884 to 22.65	Yes	****
E. coli DH5α vs. E. coli DH5α dNTPs	16.27	0.6500	15.62	2.363	7.362 to 23.87	Yes	***

8.28.A							
Compared Strains	Mean 1	Mean 2	Mean Diff.	SE of Diff.	95% CI of Diff.	Significant?	Degree of Significance
E. coli DH5α vs. E. coli O14:K7	21.65	3.233	18.42	2.727	8.975 to 27.86	Yes	****
E. coli DH5α vs. E. coli K12	21.65	10.27	11.38	2.727	1.941 to 20.83	Yes	*
E. coli DH5α vs. P. fluorescens	21.65	1.700	19.95	2.727	10.51 to 29.39	Yes	****
E. coli DH5α vs. A. tumefaciens	21.65	2.067	19.58	2.727	10.14 to 29.03	Yes	****
E. coli DH5α vs. B. subtilis	21.65	4.900	16.75	2.727	7.308 to 26.19	Yes	***
E. coli DH5α vs. S. cerevisiae	21.65	3.167	18.48	2.727	9.041 to 27.93	Yes	****
E. coli DH5α vs. E. coli DH5α dNTPs	21.65	0.7500	20.90	3.092	10.19 to 31.61	Yes	****

Appendix B - Chapter 2

B.1 TriCarb Liquid Scintillation Counter data for saturation binding assay

The 10 nM, 30 nM, 60 nM, 100 nM, 300 nM, 600 nM and 1000 nM samples were counted in descending order for all assays.

Count Conditions

Nuclide: 32P
Quench Indicator: SIS
External Std Terminator (sec): n/a
Pre-Count Delay (min): 0.50
Quench Set: n/a
Count Time (min): 10.00
Count Mode: Normal
Assay Count Cycles: 1 Repeat Sample Count: 1
#Vials/Sample: 1 Calculate % Reference: Off

Background Subtract

Background Subtract: Off
Low CPM Threshold: Off
2 Sigma % Terminator: On - Any Region

Regions	LL	UL	2Sigma % Terminator
A	5.0	1700.0	0.05
B	50.0	1700.0	0.05
C	0.0	0.0	0.05

Count Corrections

Static Controller: On Luminescence Correction: Off
Colored Samples: n/a Heterogeneity Monitor: n/a
Coincidence Time (nsec): 18 Delay Before Burst (nsec): 75

Cycle 1 Results

Count Time	CPMA	SIS	MESSAGES	A:2S%
10 10.00	2.55803e+004	22.61		0.40
30 10.00	1.31788e+005	23.70		0.17
60 10.00	1.34603e+005	23.03		0.17
100 10.00	1.87066e+005	23.26		0.15
300 10.00	2.92621e+005	23.12		0.12
600 10.00	3.39546e+005	22.65		0.11
1000 10.00	4.30857e+005	23.10		0.10

Figure A1. Data collected for May 11, 2015 binding assay.

Count Conditions

Nuclide: 32P
Quench Indicator: SIS
External Std Terminator (sec): n/a
Pre-Count Delay (min): 0.50
Quench Set: n/a
Count Time (min): 10.00
Count Mode: Normal
Assay Count Cycles: 1 — Repeat Sample Count: 1
#Vials/Sample: 1 — Calculate % Reference: Off

Background Subtract

Background Subtract: Off
Low CPM Threshold: Off
2 Sigma % Terminator: On - Any Region

Regions	LL	UL	2Sigma % Terminator
A	5.0	1700.0	0.05
B	50.0	1700.0	0.05
C	0.0	0.0	0.05

Count Corrections

Static Controller: On — Luminescence Correction: Off
Colored Samples: n/a — Heterogeneity Monitor: n/a
Coincidence Time (nsec): 18 — Delay Before Burst (nsec): 75

Cycle 1 Results

Count Time	CPMA	SIS	MESSAGES	A:2S%
(10) 10 10.00	3.76347e+004	21.87		0.33
10 10.00	4.40501e+004	22.66		0.30
30 10.00	1.37910e+005	23.28		0.17
60 10.00	1.51770e+005	23.14		0.16
100 10.00	1.40906e+005	23.03		0.17
300 10.00	3.01104e+005	23.36		0.12
600 10.00	3.55162e+005	23.35		0.11
1000 10.00	4.79322e+005	23.72		0.09

Figure A2. Data collected for May 15, 2015 binding assay. Two 10 nM samples were taken (first two entries)

Count Conditions

Nuclide: 32P
Quench Indicator: SIS
External Std Terminator (sec): n/a
Pre-Count Delay (min): 0.50
Quench Set: n/a
Count Time (min): 10.00
Count Mode: Normal
Assay Count Cycles: 1 — Repeat Sample Count: 1
#Vials/Sample: 1 — Calculate % Reference: Off

Background Subtract

Background Subtract: Off
Low CPM Threshold: Off
2 Sigma % Terminator: On - Any Region

Regions	LL	UL	2Sigma % Terminator
A	5.0	1700.0	0.05
B	50.0	1700.0	0.05
C	0.0	0.0	0.05

Count Corrections

Static Controller: On — Luminescence Correction: Off
Colored Samples: n/a — Heterogeneity Monitor: n/a
Coincidence Time (nsec): 18 — Delay Before Burst (nsec): 75

Cycle 1 Results

Count Time	CPMA	SIS	MESSAGES	A:2S%
10nM 10.00	1.75400e+004	22.35		0.48
30nM 10.00	6.88546e+004	22.99		0.24
60nM 10.00	6.42351e+004	23.18		0.25
100nM 10.00	9.64554e+004	19.06		0.20
300nM 10.00	2.37945e+005	23.26		0.13
600nM 10.00	2.22756e+005	18.78		0.13
1000nM 10.00	4.26898e+005	23.73		0.10

Figure A3. Data collected for May 22, 2015 binding assay.

Count Conditions

Nuclide: 32P
Quench Indicator: SIS
External Std Terminator (sec): n/a
Pre-Count Delay (min): 0.50
Quench Set: n/a
Count Time (min): 10.00
Count Mode: Normal
Assay Count Cycles: 1 — Repeat Sample Count: 1
#Vials/Sample: 1 — Calculate % Reference: Off

Background Subtract

Background Subtract: Off
Low CPM Threshold: Off
2 Sigma % Terminator: On - Any Region

Regions	LL	UL	2Sigma % Terminator
A	5.0	1700.0	0.05
B	50.0	1700.0	0.05
C	0.0	0.0	0.05

Count Corrections

Static Controller: On — Luminescence Correction: Off
Colored Samples: n/a — Heterogeneity Monitor: n/a
Coincidence Time (nsec): 18 — Delay Before Burst (nsec): 75

Cycle 1 Results

Count Time		CPMA	SIS	MESSAGES	A:2S%
10 nM	10.00	1.18761e+004	18.88		0.58
30 nM	10.00	4.03203e+004	18.82		0.31
60 nM	10.00	6.77136e+004	19.06		0.24
100 nM	10.00	3.28488e+004	19.36		0.35
300 nM	10.00	8.93206e+004	23.20		0.21
600 nM	10.00	1.30015e+005	23.52		0.18
1000 nM	10.00	1.90942e+005	23.41		0.14

Figure A4. Data collected for May 26, 2015 binding assay.

Count Conditions

Nuclide: 32P
Quench Indicator: SIS
External Std Terminator (sec): n/a
Pre-Count Delay (min): 0.50
Quench Set: n/a
Count Time (min): 10.00
Count Mode: Normal
Assay Count Cycles: 1 — Repeat Sample Count: 1
#Vials/Sample: 1 — Calculate % Reference: Off

Background Subtract

Background Subtract: Off
Low CPM Threshold: Off
2 Sigma % Terminator: On - Any Region

Regions	LL	UL	2Sigma % Terminator
A	5.0	1700.0	0.05
B	50.0	1700.0	0.05
C	0.0	0.0	0.05

Count Corrections

Static Controller: On — Luminescence Correction: Off
Colored Samples: n/a — Heterogeneity Monitor: n/a
Coincidence Time (nsec): 18 — Delay Before Burst (nsec): 75

Cycle 1 Results

Count Time		CPMA	SIS	MESSAGES	A:2S%
10	10.00	1.97542e+003	22.10		1.42
30	10.00	6.07492e+003	23.51		0.81
60	10.00	4.37512e+003	22.35		0.96
100	10.00	6.60381e+003	22.75		0.78
300	10.00	1.79640e+004	23.03		0.47
1000	10.00	3.01873e+004	22.02		0.36

Figure A5. Data collected for June 2, 2015 non-specific binding assay.