

“Some folk’ll never eat a skunk
But then again, some folk’ll...”
-Quote from The Simpsons

Some of the most valuable lessons are learned by those who are both willing
to get their hands dirty, and have a sense of humour about it.

University of Alberta

Generation of DNA aptamers against whole *Campylobacter jejuni* cells

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Medical Sciences —

Laboratory Medicine and Pathology

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Spring 2012

Edmonton, Alberta

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ISBN: 978-0-494-87866-8

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Abstract

DNA aptamers were generated against two whole bacterial targets, using two sets of Cell-Systematic Evolution of Ligands by Exponential Enrichment (Cell-SELEX) modified to contain a target switch step. Eight and twelve rounds of selections were carried out with *C. jejuni* and a strain of *E. coli* DH5 α pgl engineered to express a *C. jejuni* surface carbohydrate, respectively. Binding of aptamer pools was estimated using flow cytometry. Cloning of aptamer pools and sequence analysis allowed the isolation and synthesis of individual aptamers. Flow cytometry was done to assess aptamer binding. Two aptamer sequences, 10C+E-31 and 12C+E-30, appeared twice in the *E. coli* set. Two other sequences, 12C+E-25 and 12C+E-26, had K_d values in the low nanomolar range. The specificity of 12C+E-26 was further assessed, and was observed to bind non-target bacteria as well. Further synthesis and testing are required to obtain aptamers that could be useful for clinical and/or environmental analyses.

Acknowledgements

I would like to thank the following people for their contribution to my completion of this degree:

Dr. Camille Hamula (for her continuous guidance and assistance throughout my graduate work)

Dr. Monika Keelan

Dr. Jeff Fuller

Dr. Elaine Leslie

Jessica Boyd, Emily Chan, Lydia Chen, Anthony McKnight-Whitford, K. MacConkalonkey, Birget Moe, and everyone else in the division of Analytical & Environmental Toxicology, for their friendship, support, and amazing sense of humour.

Katerina Carastathis & Cheryl Titus for being able to keep track of EVERYTHING

Dr. X. Chris Le

Table of Contents

Chapter 1. Introduction	1
1.1 C. jejuni Infection.....	1
1.2 Target Identification using Aptamers and SELEX.....	3
1.2.1 Aptamers	3
1.2.2 SELEX	5
1.3 Literature Review of Previous Work	6
1.3.1 Common Aptamer Targets.....	6
1.3.2 Small Molecule Aptamer Targets	7
1.3.3 Modifications of Traditional SELEX.....	9
1.3.4 E. coli DH5 α pgl Cells and the C. jejuni Cell Surface	11
1.4 Project Rationale	12
1.5 Research objectives	13
Chapter 2. Methodology	14
2.1 Bacterial Strains and Growth	14
2.2 Polymerase Chain Reaction (PCR)	17
2.3 SELEX	19
2.4 Detection of Carbohydrate via Western Blot.....	22
2.5 Flow Cytometry of Aptamer Pools	24
2.6 Cloning.....	28
2.7 Sequence Analysis & Synthesis of Labelled Aptamers	31
2.8 Flow Cytometry of Individual Aptamer Sequences.....	33
2.9 Kd Analysis of Selected Individual Aptamer Sequences via Flow Cytometry.....	34
Chapter 3. Results and Discussion.....	40

3.1	Polymerase Chain Reaction	40
3.2	SELEX	40
3.3	Detection of Carbohydrate via Western Blot	42
3.4	Flow Cytometry of Aptamer Pools	42
3.5	Cloning & Sequence Analysis.....	45
3.6	Flow Cytometry of Individual Aptamer Sequences & Kd Analysis.....	47
Chapter 4. Summary, Conclusions, and Future Work		67
4.1	Summary and Conclusions.....	67
4.2	Future Work	70
4.3	Potential Uses for Aptamers.....	73
References.....		77
Appendix.....		87

List of Tables

Table 3.5.1. Summary table of cloning and sequencing results.....	62
Table A.1. Summary of predicted secondary structures and Gibbs Free Energy (kcal/mol) values (ΔG) of aptamers sent for sequencing for round pool 8C of <i>C. jejuni</i> selections.....	87
Table A.2. Summary of predicted secondary structures and Gibbs Free Energy (kcal/mol) values (ΔG) of aptamers sent for sequencing for round pool 10C+E of DH5 α ggl selections.....	91
Table A.3. Summary of predicted secondary structures and Gibbs Free Energy (kcal/mol) values (ΔG) of aptamers sent for sequencing for round pool 12C+E of DH5 α ggl selections.....	93
Table A.4. Sequences of aptamers selected for synthesis.....	95

List of Figures

Figure 2.3.1 Illustration of Cell-SELEX method used, including counter-selection and target switch steps.....	36
Figure 2.5.1 Illustration of side and forward scatter created by cell size, shape, and complexity.....	37
Figure 2.5.2 (A & B). Sample FACScan histogram plot of frequency of fluorescent events versus intensity of fluorescence.....	38
Figure 2.9.1 Example of a typical aptamer-bacteria binding curve, further modified to show estimation of Bmax, ½ Bmax, and Kd.....	39
Figure 3.1.1 (A & B). Gel electrophoresis photograph of 10 ng and 100 ng DNA aptamer library template,	53
Figure 3.2.1 (A-C). Gel electrophoresis photographs showing amplification of round 1 of DH5 <i>apgl</i> SELEX bound and unbound aptamer and control eluates....	54
Figure 3.3.1 (A-E). Western blot images of carbohydrate expression.....	55
Figure 3.4.1 (A & B). Flow cytometry analysis of aptamer pools of rounds 1-12 binding to DH5 <i>apgl</i> cells.....	57
Figure 3.4.2 (A & B). Flow cytometry analysis of aptamer pools of rounds 1, 3, 4, and 8 binding to <i>C. jejuni</i> cells.....	58

Figure 3.4.3 (A & B). Flow cytometry analysis of aptamer pools of rounds 1-12 binding to <i>DH5αpgl</i> cells, with library subtraction and separated by incubations.....	59
Figure 3.5.1 Gel electrophoresis photograph of assorted sizes of inserts obtained after cloning and plasmid digestion.....	60
Figure 3.5.2 Gel electrophoresis photograph of inserts obtained for several 8C colonies.....	61
Figure 3.5.3. Electropherogram of a sample DNA aptamer sequence.....	63
Figure 3.6.1 (A-C). Flow cytometry binding analysis of synthetic aptamers from <i>DH5αpgl</i> SELEX rounds 10 and 12 C+E and <i>C. jejuni</i> SELEX round 8C.....	64
Figure 3.6.2. Flow cytometry binding analysis of synthetic 12C+E-26, incubated with various bacteria to assess aptamer specificity.....	65
Figure 3.6.3 (A-C). Aptamer binding to <i>DH5αpgl</i> cells with increasing aptamer concentration of random DNA oligonucleotide library, and synthetic aptamers 12C+E-26 and 12C+E-25.....	66

List of Abbreviations

AMD = Age-related Macular Degeneration

ATP = Adenosine triphosphate

BB = Binding Buffer

BHI = Brain Heart Infusion

Bmax = Maximum binding affinity

bp = base pair(s)

BSA = Bovine Serum Albumin

C set = Set of aptamers from the *C. jejuni* SELEX set that should bind to *C. jejuni*.

C+E set = Set of aptamers from the *E. coli* DH5 α gl SELEX set that should bind to both *C. jejuni* and *E. coli* DH5 α gl.

CE = Capillary Electrophoresis

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic Acid

E+C set = Set of aptamers from the *C. jejuni* SELEX set that should bind to both *C. jejuni* and *E. coli* DH5 α gl.

E set = Set of aptamers from the *E. coli* DH5 α gl SELEX set that should bind only to *E. coli* DH5 α gl.

ELISA = Enzyme-Linked Immunosorbent Assay

FACS = Fluorescence-Activated Cell Sorting

FDA = Food and Drug Agency

FSC = Forward Scatter

ΔG = Change in Gibbs Free Energy

HRP = Horse radish peroxidase

kDa = kilodaltons

K_d = Dissociation constant

LB = Luria Bertani

μ M = micromolar

mM = millimolar

MUC1 = Mucin 1, cell surface associated

nM = nanomolar

nt = nucleotide(s)

OD₆₀₀ = Optical Density at 600 nm

PBS = Phosphate Buffered Saline

PBST = Phosphate Buffered Saline Tween-20

PCR = Polymerase Chain Reaction

pmole = picomole

PVDF = Polyvinylidene Fluoride

RNA = Ribonucleic Acid

SELEX = Systematic Evolution of Ligands by Exponential Enrichment

SPR = Surface Plasmon Resonance

SSC = Side Scatter

UV = Ultra Violet

VEGF = Vascular Endothelial Growth Factor

Chapter 1. Introduction

1.1 *C. jejuni* Infection

Campylobacter jejuni is a common cause of bacterial gastroenteritis, and can be transmitted through fecal contaminated food and water (4, 15, 21). Infections are rarely fatal, and symptoms include bloody or non-bloody diarrhea, nausea, vomiting, and fever and are often left untreated as they typically resolve within one week without intervention (4, 21). More serious side effects can include Guillain-Barre' Syndrome and arthritis (4, 21, 55). Guillain-Barré Syndrome is a disorder that may be triggered after a *Campylobacter* infection. It results in inflammation of nerves and ultimately paralysis. The syndrome is rare however, and is usually reversible (15, 21).

According to the Public Health Agency of Canada, the highest incidences of *Campylobacter* cases reported between 2000 and 2004 were in the provinces of British Columbia, Alberta, and Ontario, with the highest incidence in British Columbia (1). The increased incidences however, may be lower in some provinces due to lower reporting of cases. British Columbia (BC), Alberta (AB), and Ontario (ON) likely have higher testing capacities (ability to perform more tests) than the other provinces, which may influence diagnosis and reporting of cases. *Campylobacter* is the most common cause of bacterial gastroenteritis in British Columbia (55). According to the British Columbia Summary of Reportable Diseases in 2009 (55), 1750 cases of *Campylobacter*-associated gastroenteritis were diagnosed and reported in BC in 2009. Highest rates were seen in 1-4 year olds (48.8 per 100,000 population), 25-29 year olds (49.5 per 100,000 population) and 20-24 year old adults (54.2 per 100,000 population). Provincial (BC) and national rates declined between 2000 and 2004, but have not changed much between 2004 and 2009 (4, 55). The rate of campylobacteriosis in

BC has been consistently higher than the national rate since 2000 (4, 55). The Public Health Agency of Canada also made note that campylobacteriosis rates are under-reported in Canada (4), which may suggest a need for improved methods of detection or identification.

Current *Campylobacter* detection and identification begins with culturing of the organism from stool samples or environmental samples. Once colonies are isolated via culturing, they are examined for *Campylobacter* morphology, subjected to gram staining and biochemical tests, such as oxidase and hippurate, though some strains may produce variable results (14, 40, 46, 54, 70).

Enumeration is only performed on retail samples, after positive results for *Campylobacter* are produced. The culturing and enumeration method recommended by the Public Health Agency of Canada for *Campylobacter* in retail samples (2), has a sensitivity of 0.3 MPN/g from 50g of meat. Many samples, however, have levels of *Campylobacter* below this limit (2). Due to the ability of *Campylobacter* to enter a viable but non-culturable (VBNC) state, and inability of culture methods to detect low numbers of cells in samples, isolation of *Campylobacter* can sometimes be difficult (1, 40, 54, 70). This may result in decreased detection, diagnosis, and reporting.

Clinical and environmental isolates identified as suspected or probable *Campylobacter*, may also be subjected to a polymerase chain reaction (PCR) assay that identifies *Campylobacter* at the genus level (45, 70), or at the species level (14, 41, 46). These processes can be laborious and costly. Many public health laboratories often forward suspected *Campylobacter* isolates or stools to federal or provincial reference laboratories for identification and/or typing as they do not have the resources (such as staff, equipment, funding) to carry out such tests themselves. Since many laboratories do not have the capacity to do this testing, and detection/identification itself can be challenging, a number of *Campylobacter* cases may not be detected or reported. Therefore, a sensitive,

quick, easy, and cost effective assay for detection/identification of *Campylobacter* or its species is not currently available, and could potentially be very useful for clinical and environmental samples.

In May and June 2000, an outbreak of *Campylobacter* and *E. coli* 0157:H7 occurred in Walkerton, ON. There were 116 confirmed cases of campylobacteriosis associated with the outbreak (16, 19, 36, 37, 59). The bacteria were from farm animal feces and were found in the town's main water supply. Although the water supply was both filtered and chlorinated, the heavy rains diluted the chlorine and overloaded the filtering capacity, thus allowing contaminating organisms to survive and infect the Walkerton population (16, 19, 36, 37, 59). This incident highlighted the need for either a more effective water treatment facility, or for improved quality assurance testing in sub-par facilities during periods of high-risk, such as rain storms, which threaten to overthrow the water treatment capacity.

Therefore, improved methods of *Campylobacter* detection and identification could be useful for quality assurance testing, as well as for improving the reporting of disease incidence. For areas such as Walkerton, quality assurance tests could be performed for organisms known to be a threat in this area, such as *Campylobacter*, *E. coli*, and other organisms found commonly in feces of farm animals. The possession of a kit for the rapid detection of these organisms could be useful here.

1.2 Target Identification using Aptamers and SELEX

1.2.1 Aptamers

Aptamers are short segments of DNA or RNA, typically 40-80 nt long, which are specific for a molecular target. The target may be a protein, carbohydrate, small

molecule, another nucleic acid, or even a whole cell or organism (30, 31, 32, 33, 42). Aptamers are produced from an assortment of oligonucleotide sequences. Oligonucleotides that bind the desired target are then termed aptamers. Aptamers are usually selected for their targets via several rounds of Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Aptamers of high specificity and affinity for the target are selected via several rounds of incubation with the target (18, 23, 24, 34, 47, 49, 52, 68).

DNA aptamers are considered more favourable than RNA aptamers, as DNA is more stable than RNA. DNA has 1 less hydroxyl group in its ribose than does RNA, which makes it less susceptible to hydrolysis. DNA aptamers offer greater longevity and cost-effectiveness as a potential reagent or probe to be used for detection of a target. As opposed to antibodies and other proteins that are capable of binding targets via strong ionic bonds; aptamers primarily bind to targets through their secondary structure, such as stem-loop and hairpin structures, and weaker hydrogen bonding (42).

Many aptamers have been used in place of antibodies for detection studies and diagnostic tests (5, 28, 42, 69, 73, 74). For example, Zhao et al (74) used previously generated aptamers in Capillary Electrophoresis (CE) columns in place of antibodies, for the separation and detection of proteins. Here, DNA aptamers specific for cytochrome C and thrombin were biotinylated and immobilized on a column coated with streptavidin, to facilitate the immobilization. This allowed the separation of cytochrome C and thrombin from other proteins. DNA aptamers are more stable than antibodies as well, and are cheaper to produce via polymerase chain reaction (PCR) amplification, rather than via the animal requirement for antibody production. More importantly, the use of aptamers does not necessitate knowledge of the identity of the target, nor does it always require purification of the target (22, 31, 33). The generation of antibodies however, requires prior knowledge of the target's identity, purification of the target, and

incubation within an animal model to acquire anti-target antibodies. Aptamers could therefore be a more desirable reagent or probe than antibodies.

1.2.2 SELEX

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is a technique that is commonly used to select aptamers that bind to a target molecule. This target molecule may be another nucleic acid, a protein, carbohydrate, a chemical, or even a whole cell or organism (30, 31, 32, 33, 42).

There are several different types of SELEX to generate aptamers against a target molecule, including traditional SELEX, cell-SELEX, and Capillary Electrophoresis (CE) SELEX(18, 20, 23, 29, 30, 31, 32, 233, 38, 42, 50, 52, 53, 61, 63, 64, 68, 73, 74). Normally, an assortment of random oligonucleotide sequences are added to a target molecule, and allowed to incubate, to allow any potential binding to occur. These oligonucleotides are of unknown specificity and affinity for the target. The goal is to select aptamers that are specific for that molecule. Unbound oligonucleotide sequences are removed (via washes, column separation, etc) and bound oligonucleotide sequences are eluted and retained. Typically, the bound oligonucleotide sequences (aptamer pools) are amplified via polymerase chain reaction (PCR) and added to the target for further rounds of selection. The number of rounds chosen varies greatly. Typically, the relative binding affinity of the aptamer pools is estimated after each round, often via measurement of fluorescence or some other signal intensity. The rounds are continued until a satisfactory affinity is obtained. The potential affinity can vary depending on the target. Typically, aptamers can have binding affinities (dissociation constants - K_d s) from the molar range, if binding is very weak, to the picomolar range if binding is very strong. Protein targets with strong binding can have K_d s in the low nanomolar (nM) to picomolar (pM) range (24, 34, 47, 49,

68); more challenging targets, such as small molecules, with strong binding have had Kds in the micromolar (μM) range (24, 34, 52). The act of amplifying the aptamers generates a higher copy number of sequences which must compete for binding to the target molecules. Aptamers with higher affinity and specificity for the target are more likely to remain bound than those with lower affinity. This will increase the likelihood of retention of higher affinity aptamers, while the lower affinity aptamers are washed away. Therefore with each round of selection, aptamers with higher affinity and specificity for the target will be selected.

1.3 Literature Review of Previous Work

1.3.1 Common Aptamer Targets

Typical aptamer targets include proteins and nucleic acids; aptamer activity was discovered through the study of RNA ribozymes. These studies lead to the appreciation that oligonucleotide libraries could be used in combination with a plethora of targets (42).

Protein targets are very common. For example, the works of Hamula et al (30, 31, 32) and Zhao et al (74) have focused on generating or using DNA aptamers against protein targets. Hamula et al generated DNA aptamers specific for surface proteins of whole live bacterial cells, such as *Streptococcus pyogenes* and *Lactobacillus acidophilus*, as a means of microbial detection (30, 31, 32). Zhao et al used previously generated aptamers, specific for cytochrome C and thrombin for the purpose of protein separation (74).

An example of a protein binding RNA aptamer with clinical use is Macugen, also known as Pegaptanib, a pegylated aptamer that binds the VEGF 165 protein (28, 69). Macugen was approved for the treatment of wet age-related macular

degeneration (wet AMD) by the FDA in 2004 (5). Wet AMD is characterized by the abnormal growth of fragile blood vessels in the eye, which are prone to leakage into the retina, causing damage to the macula and resulting in deterioration of vision (5, 28, 69). VEGF is a member of the platelet derived growth factors involved in angiogenesis (27, 28). Binding of the aptamer to the VEGF protein is proposed to decrease the ability of the protein to trigger angiogenesis in the retina (27, 28). Studies involving administration of the aptamer over 2 years, have shown slowed vision loss in patients with wet AMD (5).

Another clinically promising aptamer is the anti-cancer aptamer – AS1411. The aptamer is believed to inhibit DNA replication of tumor cells, and has shown promising results from *in vivo* mouse studies (29, 39). Effects appeared to include delayed tumor growth. The aptamer was then administered to patients with advanced tumors. Similar effects on stabilization of tumor progression were noted in several patients, with no side effects reported (29, 39).

1.3.2 Small Molecule Aptamer Targets

It is more challenging to generate aptamers against small molecules due to smaller surface area for binding and the presentation of less potential binding sites (73). Nonetheless, aptamers have been generated against such small molecules as amino acids, antibiotics, nucleobases like ATP, and cocaine. Aptamers have also been generated against matrices and dyes to assist in purification and labeling (23, 2438, 42, 52, 64).

Both RNA and DNA aptamers were generated against ATP in 1993 and 1995, respectively (38, 64). The RNA and DNA aptamers were found to have similar binding affinities for ATP, and they were both found to bind at the same location. Despite the similarities in binding, the DNA and RNA aptamers had different sequences and secondary structures. To test whether similarities in sequence

account for binding similarities, Huizenga et al created a DNA aptamer from the RNA sequence, and an RNA aptamer from the DNA sequence. Both were incubated with ATP, but neither sequence recognized the molecule. Though the sequences were similar, binding properties were observed to be different. Binding affinity was therefore not merely influenced by aptamer sequence, but also by secondary structures and other interactions.

In 2001, Stojanovic et al (63) generated a DNA aptamer specific for cocaine. They then divided the aptamer into 2 subunits and labelled one with a fluorophore and one with a quencher. Fluorescence was emitted when no cocaine was bound to the subunits. When cocaine was bound, the structure changed to bring the fluorophore and quencher together. Thus, quenching of the signal was indicative of the presence of cocaine. This provided promising preliminary results for the use of aptamers as biosensors.

Carbohydrate targets can be more of a challenge than those of protein, as both DNA aptamers and the carbohydrate are negatively charged. This likely results in more resistance to aptamer-target binding than between DNA aptamers and protein targets, which may be neutral or positively charged. There has been less published success in the generation of aptamers against carbohydrate targets. Those that were successful were mostly RNA aptamers, or DNA aptamers interacting through a positively charged modification or functional group (42, 50). Despite the fact that carbohydrates can be challenging targets, carbohydrates remain of investigative interest as cell surface carbohydrates play a role in cell-recognition and signalling, cell-cell interactions, and disease progression (58, 60, 62, 72)

In 1998, Yang et al (73) generated DNA aptamers against cellulose and cellubiose. A pool of random DNA sequences was passed through a cellulose column several times, bound aptamers were eluted, and amplified. These

aptamers were found to have low affinity for other similar disaccharides, suggesting DNA aptamers are capable of differentiating between closely related carbohydrates.

In 2003, Masud et al (50) generated DNA aptamers against sialyllactose. Here, the DNA aptamers were modified to contain an amino linker, which was positively charged. This work suggested that the positive charge facilitated binding, which further supports the theory that DNA-carbohydrate interactions are unfavorable due to repulsive forces.

Strong aptamer binding is enhanced by the presence of aromatic rings and charged groups (42). Carbohydrates lack these and also carry a net negative charge. Therefore, unless modifications are introduced, the opportunity for repulsive forces and weaker binding forces make DNA aptamer-carbohydrate interactions unfavourable.

1.3.3 Modifications of Traditional SELEX

Traditionally, SELEX is performed by adding a pool of random oligonucleotide sequences to a purified target (30, 31, 32, 33, 42, 47, 68). The target is typically known before-hand and is acquired in purified form and incubated with aptamers.

Many modifications have been made to the traditional SELEX procedure. Two of the more popular methods are Capillary Electrophoresis (CE) –SELEX and Cell-SELEX. CE-SELEX, as detailed in the published protocol of Mosing and Bowser (53) involves the addition of a portion of random DNA oligonucleotide library to the target, the identity of which is known before-hand and obtained in purified form. Bound sequences are amplified via PCR, purified, denatured, and re-added to the target for the next round of selection. Here, CE is used to separate the bound sequences from the unbound. The aptamers and target are allowed to pass

through an open capillary column filled with electrolyte buffer, as in standard capillary electrophoresis. The separation is based on the size-to-charge ratio of the molecules. The oligonucleotide sequences, from which aptamers are selected, carry negative charges. The bound aptamer-target complex is larger in size than the unbound sequences, and both the complex and the unbound sequences have similar negative charges. The higher size-to-charge ratio of the bound aptamer-target complex results in its separation from the unbound sequences. The bound aptamer-target complex is collected, and the aptamers are amplified for subsequent rounds of selection.

Cell-SELEX was designed to bypass the need for a known and purified target molecule for aptamer selection. The technique is similar to conventional SELEX in that a pool of oligonucleotide sequences is incubated with target molecules (or target cells), then the unbound sequences are separated from the bound, and the bound aptamers are kept, amplified, and added to the target again for several rounds of selection (47, 53).

Cell-SELEX differs from traditional SELEX in that the target is a whole cell. Cell-SELEX has been used to generate aptamers to both mammalian and bacterial cells. Aptamers have been generated against microbes such as bacteria, viruses, and parasites, as well as eukaryotic cells such as yeast, mammalian, stem, and tumor cells (18, 20, 29, 30, 31, 32, 39, 52, 61).

Cancer cells have been a popular target in the literature (20, 29, 31, , 39, 52, 61). In 2003, Daniels et al generated a DNA aptamer against tenascin-C, a previously discovered tumor marker in the tumor matrix, using Cell-SELEX on glioblastoma cell line monolayers as a target (20, 29, 61).

In 2008, Hamula et al (30, 31, 32) published their novel technique of using Cell-SELEX against live bacterial cells. Here, separation of bound and unbound aptamers was simplified via mere centrifugation of cells. DNA aptamers were successfully generated against cell surface proteins on both *L. acidophilus* and *S. pyogenes*.

Using Cell-SELEX, the exact identity of the target on the bacterial cell surface is not known. Identity of the target is not crucial to the success of the selections. Specificity and binding affinity of the aptamers can still be assessed without knowing the identity of the specific target molecule (30, 32, 52).

In summary, section 1.3 demonstrates that aptamers have been proven to be useful in both clinical and research settings. In research, they have been used as probes and ligands for the identification or separation of target molecules. Clinically, they have been shown to have potential roles in the diagnosis or identification, and treatment of disease.

1.3.4 *E. coli* DH5apgl Cells and the *C. jejuni* Cell Surface

Many bacteria have protein glycosylation pathways, whereby one or more carbohydrates are attached to a protein. Glycosylation can protect proteins from degradation, or encourage proper protein folding for example. Glycosylation of bacterial surface proteins can contribute to the adherence, immunogenicity, virulence or pathogenicity of an organism (34, 55, 56, 58, 64, 65). Both pili and flagella may contain glycoproteins (25, 34, 55, 56, 64, 65, 71). *Campylobacter* has glycosylated flagellins which are involved in serospecificity (65). O-linked glycosylation is rather common, whereby carbohydrates are attached to oxygens of amino acids of the peptides, or to lipids. N-linked glycosylation is more rare, and involves binding to a nitrogen molecule in certain amino acid sequences. *C. jejuni* has an N-linked protein glycosylation system, which contributes to its pathogenicity (25, 34, 55, 56, 64, 65, 71).

The *pgl* gene cluster is responsible for N-glycosylation of proteins in *C. jejuni*. In 2004, Feldman et al (25) ligated this gene cluster into the pACYC184 vector and transformed *E. coli* DH5 α cells. The plasmid also conferred chloramphenicol resistance. The purpose was to study glycosylation pathways and investigate

whether antibacterial vaccines could be generated against organism-specific O-antigen components. These engineered *E. coli* DH5 α *pgl* cells therefore over-expressed the *C. jejuni* surface glycoprotein responsible for pathogenicity. Therefore, this engineered strain could also be useful as a target for generating aptamers against *C. jejuni*, or the *C. jejuni* surface carbohydrates themselves.

1.4 Project Rationale

The novel method of Cell-SELEX using live bacterial cells, and centrifugation to separate bound and unbound DNA sequences, has previously been tested with success by Hamula et al (30, 31, 32) against *L. acidophilus* and *S. pyogenes*. The extendability of this novel method needs to be tested with other bacterial cell surface targets and other bacteria. This project will use cell-SELEX to target *C. jejuni*.

Campylobacter jejuni is a clinically relevant bacterium which produces a considerable level of carbohydrate on its surface. A non-pathogenic strain of *E. coli* DH5 α (*E. coli* DH5 α *pgl*) has been engineered to over-express the *C. jejuni* *pgl* gene responsible for surface presentation of the O antigen, by the Feldman lab (25, 71) at the University of Alberta. Therefore these two bacterial models, and their surface molecules, were chosen as targets against which to generate DNA aptamers. The desired bacterial target is *C. jejuni* and the putative surface target is a carbohydrate.

The above live Cell-SELEX method will be slightly modified, whereby two sets of SELEX will be performed on two bacterial targets in order to improve yield of aptamers against both *C. jejuni* and a *C. jejuni* surface carbohydrate. *C. jejuni* ATCC 33560, and the *E. coli* DH5 α *pgl* engineered to express the *C. jejuni* carbohydrate of interest, will be used as targets in separate sets of selections. There will also be the addition of a target-switch step: During later rounds of

selection, the target of each set will be switched with that of the other set. Aptamers generated may be useful for bacterial detection in clinical and environmental settings.

The hypothesis is that the addition of the target switch step to the Cell-SELEX method, between *C. jejuni* ATCC 33560 and *E.coli* DH5 α gl, enhances selection of DNA aptamers with high specificity and affinity for *C. jejuni* itself and/or a cell surface carbohydrate.

1.5 Research objectives

The primary objective is to generate DNA aptamers with high selectivity and binding affinity to intact *C. jejuni* cells using a Cell-SELEX technique. The secondary objective is to attempt to generate DNA aptamers against a cell surface carbohydrate. This will primarily be attempted through a target-switch step during selections.

Aptamers will be evaluated for their ability to bind to both *C. jejuni* ATCC 33560 and *E. coli* DH5 α gl. A preliminary evaluation of specificity will assess binding with unrelated, non-target bacteria. Efficacy of selections, via specificity and binding affinity of the selected aptamers, will be assessed using flow cytometry.

Chapter 2. Methodology

2.1 Bacterial Strains and Growth

The engineered strain of *E. coli* DH5 α *pgl* was obtained from Dr. Mario Feldman (Biological Sciences Building, University of Alberta, Edmonton, Canada) (25, 71). It was engineered to over-express the *C. jejuni pgl* gene responsible for surface presentation of the O antigen. Therefore, this bacterium will provide a rich amount of surface carbohydrate as a target.

The engineered *E. coli* strain DH5 α *pgl* was grown on Luria Bertani (LB) agar containing 20 μ g/ml chloramphenicol (Fisher, Fairlawn, NJ, USA), and in LB broth containing 34 μ g/ml chloramphenicol and incubated for 16 hours at 37°C, as specified by Feldman et al (25).

Un-engineered *E. coli* strain DH5 α , obtained from the Invitrogen TOPO® TA Cloning Kit for Sequencing with One Shot® MAX Efficiency™ DH5 α -T1R *E. coli* (Invitrogen, Carlsbad, CA, USA) (10) was also used as a control, without chloramphenicol resistance, to assure the antibiotic in the plates was effective to selectively grow the engineered bacteria expressing the desired aptamer surface carbohydrate target. This un-engineered strain was grown on LB agar and in LB broth and incubated under aerobic conditions for 16 hours at 37°C. This strain was also used in the counter selection SELEX step for the engineered *E. coli* DH5 α *pgl* strain.

Campylobacter jejuni strain ATCC 33560: A subculture was obtained on a blood agar plate from Dr. Jeff Fuller in the Provincial Laboratory (Walter C. MacKenzie Health Sciences Centre, Edmonton, AB, Canada). It was grown under microaerobic (reduced oxygen) conditions on Brain Heart Infusion (BHI) agar and in BHI broth, both containing 10 μ g/ml of both vancomycin (Fisher, NJ,

USA) and amphotericin B (Fisher, Solon, Ohio, USA) to discourage growth of common contaminants, gram positive bacteria and fungi, respectively. Cultures were grown over 24-48 hours at 37°C, using a CampyGen pak (Oxoid, Basingstoke, Hampshire, England) in an anaerobic jar or in W-zip seal pouches (Oxoid, Basingstoke, Hampshire, England). To confirm that the growth on the plates was indeed *Campylobacter*, each liquid subculture used per SELEX round was re-plated on BHI agar, and grown over 48 hours at 37°C. Growth was subjected to the oxidase and urease biochemical tests, and a latex agglutination test containing latex beads coated with antibodies specific to *Campylobacter* species (3) were used (Oxoid, Basingstoke, Hants, UK). *Helicobacter* species were being grown in the lab during the same period of time. The *Helicobacter* species were both oxidase and urease positive. As a precautionary measure, cultures that were oxidase positive and urease negative, as well as causing agglutination of latex beads were considered to be *Campylobacter* positive.

E. coli strain ATCC 25922: A subculture of the organism was obtained from Dr. Jeff Fuller in the Provincial Laboratory, and grown on LB agar or in LB broth for 16 hours at 37°C. This strain was used in the counter selection SELEX step for the *C. jejuni* SELEX.

Glycerol stocks were prepared from both liquid subcultures as well as from agar plates (for *C. jejuni* only) by adding 150 µl glycerol (Sigma, Oakville, ON) to 850 µl of liquid subculture, or by adding 850 µl of appropriate broth, as detailed above for each bacterium, to resuspend growth on agar plates. Stocks were evenly suspended and snap-frozen using dry ice and ethanol bath, then stored at -80°C.

Other bacteria were used post-SELEX during flow cytometry analysis of relative binding affinity of synthetic aptamers to bacteria other than the *C. jejuni* or *E. coli* DH5 α *agl* target bacteria. These bacteria included *H. pylori* patient strain A158, *S. pyogenes* M1, *S. bovis*, *E. coli* K12, as well as the *E. coli* ATCC 25922 strain and

the un-engineered *E.coli* DH5 α . *H. pylori* patient strain A158 was supplied by Dr. Monika Keelan, which was grown under microaerobic conditions on Brain Heart Infusion (BHI) + 5% Sheep's blood agar plates (Oxoid, Nepean, ON) for 48 hrs at 37°C, using a CampyGen pak (Oxoid). *S. pyogenes* M1 was grown aerobically on Sheep's blood agar (Oxoid) plates and BHI broth for 16 hrs at 37°C. *S. bovis* was grown aerobically on BHI (BD Difco, NJ, USA) agar plates and BHI broth for 16 hrs at 37°C.

Un-engineered *E.coli* strain DH5 α and *E. coli* strain ATCC 25922 were chosen, as targets in the counter-selection rounds, to test specificity of aptamers to *E. coli*. During counter-selection, these bacteria are used as targets and bound aptamers are discarded. Cross-reactivity of aptamers with these bacteria is indicative of the efficacy of counter-selection and its wash steps, through separation of bound from unbound aptamers. Specificity testing using other bacteria was performed based on availability of other bacteria in the lab, as a preliminary step.

Binding to other bacteria indicates that aptamers selected may have high affinity, but low specificity, for the target bacteria. Also, the un-engineered *E.coli* DH5 α strain does not express the *C. jejuni* surface carbohydrate. Therefore, binding of aptamers to the engineered *E. coli* DH5 α *agl* but not to the un-engineered strain would suggest an increased likelihood that aptamers may have been generated against the surface carbohydrate. Binding of aptamers to *C. jejuni* but not the other bacteria would provide promising preliminary evidence of specificity for *Campylobacter*. Binding to *C. jejuni* and the engineered *E. coli*, but not other bacteria would suggest aptamers may be specific for the *C. jejuni* surface carbohydrate.

2.2 Polymerase Chain Reaction (PCR)

Amplification of Library DNA was based on the protocol used by Hamula et al (31, 32). The protocol was optimized by varying the concentrations of magnesium from 1.0 mM to 2.0 mM, template from 10 ng and 100 ng, and polymerase from 0.5 U to 2.0 U. Optimal annealing temperature was also assessed between 53°C and 69°C, and cycle number from 1 to 19 cycles. The effect of addition of dimethyl sulfoxide (DMSO, 1 µl per 50 µl reaction volume) was also assessed, as it can aid in the decrease of non-specific amplification.

Pools of 80 nt aptamers were amplified using 20 bp non-specific primers purchased from Integrated DNA Technologies Inc. (IDT, California, USA). The sequence of the forward primer is 5'-AGCAGCACAGAGGTCAGATG-3'; the sequence of the reverse primer is 5'-TTCACGGTAGCACGCATAGG-3'. Random 40 bp sequences were amplified between the two 20 bp primers, thus generating aptamers of 80 bp and various different sequences.

DNA was amplified by the polymerase chain reaction (PCR) in 100 µl reaction volumes. 1.0 EU of Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 1X reaction buffer (Invitrogen, Carlsbad, CA, USA), 2 mM MgCl₂ (Invitrogen, Carlsbad, CA, USA), 0.4 µM primers (IDT, California, USA), and 0.8 mM dNTPs (Fermentas Canada Inc., Burlington, ON) were added to 10 µl of approximately 10-100 ng of DNA aptamers (eluted from the previous round of SELEX). 2 µl of dimethyl sulfoxide (Sigma, Saint Louis, MO, USA) was added as well to decrease misamplification. Cycling was carried out with an initiation step of 5 minutes at 94°C, followed by 14-19 cycles (depending on round and DNA yield, based on yields from previous rounds and amplifications) of DNA denaturation for 30 seconds at 94°C, 30 seconds at a primer annealing temperature of 69°C, and primer extension for 30 seconds at 72°C, followed by a final extension step of 5 minutes at 72°C.

As the amplified product was 80 bp, it is too small to run and separate the fragments on an agarose gel. A polyacrylamide gel offers much better resolution than would an agarose gel. The amplified PCR products were visualized on a 7.5% polyacrylamide gel (40% 29:1 bis acrylamide, BioRad, Hercules, CA, USA) in a BioRad Protean III unit (BioRad, Hercules, CA, USA), run at 100-120V. Gels were stained with a 1 mg/ml ethidium bromide solution (Sigma-Aldrich Canada Ltd., Oakville, ON) after electrophoresis, and then visualized on either a basic UV transilluminator or the GE Healthcare IQ350 digital imager (GE Healthcare Bio-Sciences Corp., NJ, USA).

PCR products were purified using the Qiagen MinElute PCR Purification kit (Qiagen, Maryland, USA), according to manufacturer's instructions (6). The kit includes columns which bind to double stranded DNA, and facilitate the removal of primers, salts, and other left-over PCR reagents which may interfere with subsequent reactions. Reagents used were supplied in the kit, though composition of buffers was not disclosed due to their proprietary nature. Briefly, 5 volumes of Buffer PB were added per 1 volume of PCR reaction (100 μ l each), then mixed and added to a MinElute column and centrifuged for 1 minute at 10,000 xg to bind the DNA to the column. Flow-through was discarded and 750 μ l Buffer PE was added to the column and centrifuged at 10,000 xg for 1 minute to wash the DNA. Flow-through was discarded and the column was spun at 10,000 xg for 1 minute to remove any residual buffer. The column was placed in a new 1.5 ml microcentrifuge tube and DNA was eluted by adding 12 μ l 1x Binding Buffer (50mM Tris-HCl (pH7.4); 1mM MgCl₂; 100mM NaCl; 5mM KCl), incubating 1 minute at room temperature, then centrifuging 1 minute at 10,000 xg. Eluted DNA was then used in the next round of selections. The double stranded purified PCR products were then heat denatured (94°C) to produce single stranded DNA aptamers to facilitate binding of DNA to target molecules during SELEX.

Prior to flow cytometry analysis, 2.0 μ l of the amplified and purified bound aptamer pools was re-amplified with fluorescent (fluorescein labelled) primers (same sequences as above, but with a fluorescent 5' 56-FAM tag, a fluorescein label (IDT, California, USA)), at 12 cycles under the same PCR and cycling conditions as above.

DNA concentrations of amplified, purified double stranded DNA aptamers were then measured by spectrophotometric analysis (measuring absorbance at 260 nm, with an OD₂₆₀ of 1 equivalent to 50 μ g/ml double stranded DNA) using a BioRad SmartSpec 3000 spectrophotometer (BioRad, Hercules, CA, USA) or a Perkin Elmer Lambda LS 35 spectrophotometer (Perkin Elmer, Massachusetts, USA). DNA concentration is calculated by multiplying the absorbance value at 260 nm, by the dilution factor, and 50 μ g/ml. 100 pmole of purified aptamers were used for the next round of SELEX. 100-200 pmole purified fluorescent aptamers were used for flow cytometry analysis of fluorescence shifts in binding. The amplified fluorescent aptamers were then incubated with bacteria of interest and subjected to flow cytometry analysis.

2.3 SELEX

The SELEX protocol used was based on that used by Hamula et al (31, 32), with some modifications. The amount of starting oligonucleotide library used in the first round of selections was 266.3 nmole for the *E. coli* DH5 α pgl set, and 284.7 nmole for the *C. jejuni* set. In the first round of selections, 10⁸ cells/ml was used of each bacterial target. All subsequent rounds used 100pmole of amplified and purified aptamers from the previous round, and 10⁷ cells/ml of each target bacterium.

In each round of selection, cells were first washed to remove media, in 250 to 500 μ l 1x Binding Buffer (50mM Tris-HCl (pH7.4); 1mM MgCl₂; 100mM NaCl;

5mM KCl) followed by centrifugation at 4°C at 5000 or 8000 xg for *E. coli* or *C. jejuni* cells, respectively. Aptamers and cells, as well as controls consisting of cells only (to rule out contamination of reagents with DNA), were allowed to incubate for approximately 50 minutes to 1 hour. Cells were then washed 3 times, with 250 to 500 µl 1x Binding Buffer and pelleted via centrifugation at 4°C for 10 minutes at 5000 xg for *E.coli* cells or 8000 xg for *C. jejuni* cells. The supernatant from these washes should have contained the unbound aptamers. Aptamers with high affinity for the cells should remain bound to cells at this point. Cells were then resuspended in 100 to 250 µl 1x PCR Reaction Buffer (Invitrogen, Carlsbad, CA, USA) and transferred to a new tube, in order to remove any aptamers that may have bound to the tube itself. Heat denaturation at 94°C was then carried out for 5 minutes to remove any bound aptamers from the cells. Tubes were immediately placed on ice for 10 minutes, then centrifuged at 4°C for 10 minutes at 5000 xg for *E. coli*, or 8000 xg for *C. jejuni* cells. Both the supernatant and remaining cell fraction were kept and amplified for the next round of selection.

Twelve Rounds of SELEX were carried out on *E. coli* DH5α *pgl* and 8 Rounds of SELEX were carried out on *C. jejuni* ATCC 33560 (Figure 2.3.1). Counter-selection was done between Rounds 3 and 4 for each bacterium. The objective of this step was to reduce cross-reactivity of aptamers to the target bacterium. However, another step was added, differing from the method by Hamula, et al. A target-switch step was also included in the SELEX for both bacteria, whereby the target bacterium was switched to either *C. jejuni* ATCC 33560 (during SELEX initiated with *E. coli* DH5α *pgl*) or to *E. coli* DH5α *pgl* (during SELEX initiated with *C. jejuni* ATCC 33560). The objective of this step was to enhance specificity of aptamers for the desired target, namely the surface carbohydrate molecule.

For the *E. coli* DH5α *pgl* SELEX, un-engineered *E. coli* DH5α were used as the counter-selection target. This strain was chosen since it is theoretically identical

in surface expression to the engineered strain, except for the *C. jejuni* carbohydrate target surface expression. Since they should be identical except for the carbohydrate expressed, any aptamers from round 3 that binds the engineered strain, but not the un-engineered strain in counter-selection, should be specific for the *C. jejuni* surface carbohydrate, and are therefore, more likely to also bind *C. jejuni*.

The target-switch step for *E. coli* DH5 α *pgl* was carried out between rounds 4 and 5, and utilized *C. jejuni* ATCC 33560 as the new target. Aptamers that bound both bacteria were kept, and denoted as the C+E set. Aptamers that did not bind the *C. jejuni* ATCC 33560, found in the unbound fraction/supernatant, were also kept and denoted as the E set. This target-switch step should identify which aptamers bind the desired *C. jejuni* surface carbohydrate on actual *C. jejuni* cells, and which aptamers bind the *E. coli* DH5 α *pgl* surface carbohydrate, but not actual *C. jejuni* cells. This step is important, since the *E. coli* DH5 α *pgl* may express the surface carbohydrate in a slightly altered conformation than that on the surface of actual *C. jejuni*. Therefore, the target-switch step is also important to ensure that the cloned surface carbohydrate expressed on the *E. coli* DH5 α *pgl* surface is the same in structure to the intact *C. jejuni* surface carbohydrate. All remaining rounds of SELEX initiated with *E. coli* DH5 α *pgl* were carried out using *E. coli* DH5 α *pgl* as the target bacterium.

For the *C. jejuni* ATCC 33560 SELEX, the reference strain of *E. coli* ATCC 25922 was used as the counter-selection target. This *E. coli* reference strain is considered non-pathogenic, and will likely be similar to the non-pathogenic *E. coli* strains found to be largely present in stool. Since *C. jejuni* is commonly transmitted to humans through fecal contamination, any aptamers to *C. jejuni* should not cross-react with a normal component of stool, to prevent false positive identification of *C. jejuni*.

The target-switch step for *C. jejuni* ATCC 33560 was between rounds 5 and 6, and utilized *E. coli* DH5 α *pgl* as the new target. This step should identify which aptamers bind the desired *C. jejuni* surface carbohydrate (expressed on the surface of *E. coli* DH5 α *pgl*), and which aptamers bind some other molecule on the *C. jejuni* surface, carbohydrate or non-carbohydrate in identity. Aptamers that bound both bacteria were kept, and denoted as the E+C set. Aptamers that did not bind the *E. coli* DH5 α *pgl*, were also kept and denoted as the C set. During the next round of selection (Round 6), the E+C set were added to *E. coli* DH5 α *pgl* again, to attempt to increase affinity of the *C. jejuni* aptamers to the carbohydrate surface target. The C set, however was added to *C. jejuni* in this round, as they do not bind to the *E. coli* DH5 α *pgl*. All remaining rounds were carried out using *C. jejuni* ATCC 33560 as the target bacterium.

2.4 Detection of Carbohydrate via Western Blot

Prior or concurrent to every 1-3 rounds of SELEX, the same liquid culture of bacteria to be used for the SELEX was also digested and subjected to Western Blotting to confirm surface expression of carbohydrate. This verified the presence of the desired target of aptamer selection. One ml bacteria were used at a concentration of 10^7 - 10^8 cells/ml. The protocol followed was used by Feldman et al (25) to ensure the engineered *E. coli* DH5 α *pgl* was expressing the desired gene product. Bacteria were centrifuged at 5,000 xg for 10 minutes and re-suspended in 100 μ l Laemmli Buffer (62.5 mM Tris-HCl, pH6.8; 25% glycerol; 2% SDS; 0.01% bromophenol blue) (BioRad, Hercules, CA, USA), with 5% v/v β -mercaptoethanol, heated for 12 minutes at 94°C, then 20 μ g of 20 mg/ml Proteinase K (Invitrogen, Carlsbad, CA, USA) was added per OD₆₀₀ of 0.75 bacteria ($\sim 7.5 \times 10^8$ cells/ml), and incubated at 50°C overnight to digest protein. Digests (OD₆₀₀ of 0.25 or ($\sim 2.5 \times 10^8$ cells/ml) were then run on a pre-made 15% Tris-HCl gel (5% stacking gel) (BioRad, Hercules, CA, USA) at 100V for 1 hour

and transferred to a Polyvinylidene Fluoride (PVDF) membrane (BioRad, Hercules, CA, USA) at 100V for 1.5 hours in a BioRad Protean III unit (BioRad, Hercules, CA, USA). Membranes were blocked overnight at 4°C or for 2 hours at room temperature with 1x Phosphate Buffered Saline Tween-20 (PBST) + 5% Bovine Serum Albumin (BSA). Membranes were then incubated for 1 hour each with Biotinylated Soybean Agglutinin (Vector Laboratories, Inc, Burlingame, CA, USA) as the primary antibody, and Goat Anti-biotin Peroxidase Antibody (Sigma, Saint Louis, MO, USA) as the secondary antibody. Detection was carried out using Pierce Super Signal West Pico Chemiluminescent Substrate for horse radish peroxidase (HRP) (Fisher, Rockford, IL, USA) and/or ECL Plus Western Blotting Detection System with a substrate (lumigen PS-3 acriden) also catalyzed by HRP (GE Healthcare, Buckinghamshire, UK) chemiluminescence kits. For the Pierce kit, the two solutions were mixed 1:1 and added to the membrane. For the ECL kit, solutions A and B were mixed 40:1 respectively and added to the membrane. Membranes were then exposed to X-ray film, typically for 1-5 seconds. Imaging was carried out using either an X-ray film developer and a GE Healthcare IQ350 digital imager.

The primary antibody is a biotinylated mannanose binding lectin, which binds to most carbohydrates non-specifically (58). Specifically, it binds oligosaccharides with α & β N-acetylgalactosamine residues – which the target surface carbohydrate in both *C. jejuni* and the engineered *E. coli* DH5 α *pgl* contain (25, 71). The secondary antibody binds biotin, so it will bind the primary antibody, as well as the biotinylated molecular weight (broad range) standards (BioRad, Hercules, CA, USA), used to estimate fragment size.

Membranes were stained with 1x Ponceau S stain (0.1% Ponceau S powder; 50% v/v acetic acid) (BioRad, Hercules, CA, USA), to confirm that protein digestion had occurred. As a digestion control, a commercial *E. coli* protein lysate (BioRad, Hercules, CA, USA) was also loaded on gels and detected with 1x Ponceau S

(0.1% Ponceau S, 5% acetic acid) stain (Thermo Fisher Scientific, Fairlawn, NJ, USA).

2.5 Flow Cytometry of Aptamer Pools

Since flow cytometry was the main method by which aptamer specificity and affinity was assessed, this technique is described here in more detail.

Fluorescence-Activated Cell Sorting (FACS) is a type of flow cytometry by which cells are sorted individually, excited by a laser, and any fluorescence they emit as a result, as well as light scatter, is detected and recorded. A liquid sample is introduced through a probe into a cuvette, on which a blue argon laser (488 nm) is focused. Therefore, an appropriate fluorescent label must be selected so that it can be excited by the 488 nm light source emitted by the blue laser (11, 43).

Once the sample enters the cuvette filled with sheath fluid (Phosphate Buffered Saline), typically one cell at a time, the cell scatters the laser light. Forward scatter (FSC) is an indicator of cell size, as bigger cells will cause a greater angle of diffraction than smaller cells. This diffracted light is then detected by a detector in line with the blue laser. Side scatter (SSC) is an indicator of cell granularity or surface smoothness or roughness. A more granular or rough cell will scatter light in more directions than a smooth cell. A detector is placed perpendicular to the incident light to detect side scatter (11, 43). Specific fluorescence is a result of the cells bound to fluorescently labelled aptamer, and is indicative of degree of binding or amount of binding sites. Fluorescence is detected using another detector perpendicular to the incident light source. This light is first filtered through either a green, orange, or red filter, depending on the wavelength of fluorescence emitted by the fluorescent dye. For example, since the fluorescein of labelled aptamers emits green light, the green filter would be used. Light detected is converted into an electrical signal via a photomultiplier tube, consisting of a series of dynodes, the first of which releases electrons when

excited by light, which then excites neighbouring dynodes, releasing even more electrons, and so on, thus amplifying or multiplying the original signal.

This signal is then sent to a computer, containing software (CellQuest) that will record the signal, and generate a cell scatter plot and a histogram plot from the results. The cell scatter plot shows side scatter versus forward scatter, allowing one to discern between relative cell sizes and internal complexity (11, 43). The histogram plots frequency (number of fluorescent events counted) versus intensity of fluorescence, allowing visualization of relative fluorescence intensity shift from background (Figure 2.5.2A).

Control cells, cells that are unlabeled, emit a background level of fluorescence which needs to be subtracted from fluorescence detected from experimental cells bound to fluorescently labelled aptamer. The equipment's software detects any fluorescence and displays a cell scatter plot showing fluorescence detected from side scatter versus forward scatter. Different cells will produce different and characteristic scatter plots. The software also plots frequency of fluorescent events versus fluorescence intensity as a histogram. A more extreme shift in fluorescence intensity from that of the control cells, is suggestive of stronger binding. The software and equipment settings were optimized for both *E. coli* DH5 α and *C. jejuni* ATCC 33560 cells that were unlabelled, to serve as a background signal for each bacteria. The histogram peak was centred at approximately 10^0 , or the middle of the first decade, as is considered standard protocol for the setup of this software (11, 43). Since samples containing cells and labelled aptamers should not exhibit fluorescence below that of the unlabelled cells only, this placement allows the full histogram to be viewed, while leaving the most room on the plot for shift from background (Figure 2.5.2B).

A marker is set at the tail end of the histogram, and fluorescence within the gate and marker is given as a % gated value, indicative of % shift from background.

Therefore, the % gated value is indicative of amount of fluorescence above background. The values plotted for both cell scatter and histogram plots are unitless, and serve only as a means to gauge relative changes in fluorescence (11, 43).

Gates were set to surround the control cell scatter only, and to include both living and dead cells, with the upper limit of the gate being just above the top of the control cell scatter, in order to eliminate interfering fluorescence from other molecules/substances which may be heavier. Any fluorescence detected within the gate is read and illustrated by the software as a histogram, and any fluorescence above the background/control level is illustrated as a histogram shifted right from the location of background.

Flow Cytometry was done on aptamer pools for each round of selection, after all rounds were completed. Each aptamer pool was amplified under the conditions as previously given (section 2.2), using fluorescently (fluorescein) labelled primers, at 12 cycles, and using 2 μ l of template diluted 1 in 100. This concentration was shown during optimization to use the least amount of template while maximizing PCR yield. The template used was DNA from each SELEX round pool, amplified and purified previously with non-fluorescent primers.

Flow Cytometry was carried out using a Beckton Dickinson FACScan (San Jose, CA, USA) in the Faculty of Medicine and Dentistry Flow Cytometry Facility (University of Alberta, Edmonton, AB, Canada). DNA aptamers were amplified with fluorescent primers to allow the amplified aptamers to serve as the fluorescent label. The fluorescent dye in the primers was fluorescein, which absorbs light at 495 nm - similar to the peak emission (488 nm) of the blue argon ion laser. Therefore, it is a good dye for flow cytometry. Once excited, fluorescein emits maximum fluorescence at 519 nm, which is detected in the green light range (11, 43).

Fluorescence intensities for both *E. coli* DH5 α *pgl* and *C. jejuni* ATCC 33560 were measured using the same instrument settings. The blue argon ion laser (488 nm) was used to excite the fluorescein-labeled aptamers bound to cells. Peak fluorescence at 519 nm was detected using a photomultiplier tube at a right angle (90 °) with respect to the excitation light. The photomultiplier tube converted photon counts to electric current and voltage signals that were registered on a computer. The voltage of the photomultiplier tube for measuring the side scatter was set at 319 V, and the voltage of the photomultiplier tube for measuring the fluorescence (519 nm) was set at 782 V.

FACS analysis was carried out using approximately 1×10^7 cells/ml for *E. coli* DH5 α *pgl*, and $1-5 \times 10^7$ cells/ml of *C. jejuni* ATCC 33560. These concentrations were chosen in order to limit the number of target cells for aptamers to bind, while still allowing the FACScan to provide a fluorescence intensity value after 1-5s. Longer read times resulted in higher interference and less consistent readings, as the solutions would be depleted faster. Readings were taken after counting 10,000 events. Aptamers were used at concentrations between 100-250 pmole in 500 μ l total volume. Aptamer pools were heated at 94°C to denature DNA into single strands, then left to incubate with bacteria for approximately 1 hour. Bacteria were then washed once, to remove excess unbound aptamers, then subjected to flow cytometry analysis.

Shift in fluorescence from background was measured. All incubations were run in triplicate, with 3 incubations performed per aptamer pool. The 9 values obtained were averaged to obtain a single average value. Shifts obtained from a random DNA oligonucleotide library were used to subtract non-specific binding values from the shifts above background for this average value per aptamer pool.

2.6 Cloning

Cloning was carried out using the Invitrogen TOPO® TA Cloning Kit for Sequencing with One Shot® MAX Efficiency™ DH5α-T1R *E. coli* (Invitrogen, Carlsbad, CA, USA). The kit contains the TOPO vector - a high copy plasmid containing Ampicillin and Kanamycin resistance, as well as a lacZα-ccdB fusion gene (10).

The purpose of the antibiotic resistance is to allow selective growth of cloned colonies on media containing the above antibiotics to prevent contamination from other bacteria which do not contain the TOPO plasmid. The lacZα-ccdB fusion gene encodes an enzyme that is lethal to the bacteria when intact. However, the cloning site disrupts this gene, allowing the cells to survive when an insert is present. Therefore, any cells that survive on the plates containing antibiotics are very likely to contain an insert.

Inserts are amplified prior to ligation into the vector. Most polymerases without proofreading ability, such as Platinum Taq, add extra adenosine nucleotides to the 3' ends of their template strand. Therefore, the amplified DNA has adenosine nucleotides on opposite ends of each strand of the double-stranded DNA. The TOPO vector has thymine nucleotides on opposite ends of each strand of the cloning site, allowing a double-stranded PCR product to be ligated directly into the plasmid without restriction digestion or other modifications. This allows much easier and faster cloning.

After selections, aptamer pools were cloned from rounds 10 & 12, after the target switch step, for the *E. coli* DH5α *pgl* set. This set also bound *C. jejuni* ATCC33560 (Round 10C+E & Round 12C+E, where C represents binding to *C. jejuni*, and E represents binding to *E. coli*). Cloning was also done using pools from round 8 for the *C. jejuni* ATCC33560 (Round 8C) set that only bound the *C.*

jejuni. Previously amplified and purified aptamer pool DNA was diluted 1 in 100. 10-100ng of this previously amplified and purified aptamer pool DNA was amplified at 7 cycles, using 1xPCR Buffer (without Magnesium), 50mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1 μ M unlabelled primers (IDT, Coralville, IA, USA), and 1 unit of Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Aside from the above mentioned cycle number, cycling conditions were the same as previously mentioned for amplification of aptamers in section 2.2.

A 750 bp control DNA fragment, supplied with the kit, was amplified at 25 cycles according to manufacturer's instructions (10). Briefly, DNA from rounds 10C+E and 12C+E and the control 750 bp DNA were ligated into the TOPO vector after purification. For rounds 10C+E and 12C+E, purification of the entire PCR reaction was done using the Qiagen MinElute PCR purification Kit (Maryland, USA), in order to remove primers, salts, and any other contaminants (as done in section 2.2). For round 8C, the 80 bp fragment was gel purified using the Qiagen QiaexII Gel Purification Kit (Maryland, USA) according to manufacturer's instructions (7). . This was due to excess non-specific amplification of round 8C aptamers, after an initial attempt at cloning without purification, which led to a very low success rate at obtaining 80 bp aptamer inserts for this round pool. The Gel Purification kit includes silica-gel beads which bind the DNA in high salt solutions. After washing, the DNA can be eluted from the beads using a low salt buffer or water. A summary of the protocol is as follows: Reagents used were supplied in the kit, except the diffusion buffer (0.5 M ammonium acetate; 10mM magnesium acetate; 1mM EDTA, pH 8.0; 0.1% SDS). Other buffers in the kit are proprietary, and their composition is not available.. Gel bands of the desired fragment size (~80 bp) were cut from the polyacrylamide gels and weighed. One and a half volumes of diffusion buffer were added to 1 volume of gel (ie. 150 μ l for 100 mg gel slice), and incubated 30 minutes at 50°C. The supernatant was transferred to a new tube, and 6 volumes of Buffer QX1 was added per 1 volume of supernatant. The solution was vortexed and 10 μ l of QIAEX II was added and

incubated 10 minutes at room temperature, while vortexing every 2 minutes. The solution was centrifuged for 30 seconds at 10,000 xg, and the resulting pellet was washed twice with 500 µl Buffer PE. The pellet, consisting of QIAEX II beads bound to DNA, was air-dried for approximately 15 minutes and resuspended in sterile distilled and deionized water, followed by incubation at room temperature for 5 minutes. The solution was centrifuged for 30 seconds at 10,000 xg, and the supernatant containing pure 80 bp DNA was transferred to another tube, to be used for ligating into the cloning plasmid.

Cloning was carried out according to the manufacturer's protocol (10). Specifically, 2 µl of purified DNA and 1ul of the 750 bp control DNA were ligated into the TOPO vector. Controls included 1 µl TOPO vector DNA only (no insert DNA), to ensure that clones without inserts do not grow on selective plates, and 1ul of pUC19 vector DNA only to measure transformation efficiency. The vectors were then transformed into chemically competent *E. coli* DH5α-T1R cells via heat shock at 42°C for 30 seconds. However, transformed cells were then incubated at 37°C, shaking at 175-200 rpm, for 1.5 to 2 hours instead of the suggested 1 hour, as this was found to improve transformation efficiency.

Cloning reactions were made for rounds 10C+E, 12C+E, and 8C, as well as for a control 750bp DNA insert. 10-100 µl of transformed cells were plated on LB plates containing either 50 µg/ml kanamycin or 100 µg/ml ampicillin. Plasmids were extracted using the Qiagen QuickLyse® MiniPrep Kit (Qiagen, Maryland, USA) (9). The kit purifies DNA from bacterial cells, via cell lysis followed by addition to a column that binds DNA. The DNA is washed in, then eluted from (using a low salt buffer or water), the column. A summary of the protocol used is as follows: Reagents used were supplied in the kit. Buffers in the kit are proprietary, thus their composition is not available. One and a half millilitres of each overnight culture of the selected *E.coli* DH5α colonies were added to a QuickLyse Lysis Tube, and centrifuged at 13,000 rpm for 1 minute. Media was

discarded, and 400 μ l cold Complete Lysis Solution was added to the cell pellet. The solution was vortexed then incubated for 5 minutes at room temperature. The lysate was transferred to a QuickLyse spin column and centrifuged at 13,000 rpm for 1 minute. The column was washed with 400 μ l Buffer QLW and centrifuged 1 minute at 13,000 rpm. Flow-through was discarded, and the column was spun again for 1 minute at 13,000 rpm to dry. The column was placed in a clean 1.5 ml microcentrifuge tube and DNA was eluted by adding 50 μ l Buffer QLE to the column. The column was then centrifuged at 13,000 rpm for 1 minute, and eluted DNA was used for restriction digestion and sequencing. Restriction digestion of the plasmids was then carried out using 0.5 units of EcoRI (Invitrogen, Carlsbad, CA, USA). Digests were then electrophoresed, along with a Gene Ruler 100 bp ladder (Fermentas Canada Inc., Burlington, ON), to ensure that 80 bp inserts were obtained. Random DNA oligonucleotide library of known concentration was also electrophoresed, in order to estimate plasmid and insert concentration yielded. All extracted plasmids with an 80 bp insert were sent for sequence analysis.

2.7 Sequence Analysis & Synthesis of Labelled Aptamers

DNA sequencing was performed on the extracted plasmids found to contain an 80 bp aptamer insert. 150 to 225 ng (15-2.25 ng/ μ l) of plasmid DNA was sent to the TAGC Sequencing Facility (Katz Building, University of Alberta, Edmonton, AB, Canada), and sequenced only in the Forward direction using the M13 Forward primer 5'- GTAAAACGACGGCCAG -3'.

Sequence results were returned as both a sequence of nucleotides and an .abi file which was opened and viewed as an electropherogram using Finch TV version 1.4.0 sequence viewer software (Geospiza Inc., Seattle, WA, USA), to confirm that the sequences were accurate.

Useable sequences were considered to be sequences that gave a single 80 nt aptamer sequence. Sequencing results that could not be accurately read to acquire an 80nt sequence, or results that showed more than one sequence present via more than one peak per location in various locations in the electropherograms, were considered unusable.

The 20 nucleotide (nt) primer sequences at either end of the sequences were conserved, and only the 40 nt in the middle were variable. Therefore, sequences with a deletion in the 20 nt primer region were still considered to be useable if the deletion did not affect the secondary structure predicted for the sequence when the missing nucleotides were restored. For example, the 78/79 nt structure was put into OligoAnalyzer along with the 80 nt structure (the missing nucleotide(s) from which were typed into the sequence) – if the structure and ΔG values were the same for both, the sequence was still considered useable. However, if the deletion occurred within the variable 40 nt region of the aptamer sequence, the sequence was not considered useable. It is impossible to predict which nucleotide is missing from which location, and whether the aptamer was selected with the deletion during SELEX (and is therefore useable), or whether the deletion occurred as an amplification error during cloning (and may therefore have lost its specificity and affinity for the target cell(s)).

To obtain the reverse sequence, useable forward sequences were entered in the Nucleic Acid Sequence Massager Online Tool (Attotron Biotechnologies Corporation, cybertory.com), to acquire a reverse and complement sequence. Useable forward and compliment/reverse sequences were then entered into the Integrated DNA Technologies, Inc. OligoAnalyzer 3.0 online tool (IDT, Coralville, IA, USA). This tool is used for analyzing sequences and predicting secondary structures and their free energy values. The value for change in Gibbs free energy (ΔG) is considered to be an indicator of stability at room temperature for aptamers (42, 48). An aptamer ΔG value of -5 kcal/mole and lower is

considered to be stable at room temperature (31). Stability at room temperature is considered desirable for aptamers. Aptamers with enhanced stability are desirable in order to rival the application of antibodies, as well as to be effectively used in laboratory diagnostic tests on a bench-top.

Sequences with a predicted Gibbs free energy change (ΔG) of -4 kcal/mole and lower were then subjected to further classification of secondary structure. Sequences were classified based on amount of stem, loop, and/or branching. Then a few sequences were selected from each class of structure to be synthesized.

Selected sequences were synthesized with a fluorescein label by Integrated DNA Technologies Inc. (IDT, Coralville, IA, USA), and were then tested individually by flow cytometry to estimate strength of binding to target and non-target bacteria, and to estimate binding affinity (K_d). Estimation was by visual observation of histogram shift from the control cells. Aptamers that produced the greatest shift in fluorescence from the control cells (highest % Gated values), and lower cross-reactivity with non-target bacteria, were chosen for K_d analysis.

2.8 Flow Cytometry of Individual Aptamer Sequences

The Beckton Dickinson FACScan was used at the same settings as previously indicated for Flow Cytometry of Aptamer Pools (section 2.5). Since these sequences have been synthesized and ordered in purified form at a predetermined concentration, the single-stranded aptamers can be added to incubate with target bacteria without amplification or purification.

FACS analysis was carried out using *E. coli* DH5 α *pgl*, and *C. jejuni* ATCC 33560 as previously described in section 2.5. Aptamers were used at concentrations between 100-200 pmole in 500 μ l total volume. Aptamers were

allowed to incubate with bacteria for approximately 1 hour, then were washed once, then subjected to flow cytometry.

Shift in fluorescence from background was measured. Three incubations were run per aptamer, with each incubation measured in triplicate. The 9 readings were averaged to obtain a single value. Random DNA oligonucleotide library shifts were used to subtract non-specific binding values from the average value per aptamer.

2.9 Kd Analysis of Selected Individual Aptamer Sequences via Flow Cytometry

The Beckton Dickinson FACScan was used at the same settings as previously indicated for Flow Cytometry of Aptamer Pools (section 2.5).

FACS analysis was carried out using approximately 1×10^7 cells/ml for *E. coli* DH5 α *pgl*, and $1-5 \times 10^7$ cells/ml of *C. jejuni* ATCC 33560. Aptamers were used at concentrations ranging from 0 – 200 nmole in 500 μ l total volume. Aptamers were allowed to incubate with bacteria for approximately 1 hour, then were subjected to flow cytometry analysis. Cell plus aptamer incubations were not washed before Kd analysis, as there was only 1 aptamer sequence being assessed, and Kd measurements represent both bound and unbound sequences. The measurements provide an estimate of the ability of an aptamer to remain bound to its target. The dissociation constant, Kd, is inversely indicative of aptamer binding affinity. To calculate the dissociation constant (Kd), the various concentrations of the synthesized fluorescein-labeled aptamers (0-200 nM) are plotted against their corresponding fluorescence intensities. The resulting curve was sigmoid shaped, with a linear portion followed by a plateau, and the Kd is considered to be the concentration value (on the x-axis) that lies at half the value of the maximum binding (maximum fluorescence intensity value, on the y-axis) at the plateau (Figure 2.9.1).

All incubations were run in triplicate. Each of the 3 incubations was measured 3 times. A single averaged value from all 9 trials was obtained for each concentration of the aptamer. Fluorescence Intensities above background (both with and without subtraction of library values of non-specific binding) were plotted against aptamer concentration.

Summary of SELEX sets with *E. coli* DH5 α (p*gl*) & *C. jejuni* ATCC 33560

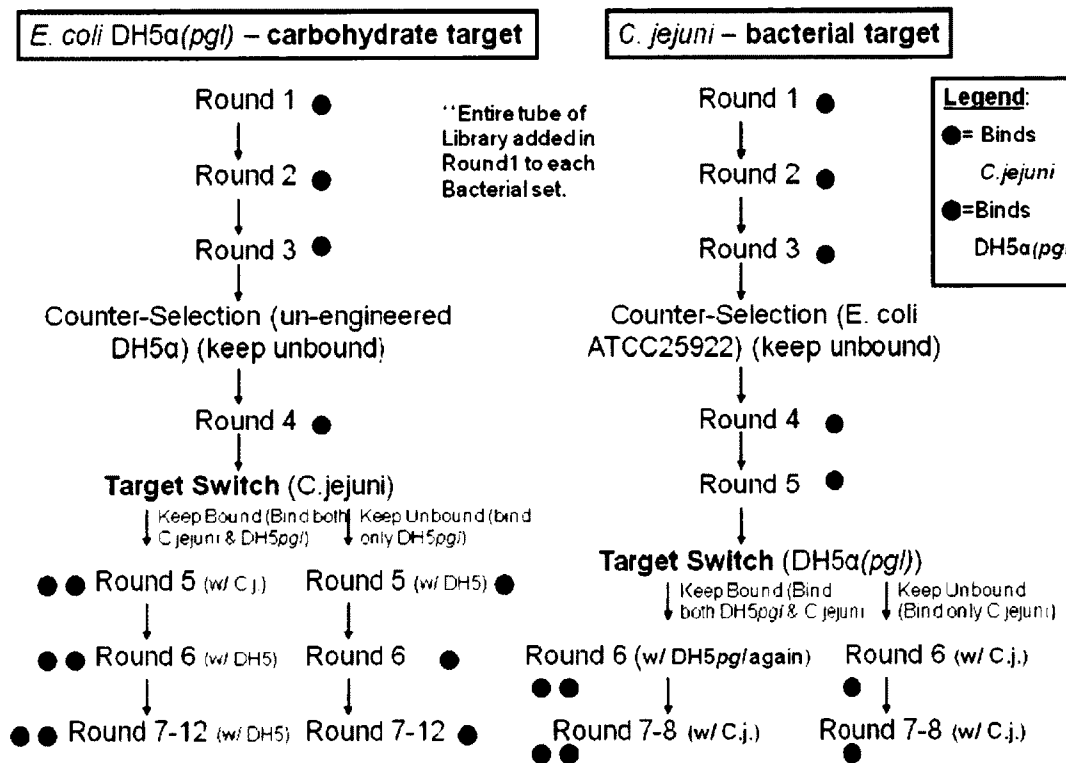


Figure 2.3.1 Illustration of Cell-SELEX method used, including counter-selection and target switch steps. Note: C.j. = *C. jejuni*; DH5 = *E. coli* DH5ap*gl*; DH5p*gl* = *E. coli* DH5ap*gl*. Red circles indicate that the aptamer pools in that round should only bind *C. jejuni* (denoted as the “C” set in the *C. jejuni* SELEX set); Blue circles indicate that the aptamer pools in that round should only bind *E. coli* DH5ap*gl* (denoted as the “E” set in the *E. coli* DH5ap*gl* SELEX set); Red and blue circles together, indicate that the aptamer pools in that round should bind both *C. jejuni* and *E. coli* DH5ap*gl* (denoted as the “E+C” set in the *C. jejuni* SELEX set, or “C+E” set in the *E. coli* DH5ap*gl* SELEX set). For example, aptamers in pool “8C” are from round 8 of the *C. jejuni* SELEX set, and are expected to bind only *C. jejuni*. Aptamers in pool “10C+E” are from round 10 of the *E. coli* DH5ap*gl* SELEX set, and should bind both *E. coli* DH5ap*gl* and *C. jejuni*. A synthetic aptamer 10C+E-30 is the 30th aptamer clone sequenced from the latter round and set.

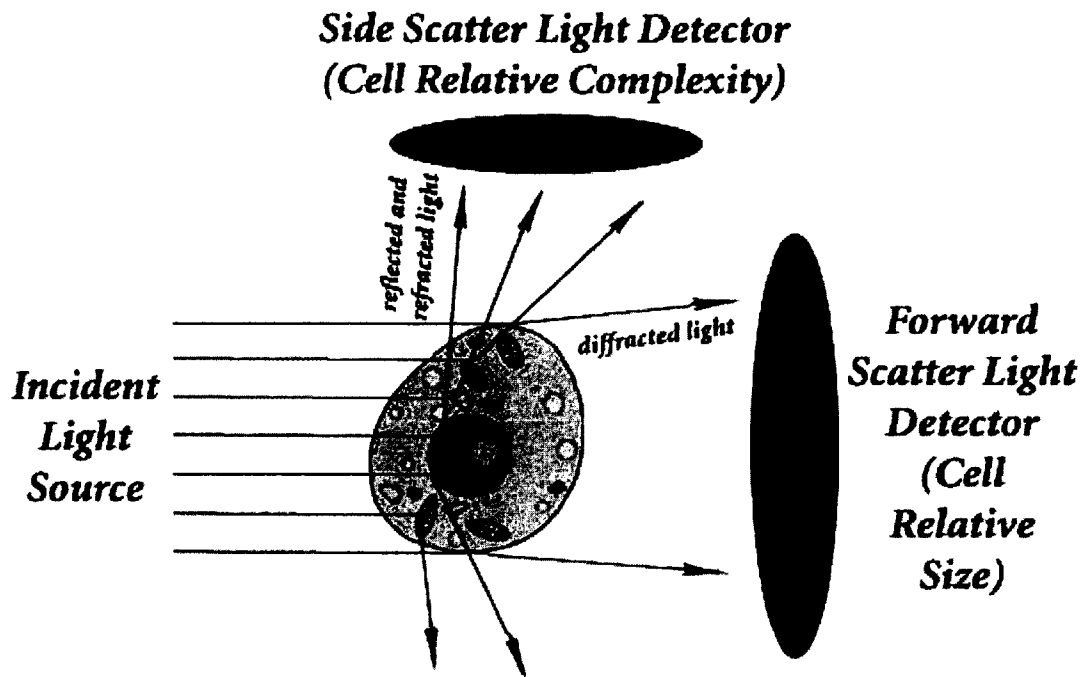


Figure 2.5.1 Illustration of side and forward scatter created by cell size, shape, and complexity. Image Source: University of Alberta Faculty of Medicine & Dentistry Flow Cytometry Facility: <http://flowcytometry.ualberta.ca/Analysis.htm> (last updated April 19, 2011).

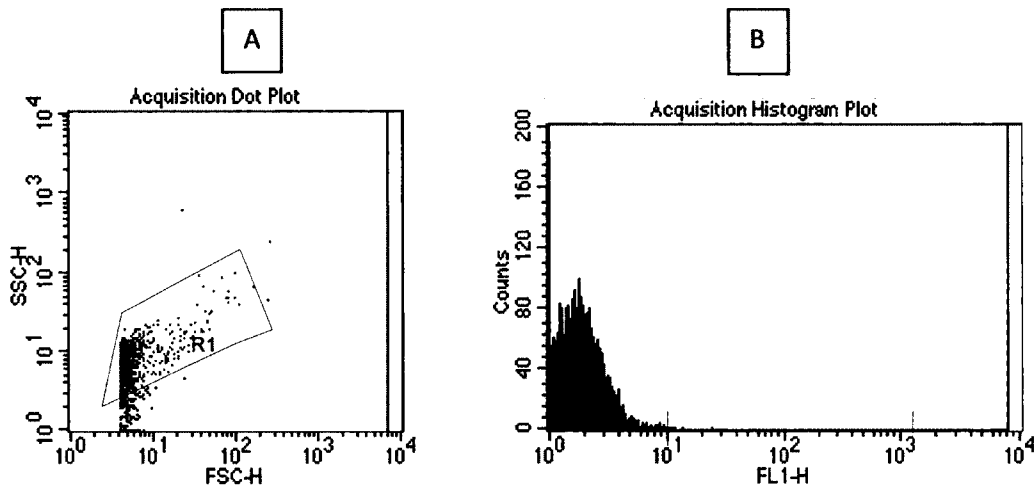


Figure 2.5.2 Sample FACScan histogram plot of frequency of fluorescent events versus intensity of fluorescence. A) The plot shows side scatter versus forward scatter, as well as creation of the gates, within which fluorescence from target cells can be detected. B) Controls containing target cells only are used to set the gates or limits for fluorescence detection. Any shift in fluorescence intensity is visible as a shift from background of cells only.

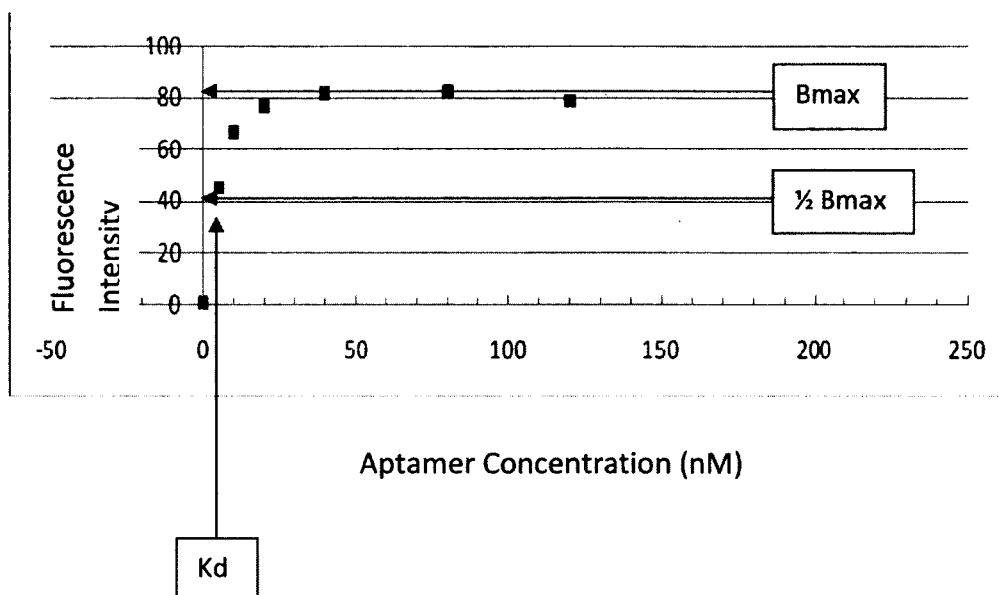


Figure 2.9.1 Example of a typical binding curve , further modified to show approximate locations of B_{max} , $\frac{1}{2} B_{max}$, and K_d .

Chapter 3. Results and Discussion

3.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) conditions were optimized to amplify the 80-nt aptamer sequences and to minimize any non-specific amplification. It was found that DNA template concentrations of 10 and 100 ng amplified well at 69°C with DMSO added (see Figure 3.1.1 panel A). Using the same conditions, 10 µl of SELEX eluate, in 10-50 reactions, was amplified at 13-18 cycles (Figure 3.1.1 panel B). When electrophoresed, these volumes were found to produce similar band intensities to those produced using 10 and 100 ng of template. Once amplified, the DNA aptamers in the eluate were purified to remove contaminants, then subjected to spectrophotometric analysis of DNA concentration, and used in the next round of SELEX.

PCR cycle number was reduced to 13-14 cycles when amplifying the DNA pool collected from the unbound fraction during the counter selection step. This is because more DNA sequences are present in the unbound fraction, and thus a higher concentration of template is present in this solution. Decreasing cycle number to 13-14 cycles partly compensated for this higher concentration without loss in yield.

3.2 SELEX

Twelve rounds of SELEX were performed for the engineered *DH5apgl* target cells with un-engineered *DH5α* cells used in counter selection. Eight rounds of SELEX were performed for the *C. jejuni* target cells with *E. coli* ATCC 25922 cells used in counter selection, as detailed in section 2.3.

During SELEX, an entire tube of random 80 nt DNA oligonucleotide library was added to target cells in the 1st round, or 100 pmole of amplified bound eluate from the previous round is added to target cells in all successive rounds of SELEX. Following incubation of cells with the DNA library (DNA pool of sequences), the cells were washed to remove unbound sequences. The wash solutions were collected and unbound sequences amplified by PCR to test how many washes were needed to remove unbound and weakly bound sequences from the cells. Figure 3.2.1A shows that the unbound sequences are predominantly present in the first wash. PCR amplifications from the solutions of the second and third washes show much weaker bands of the 80-nt sequences. Three washes were enough to significantly reduce the amount of unbound DNA sequences (Figure 3.2.1A). Therefore, 3 washes were carried out prior to heat denaturation of the bound sequences from target cells.

The bound aptamers were heat denatured once, and eluted from the cells three times. Three eluates were collected separately and PCR amplified to test the amount of the eluted aptamers. Figure 3.2.1B shows that all three elutions contain amplifiable aptamers. In principle, aptamers with stronger binding affinity are expected to be present in successive elution solutions, i.e., aptamers with weaker binding would be eluted first and aptamers with stronger binding would be eluted later.

The results of amplifiable DNA sequences in all three elutions (Figure 3.2.1B) suggest that the bound DNA sequences were not completely eluted off from the cells by heat denaturation and buffer elution. Indeed, it was found that the cells remaining in the tube after heat elution still contained an amplifiable amount of aptamers (Figure 3.2.1C). Therefore, the residual cell pellets (and fragments) were also kept and amplified, along with the other cell-bound eluates.

In counter selection and target switch steps, non-target cells for each set were incubated with DNA pools from preceding rounds. The cell-bound sequences were less favourable because these represent undesirable binding to non-target cells. The unbound fraction was kept, PCR amplified, and used for the next round of selection against the target cells. The use of alternating positive selection and counter selection steps is intended to increase the specific binding of the aptamers to the target of interest and to minimize non-specific binding.

3.3 Detection of Carbohydrate via Western Blot

Cleaved carbohydrate extracts were between 14.4 and 21.1 kDa, as found in the work of Feldman et al (25), from which the protocol was taken. Over-expression of carbohydrate was not detected in non-target bacteria via Western blotting, relative to the engineered *DH5αpgl* cells. As seen in Figure 3.3.1, a significant amount of carbohydrate is detected in the engineered cells, supporting that they are a good target cell for selection against a carbohydrate. It also appears that target cells are consistently producing a large amount of carbohydrate during each round of SELEX assessed.

3.4 Flow Cytometry of Aptamer Pools

After SELEX, aptamer pools from each round of selection were subjected to flow cytometry analysis. Pools that bound to target cells with the highest shift in fluorescence from background were assumed to exhibit stronger binding, and thus contain the most desirable aptamers. Figure 3.4.1 shows results from flow cytometry analyses of *DH5αpgl* cells incubated with fluorescently labelled aptamer pools from 12 rounds of SELEX. In a parallel set of experiments, DNA library was fluorescently labelled and used as a control for the flow cytometry analysis. When comparing the DNA aptamer pools with the DNA library, the net

increase in fluorescence intensity (Figure 3.4.1A) represents the enhanced binding of the aptamer pool to the target cells. In the selection of aptamers against the *E. coli* DH5 α ggl cells (carbohydrate target), aptamer pools from round 8C+E show a higher shift in fluorescence from background (Figure 3.4.1A) with library subtraction, aptamer pools from rounds 10C+E and 12C+E show higher fluorescence intensity (Figure 3.4.1B) without library subtraction. Since the subtraction from library produced inconsistent results, both pools 10C+E and 12C+E were selected for cloning, as they had the highest shifts before subtraction.

From the selection of aptamers against *C.jejuni* (Figure 3.4.2 A and B), the aptamer pool obtained from round 4 had the highest shift in fluorescence above background. A counter selection step was introduced between round 3 and 4. It is desirable to obtain aptamers from rounds after the counter selection because the counter selection step should increase specificity. Theoretically, affinity should also increase with each round of selection. Therefore, the latest rounds with high shifts in fluorescence intensity are more desirable. Round 8C was chosen for cloning, as it had a positive shift in fluorescence intensity, without library subtraction, and was the latest round available.

The target switch step is assumed to enhance selection for aptamers that will bind both the *C. jejuni*, and the *C. jejuni* surface carbohydrate over-expressed on *E. coli* DH5 α ggl cells. For *C. jejuni* selections, post-target switch rounds are round 6, 7, or 8. The C+E set of *E. coli* DH5 α ggl selections were chosen as they were more likely to bind both *C. jejuni* and its surface carbohydrate. The C set of *C. jejuni* selections was chosen as it was most likely to bind actual *C. jejuni*. Aptamers that bind any target on *C. jejuni* cells specifically, or that bind a surface carbohydrate are considered equally desirable in utility.

Non-specific binding of sequences in the random DNA oligonucleotide library would be expected to be roughly the same at each reading. However, the values of fluorescence intensity without library subtraction do not always follow the same relative trends as those with library subtraction. For example, in Figure 3.4.1 A and B, rounds 1 to 7 follow similar trends of increase or decrease both with and without library subtraction. Yet in round 8, the E set has slightly higher fluorescence than the C+E set without library subtraction, whereas the C+E set has much higher fluorescence than the E set with library subtraction. Also in round 6 for the E set, fluorescence is similar to rounds 5 and 7 without library subtraction, but is lower than the same rounds with library subtraction. This suggests a lack of reliability of the library subtraction, or at least of the batch of library used for those sets. Therefore, the values without library subtraction were used to select round pools for further analysis. Therefore, round pools 10C+E and 12C+E of the *E. coli* DH5 α ggl selections, and 8C of the *C. jejuni* selections were subjected to cloning and sequence analysis.

Also, 3 incubations (in separate tubes) of cells and DNA library (sequence pools) were performed for each round of SELEX. Each incubation was measured 3 times during flow cytometry. Figures 3.4.1 and 3.4.2 for both *E. coli* DH5 α ggl and *C. jejuni* selections, demonstrate large standard deviations in measurements that were averaged over the 3 incubations for each pool. However, multiple measurements within the same incubation show smaller standard deviations in averaged measurements when pools are separated into the 3 incubations (Figure 3.4.3 A and B). Though for both sets, there are smaller deviations for rounds 1-8 than for rounds 9-12. Therefore, although there is low variability in measurements for each incubation, there can be much higher variability between incubations. This is despite the fact that each incubation used target cells from the same suspension, aptamers from the same batch of purified SELEX eluate, and were performed at the same time under the same conditions with the same reagents.

3.5 Cloning & Sequence Analysis

Forty plasmids were extracted for rounds 10C+E and 12C+E each, and 60 plasmids were extracted for round 8C. Of these 40 plasmids, 26 of 40 had 80bp inserts post-digestion for 10C+E, and 22 of 40 had 80bp inserts for 12C+E. Thirty plasmids were initially extracted for 8C, after cloning inserts that were only PCR purified by the MinElute kit. These plasmids had a very low yield for 80bp inserts, with only 6 of 30 plasmids having an 80bp insert. Most inserts appeared to be misamplification after restriction digestion and visualization on a 10% polyacrylamide gel (Figures 3.5.1 and 3.5.2).

However after gel purification of only the 80bp band for 8C, followed by cloning, another 30 plasmids were extracted from clones/colonies. Of these 30 plasmids, 1 had no insert, and all 29 remaining plasmids had 80bp inserts after restriction digestion and electrophoresis. Therefore, gel purified aptamers produced a higher yield of clones that can progress to the sequencing step, than non-gel purified aptamers when misamplification is present.

Twenty six, 22, and 35 sequences were sent for sequencing from the 10C+E, 12C+E, and 8C pools respectively. Of these sequences, 29, 16, and 17 were useable for 10C+E, 12C+E, and 8C respectively (see Table 3.5.1). Useable sequences are readable sequences that are 80nt long, or are only missing nucleotides from the primer regions at either end, which are conserved sequences among each aptamer. These sequences are also derived from a single colony, producing a single sequence. For example, on some occasions plasmids were extracted from mixed colonies that appeared to be single colonies, yet the sequencing results showed that more than one sequence was present. Thus, more than one plasmid was extracted, as a plasmid will generally only have one insert. Figure 3.5.3 is representative of a typical electropherogram of a single insert, received from the sequencing facility.

All useable sequences were entered into the Integrated DNA Technologies Inc OligoAnalyzer 3.0 (Appendix Tables A.1, A.2, and A.3) online tool, to assess aptamer stability via prediction of secondary structure and value of Gibbs Free Energy. Twenty six sequences with a desirable combination of Gibbs Free Energy and secondary structure were chosen from the 8C, 10C+E, and 12C+E pools and sent for synthesis with a fluorescent label (fluorescein). These sequences (Appendix Table A.4) were subsequently tested, using flow cytometry analysis, for their binding to the target cells.

It is expected that any sequence with high affinity for its target would be selected for, over sequences with lower affinity. Therefore, many more copies of the high affinity aptamers would be present than those with lower affinity. Thus, if a sequence was seen multiple times in sequencing results, it would appear that this sequence is likely to be of higher affinity for the target cells than others, and therefore would be worthwhile to synthesize and further analyze specificity and binding affinity via flow cytometry. Generating DNA aptamers against the desired targets was likely to yield less aptamers with high specificity and affinity than would a traditional target. Thus, it was decided that at least 100 clones should be picked and extracted, to enhance the likelihood of detecting recurring sequences. Sequences 12C+E-27 and 12C+E-30 had the same sequence, and 10C+E-28 and 10C+E-31 had the same sequence. In both cases the same sequence appeared twice, out of 21 inserts that were sequenced and interpretable. These were the only sequences that appeared more than once during sequence analysis. 12C+E-30 and 10C+E-31 were therefore sent for synthesis irrespective of ΔG value or secondary structure. The work of Hamula et al (31) involving generation of DNA aptamers against a protein target on the surface of *L. acidophilus* also showed that one of the most promising aptamers was one that appeared multiple times in sequence analysis. 10C+E-31 and 12C+E-30 were analyzed for binding to *E. coli* DH5 α agl cells (Figure 3.6.1 B and C). Unfortunately, due to delays in receiving

synthetic aptamers from IDT as well as time constraints in the lab, they could not be subjected to K_d analysis. This analysis would therefore need to be performed as part of future work.

3.6 Flow Cytometry of Individual Aptamer Sequences & K_d Analysis

Results of flow cytometry analysis of shift of fluorescence from background are shown in Figure 3.6.1 A-C. Twenty four synthetic aptamer sequences were successfully subjected to flow cytometry analysis: 8 of the 8C set, 8 of the 10C+E set, and 6 of the 12C+E set. Of the 8C set, 6 were tested against *E. coli* DH5 α gl as an attempt to gauge whether they may also bind the surface carbohydrate in addition to *C. jejuni* itself. These results show that many of these selected aptamer sequences are able to bind to *C. jejuni* (Figure 3.6.1A) or to the engineered *E. coli* DH5 α gl cells (Figure 3.6.1 B and C). Several aptamer sequences from round 8C, including 8C-15complement, 8C-23, and 8C-23complement, show a 50% net increase in gated fluorescence intensity after subtraction from library and background, when tested against the target *C. jejuni* cells. This increase is comparable to the reported values from the previous successful selection of high-affinity aptamers against *L. acidophilus* (31, 32). These results suggest good binding affinity of the selected aptamer sequences 8C-15complement, 8C-23, and 8C-23complement for the target *C. jejuni* cells.

Since the "C" set represents the "unbound" fraction from the target switch step (target switched from *C. jejuni* to *E. coli* DH5 α gl), it is expected that most of the sequences in the "C" set will bind to *C. jejuni* and not *E. coli* DH5 α gl. The "E+C" set however, should contain mostly sequences that bind both organisms. The results for round 8C (Figure 3.6.1 A and C) show that in addition to binding the *C. jejuni*, several aptamers were found to bind to *E. coli* DH5 α gl as well. 8C-25complement and 8C-23complement showed positive shifts in fluorescence intensity with both *C. jejuni* and *E. coli* DH5 α gl. 8C-42complement, 8C-

48complement, 8C-49complement, 8C-59complement were only incubated with *E. coli* DH5 α pl, but showed positive shifts in fluorescence intensity. This may suggest that wash steps are subject to error. This is not surprising, as it was expected that some unbound sequences may be left in the bound fraction, and vice versa.

It is expected that the same sequences and structures, should produce similar binding. For example, forward and reverse sequences of the same aptamer, that produce the same predicted secondary structure, would be expected to have similar binding abilities to target cells. Aptamer pairs 8C-23 and 8C-23complement have the same predicted secondary structures (Table A.1) and similar ΔG values (8.2 and 7.63 kcal/mol), and also produced similar shifts in fluorescence intensity (49.1 and 50.0%) when bound to *C. jejuni* (Figure 3.6.1A). Of interest, however, is that 8C-15 and 8C-15complement have very different shifts in fluorescence from background (Figure 3.6.1 A). 8C-15complement produced a shift in fluorescence (Figure 3.6.1A) of approximately twice as much as 8C-15 (7.7% and 49.2% with library subtraction, and 47.3% and 88.8% without library subtraction). The 2 structures have almost identical ΔG values (6.05 and 6.04 or 5.84 kcal/mol) and predicted structures (Table A.1). However 8C-15complement has 2 predicted structures, the most favourable of which is not similar in structure to that of 8C-15. The 2nd most favourable structure is identical to 8C-15. The difference in shifts produced may indicate that formation of the 1st structure is indeed favoured. It may also, though less likely, suggest an error may have occurred during the measurements of fluorescence intensity. Also worth noting, is that 8C-15complement, 8C-23, and 8C-23complement had similarly high shifts in fluorescence intensity (Figure 3.6.1A) and also had similar predicted structures, in that they were composed mostly of stem and loop structure with minimal branching. These results may suggest that this type of structure may be favourable for binding to the target, and more aptamers with this type of structure could be investigated in future work.

Attempts were made to test the binding of as many aptamer sequences as possible to the engineered *E. coli* DH5 α pl cells. As synthetic aptamers are expensive to produce, only a select few sequences could be synthesized and analyzed at a time. Once those sequences were assessed, if results were not favourable, more sequences could be synthesized. Synthetic sequences take on average 3 weeks to 1 month to receive from the supplier (IDT, California, USA). Therefore, the process of synthesis and analysis can be quite time consuming. Batches of individual aptamer sequences were synthesized and each assessed for their binding. In practice, if flow cytometry analyses showed that increases in fluorescence intensity above background were very low for a given batch of aptamer sequences, another order of new aptamer sequences could be placed. While waiting for the new sequences to arrive, further characterization by flow cytometry could be performed on the more favourable sequences of the previous batch.

Attempts were also made to test the binding of individual aptamer sequences to *C. jejuni* cells. However, results were not consistent or conclusive due to problems with cell viability and time constraints in the lab. After a certain period of time with success in growing *C. jejuni*, its growth became less successful. This also coincided with the -80°C freezer failing to maintain adequate temperature around this time period. This could have led to a decreased viability of the frozen stock. Upon analysis of many aptamers with *C. jejuni* as a target, flow cytometry plots of side versus forward scatter showed most cells were detected in the gated regions where dying, dead, or less healthy cells appear. Therefore, these binding assays may not be as reliable in determining binding to surface molecules for this target, as compared to readings of other target cells which appeared healthier.

From round 10 of the selection against *E. coli* DH5 α pl, the synthetic aptamer sequence 10C+E-31 had 31.7% gated fluorescence above background without

library subtraction, and only 2.8% gated fluorescence above background with library subtraction (Figure 3.6.1B). From round 12 of the *E. coli* DH5 α gl selections, 12C+E-30 had 19.2% gated fluorescence above background without library subtraction, and -19.4% gated fluorescence above background with library subtraction (Figure 3.6.1C). This is a rather low value, and may suggest that the actual aptamer is the complement strand of this sequence, which would need to be synthesized and tested as well. The synthetic aptamer sequence 12C+E-12 had the highest increase in fluorescence (44.0% gated fluorescence) above background (without library subtraction) (Figure 3.6.1B). Flow cytometry analyses using two other aptamer sequences, 12C+E-25 (29.1% gated fluorescence) and 12C+E-26 (37.1% gated fluorescence) were performed. The aptamers 12C+E-25 and 12C+E-26 had been received from the supplier sooner and thus analyzed sooner than 12C+E-12. For the same reason, 10C+E-31 and 12C+E-30 were not subjected to Kd analysis either. Prior to obtaining the results in Figure 3.6.1A, the flow cytometry analyses with aptamer 12C+E-26 showed the highest fluorescence increase (Figure 3.6.1 B). Therefore, 12C+E-26 was also subjected to flow cytometry analysis to measure its specificity for other bacteria as well (Figure 3.6.2).

Shift in fluorescence from background was measured for synthetic aptamer 12C+E-26 against *S. pyogenes* M1, *S. bovis*, *E. coli* K12, *E. coli* DH5 α , *E. coli* DH5 α gl, *C. jejuni* ATCC33560, and *H. pylori* A158 (Figure 3.6.2). Cross-reactivity was observed with several of these bacteria, yet the highest shifts in % Gated Fluorescence (without library subtraction) were for *C. jejuni* (74.6% gated fluorescence), *H. pylori* A158 (70.01%), and *E. coli* DH5 α gl (68.2% and 67.2%), of which the *C. jejuni* and *E. coli* DH5 α gl are the desired binding targets. However, several of the other bacteria exhibited similar shifts including *S. pyogenes* M1 (53.9%), *S. bovis* (63.6%), *E. coli* K12 (66.5%), and *E. coli* DH5 α (61.7%). With library subtraction, though, *S. pyogenes* M1 (11.1%) has the highest shift and *S. bovis* had the second highest shift (9.8%). Since the DNA

aptamer library consists of several random sequences, perhaps the 2 *Streptococcus* species express more of a certain target that attracts strong or non-specific binding. Positive charges, ionic bonds, and aromatic rings also influence strong binding (42), which may be a factor as well.

For the *E. coli* DH5 α set, the "E" set represents the "unbound" fraction from the target switch step (target switched from *E. coli* DH5 α to *C. jejuni*), most of the sequences in the "E" set should bind only to *E. coli* DH5 α . The "E+C" set should contain mainly sequences that bind both organisms. Figure 3.6.2 shows that in addition to binding the *E. coli* DH5 α , 12C+E-26 was also capable of binding to *E. coli* DH5 α as well as *C. jejuni*. This suggests that counter-selection may need improvement – possibly due to wash steps – since *E. coli* DH5 α was used in counter-selection. It also provides some preliminary evidence that the target-switch step may have been effective, as the aptamer exhibited similar shifts in fluorescence intensity for both *C. jejuni* and *E. coli* DH5 α .

K_d analysis was performed on the random DNA aptamer library, 12C+E-26, and 12C+E-25. K_d was estimated as the concentration of aptamer at half the value of B_{max} (here, B_{max} is the maximum level of fluorescence). Using the curve generated without library subtraction, and its data points, K_d ranges were estimated. The value of B_{max} was divided by 2, and the aptamer concentration ranges with fluorescence intensities nearest this value were given as the K_d range. For 12C+E-26, B_{max} was 85 % gated fluorescence intensity. Therefore ½ B_{max} was 42.5% gated fluorescence. Aptamers at 2 nM had a gated fluorescence intensity of 37.2 %, and at 4 nM had a gated fluorescence intensity of 54.0%. K_d was therefore estimated to be between 2 and 4 nM. For 12C+E-25, B_{max} was 77% gated fluorescence intensity. K_d was estimated to be between 1 and 10 nM, as ½B_{max} was 38.5% gated fluorescence, and the gated fluorescence intensity of 10 nM aptamers was 69.7%. The % gated fluorescence intensities were not acquired for aptamer concentrations between 1 and 10 nM for this aptamer. For

the library, B_{max} was 82.2% and ½B_{max} was 41.1% gated fluorescence intensity. K_d was estimated to be between 0 and 5 nM, as 5 nM was the lowest concentration measured and had a gated fluorescence of 45.3%.

Variable fluorescence intensities were also noted when intensities rose above 60% Gated Fluorescence, during K_d analysis (Figure 3.6.3). Here, intensities no longer have a direct relationship with the aptamer concentration. For example, when aptamer-cell incubations are washed prior to flow cytometry, the fluorescence intensity typically increases as aptamer concentration increases, until a plateau is reached. However, unwashed incubations appear to be much more variable. For example, in Figure 3.6.3A, at 120 nM (78.6% gated fluorescence) and 200 nM (69.8%) of library, the % gated fluorescence shift goes down from 81.6% at 40 nM and 82.2% at 80 nM, rather than increasing, or holding steady at plateau. These observations of varying signal intensity may indicate a limitation of the flow cytometer itself, or of the flow cytometry assay parameters. Since cells have many potential binding sites, compared to purified molecular targets, the library may be non-specifically binding to a plethora of potential sites compared to the aptamers selected. This may cause the library shifts in fluorescence intensity to rise above those of aptamers selected, causing a false negative reading after library subtraction.

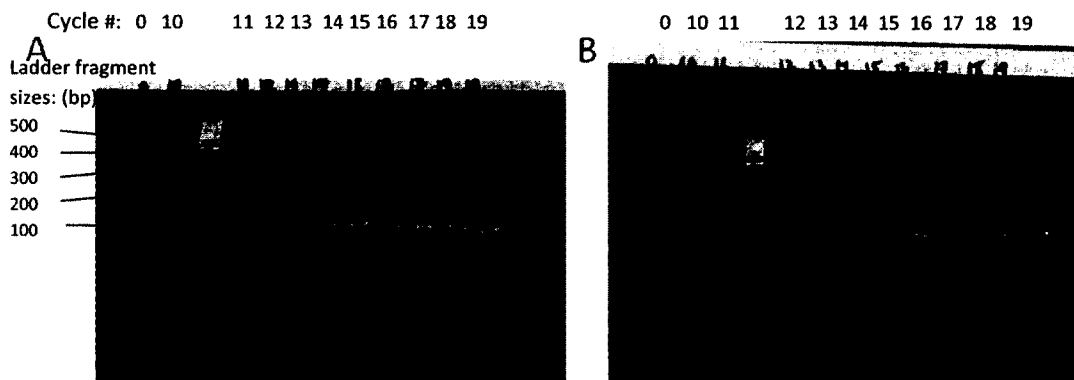


Figure 3.1.1. Gel photograph of 10 ng (A) and 100 ng (B) DNA aptamer library template, amplified at an annealing temperature of 69°C, for 10-19 cycles, with the addition of DMSO. Cycle number is written along the top of each gel over the appropriate well. Aptamers are 80bp when amplified, and fall just below the 100bp band of the 100bp ladder.

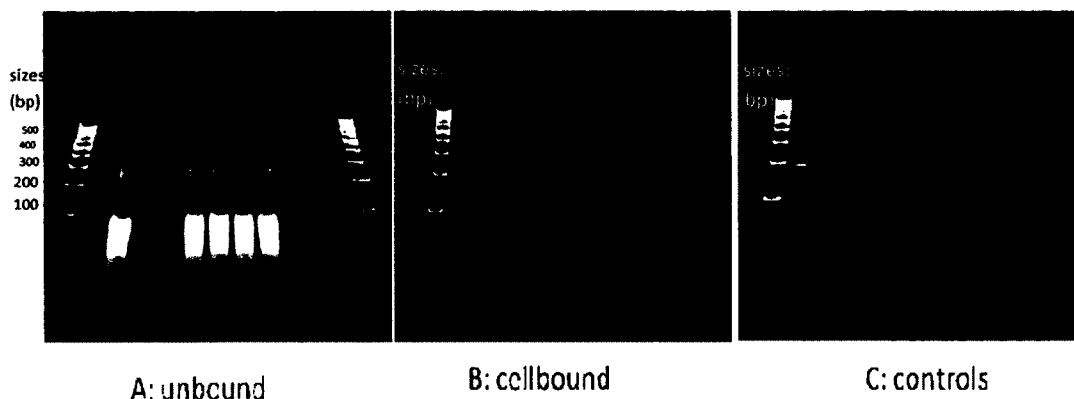


Figure 3.2.1. Gel photographs showing amplification of round 1 of DH5*apgl* SELEX unbound (Gel A) and bound (Gel B) aptamer eluates, as well as cell eluates (Gel C). Loading order is as follows: (CB = cell bound aptamer eluate; UB = Unbound eluate; 1xBB = 1x Binding Buffer)

Gel A: Empty well, Fermentas ladder, NEB ladder, Unbound eluate wash 1 (pool of 4 incubations together), Unbound eluate wash 2 pool, Unbound eluate wash 3 pool, Unbound eluate wash 1 incubation 1, Unbound wash 1 incubation 2, Unbound wash 1 incubation 3, Unbound wash 1 incubation 4, Unbound wash 2 incubation 1, Unbound wash 2 incubation 2, Unbound wash 2 incubation 3, Fermentas ladder, Empty well.

Gel B: E, Fermentas ladder, NEB ladder, Empty well, CB wash1, CB wash2, CB wash3, Empty well (ladder in next well leaked into it), NEB ladder, PCR negative control Pool (4 negatives pooled), PCR negative control Pool (from master mixes w/ 1xPCR buffer and 1xBB added to negative control tubes instead of sterile water), CB wash1, CB wash2, CB wash3, CB wash1-3 Pool.

Gel C: Empty well, ladder, NEB, leftover cells (from CB eluate): UB wash1 (incubation 1), UB wash1 (incubation 2), CB wash1 (incubation 1), CB wash1(incubation 2), Cells only (No Aptamers): UB wash1, CB wash1, No Cells/No Aptamers (LB+Cm only): UB wash1, CB wash1, Empty well, CB (leftover cells) wash1-3 Pool, Empty well, Empty well.

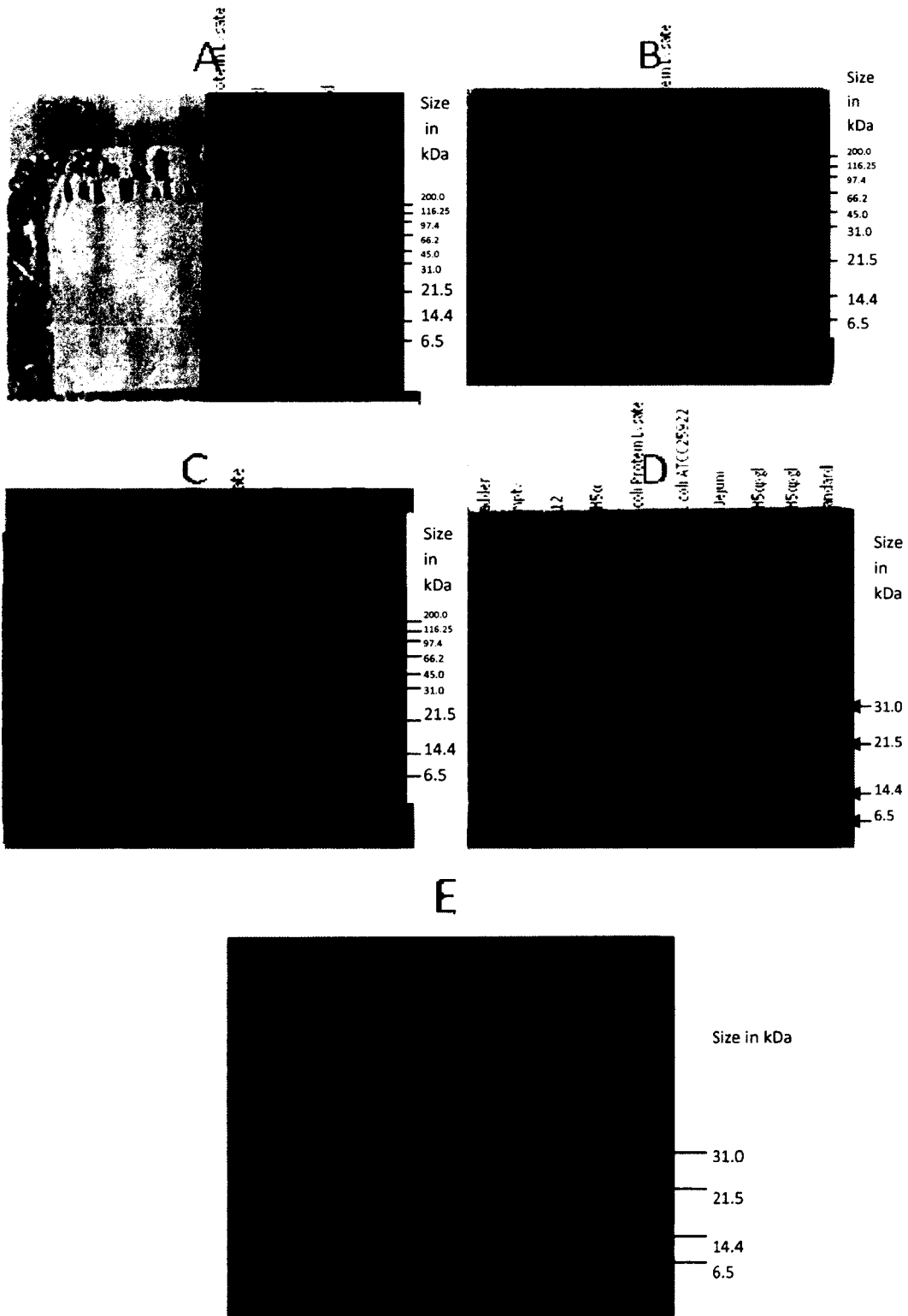


Figure 3.3.1. Images of Western blot membranes containing fractions of *E. coli* K12, *E. coli* DH5 α , *E. coli* ATCC 25922, *C. jejuni* ATCC33560, *E. coli* DH5 α agl, and *H. pylori* patient strains A158, A236, and A212 after protein digestion. Membranes were probed for presence of carbohydrates with the primary biotinylated soybean agglutinin antibody and the secondary anti-biotin antibody. Loading orders on membranes are as follows: NOTE: E = empty well.

Gel A: Round 2: Developed film has been laid over the un-stained membrane (Ponceau stain not done). Loading order: 5ul of size marker, 10ul of size marker, E, *E. coli* K12, *E. coli* DH5 α , *E. coli* protein lysate, *E. coli* DH5 α agl, E, biotinylated size marker, E.

Gel B: Counter-Selection (between Round 3&4): Developed film has been laid over the Ponceau Red stained membrane. Loading order: E, 5ul size marker, E, *E. coli* K12, *E. coli* DH5 α , *E. coli* protein lysate, E, *E. coli* DH5 α agl, biotinylated size marker, E.

Gel C: Target Switch Round: Developed film has been laid over the Ponceau Red stained membrane. Loading order: E, 10ul size marker, E, *E. coli* K12, *E. coli* DH5 α , *E. coli* protein lysate, *C. jejuni* ATCC33560, *E. coli* DH5 α agl, biotinylated size marker, E.

Gel D: Round 6 and 8: Developed film has been laid over the Ponceau Red stained membrane. Loading order of Image D: 5ul size marker, E, *E. coli* K12, *E. coli* DH5 α , *E. coli* protein lysate, *E. coli* ATCC25922, *C. jejuni* ATCC33560 (from Round 1 of *C. jejuni* SELEX), *E. coli* DH5 α agl (from Round 6), *E. coli* DH5 α agl (from Round 8), biotinylated size marker.

Gel E: Round 9 and 10: Developed film has been laid over the Ponceau Red stained membrane. Loading order of Image E: E, 5ul size marker, E, *C. jejuni* ATCC33560 (from Round 3 of *C. jejuni* SELEX), *H. pylori* A158, *H. pylori* A236, *H. pylori* A212, *E. coli* DH5 α agl (from Round 9), *E. coli* DH5 α agl (from Round 10), biotinylated size marker.

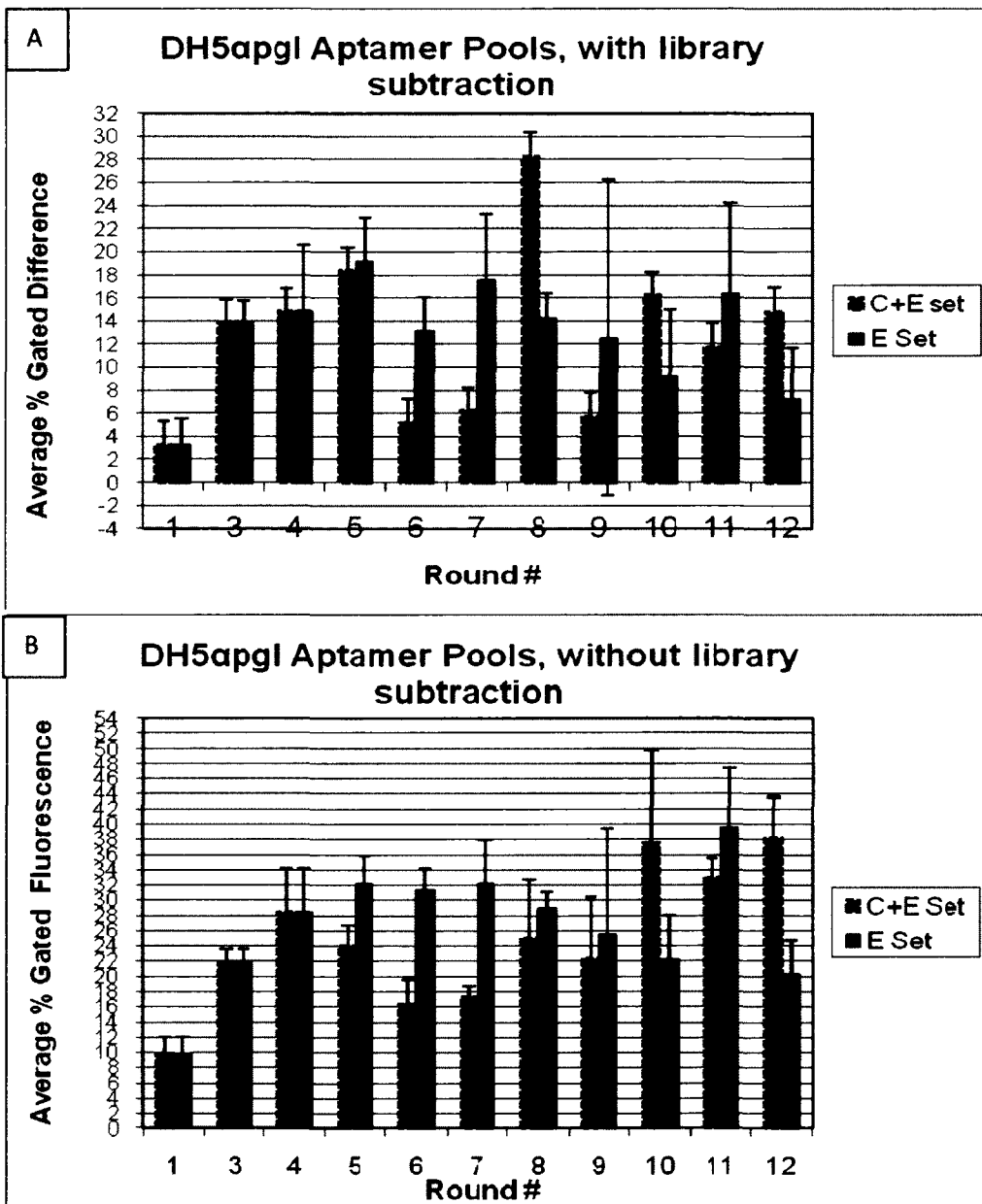


Figure 3.4.1. Results of flow cytometry analysis of aptamer pools from rounds 1-12 for *E. coli* DH5apgl SELEX before the target switch step, and C+E and E sets after the target switch step. Results are shown both with (A) and without (B) library subtraction.

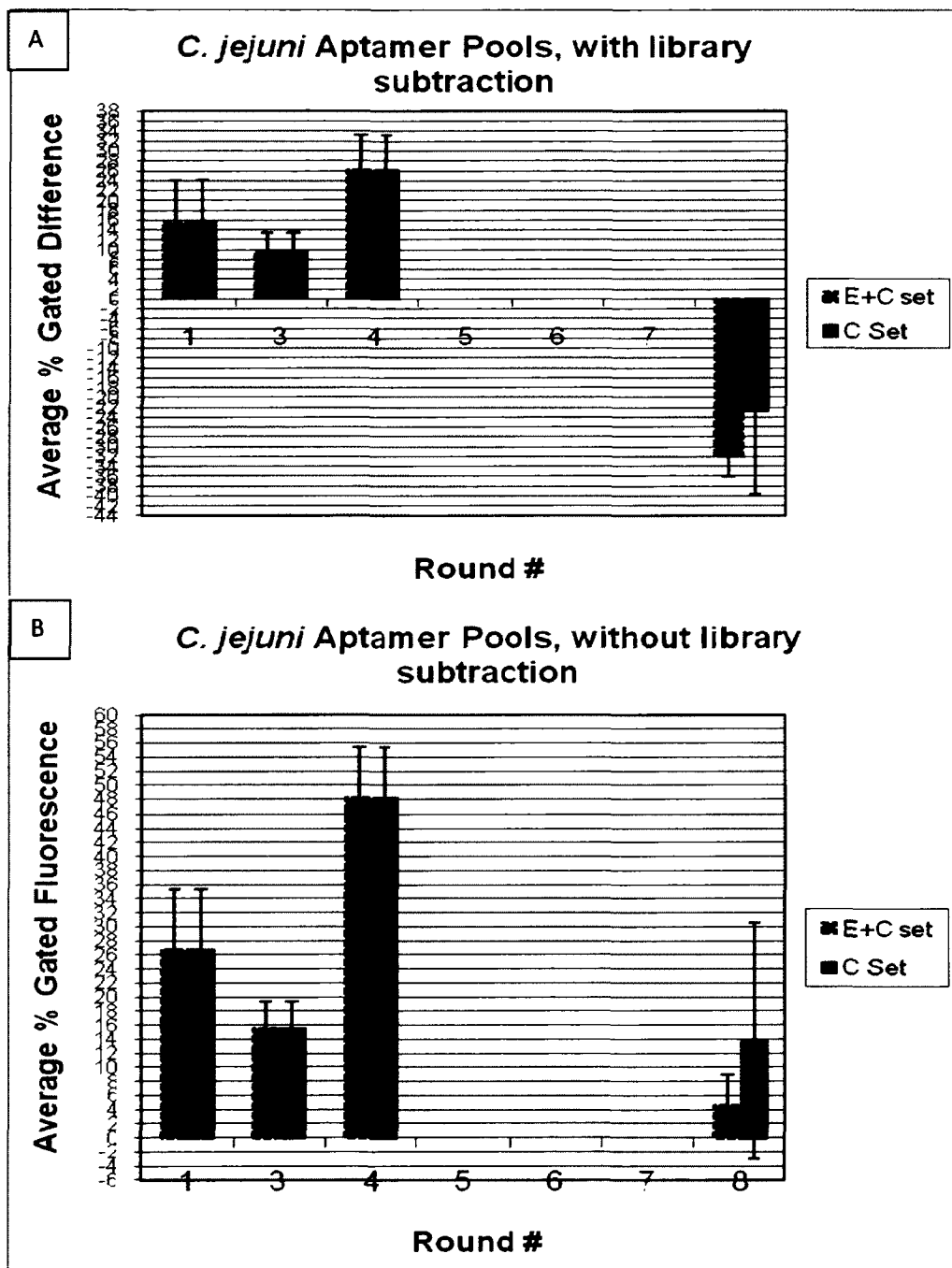


Figure 3.4.2. Results of flow cytometry analysis of aptamer pools from rounds 1, 3, 4, and 8 for *C. jejuni* SELEX before the target switch step, and E+C and C sets after the target switch step. Results are presented both with (A) and without (B) library subtraction. Standard deviation bars are also shown for each pool.

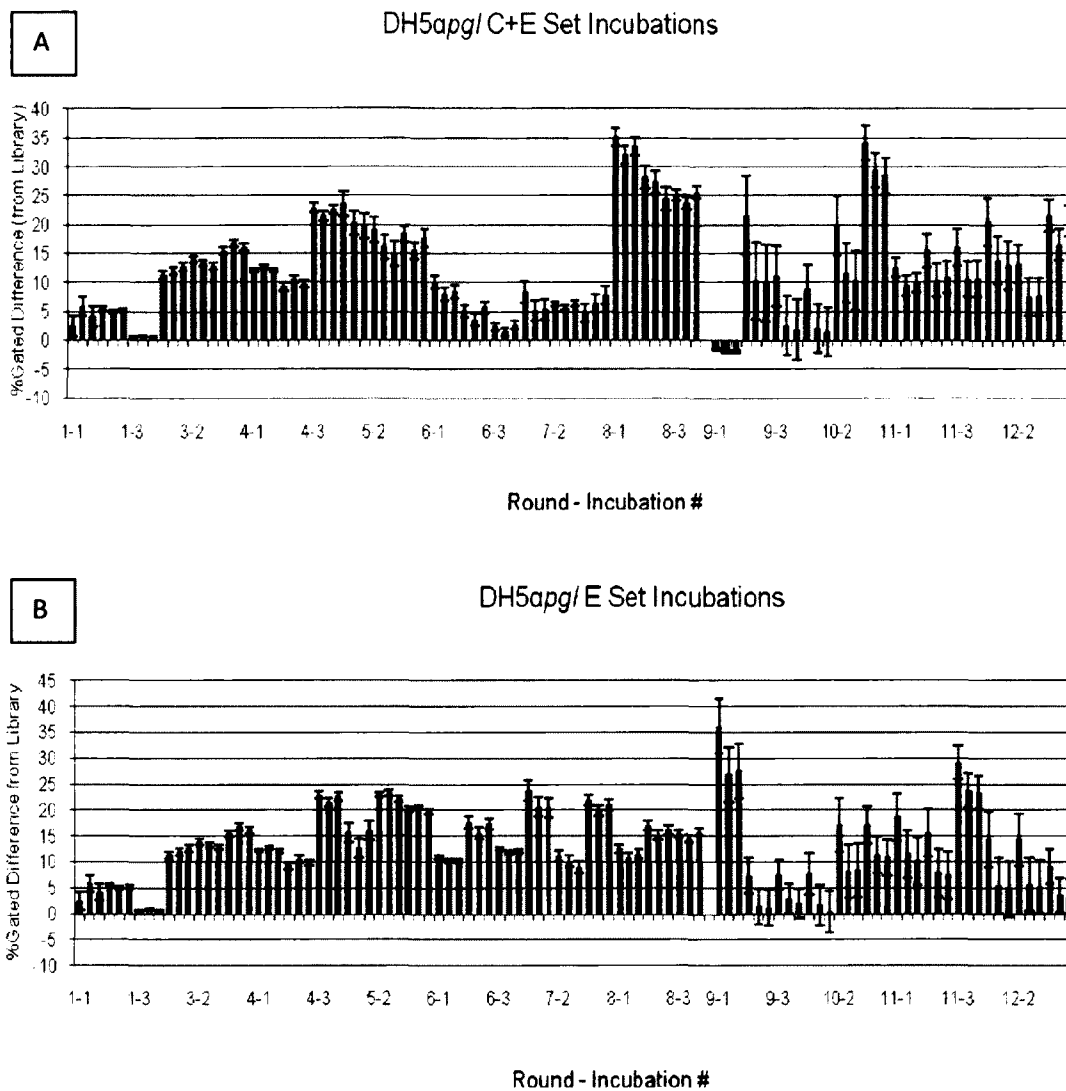


Figure 3.4.3. Results of flow cytometry analysis of aptamer pools 1-12 for *E. coli* DH5apgl. DH5apgl SELEX C+E set (A) and E set (B) with library subtraction, separated by incubations. Standard deviation bars are also shown for each incubation.

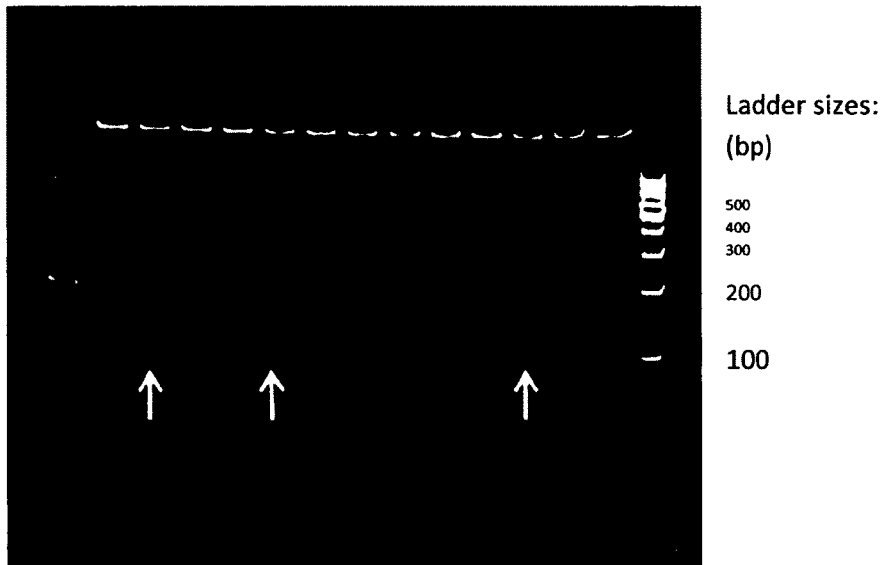


Figure 3.5.1 Gel photograph of assorted sizes of inserts obtained after cloning and plasmid digestion. The white arrow indicates the insert of the desired size (~96bp) on the gel, which can be sent for sequencing. Other bands that are not at this location are not the correct size and were not sequenced.

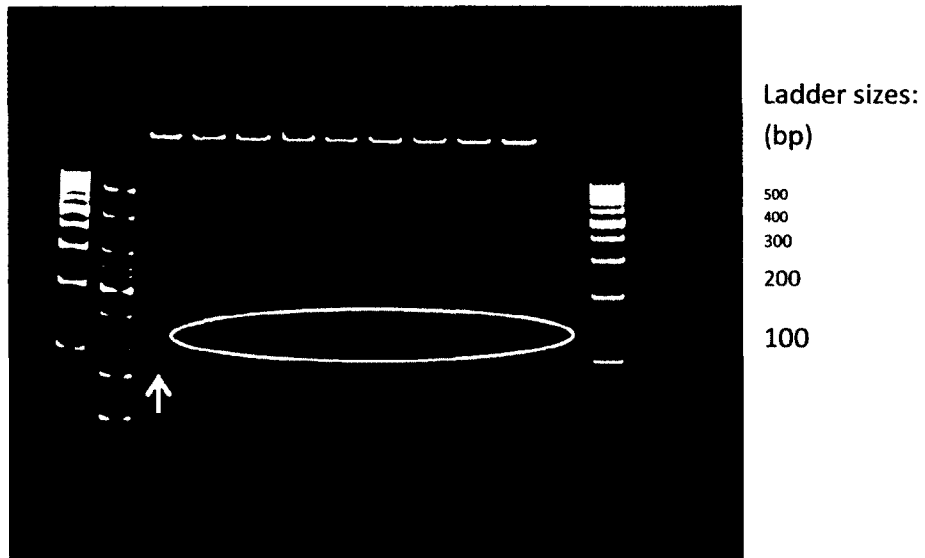


Figure 3.5.2 Gel photograph of inserts obtained for several 8C colonies, next to a 10C+E insert that is known to be of the correct size. The white arrow indicates the insert of the correct size (~96bp). The bands within the white circle are not the correct size.

Table 3.5.1. Summary table of cloning and sequencing results.

Round Pool	# plasmids extracted	# sequences sent for sequencing	# useable sequences obtained (forward strand)	# sequences w/ favourable ΔG values of about -5 kcal/mol and lower (including complement strand)	# sequences sent for synthesis (and were tested for binding)
8C	60	35	34	54	10 (8)
10C+E	40	26	21	29	9 (8)
12C+E	40	22	21	24	7 (6)
Total	140	83	76	107	26 (22)

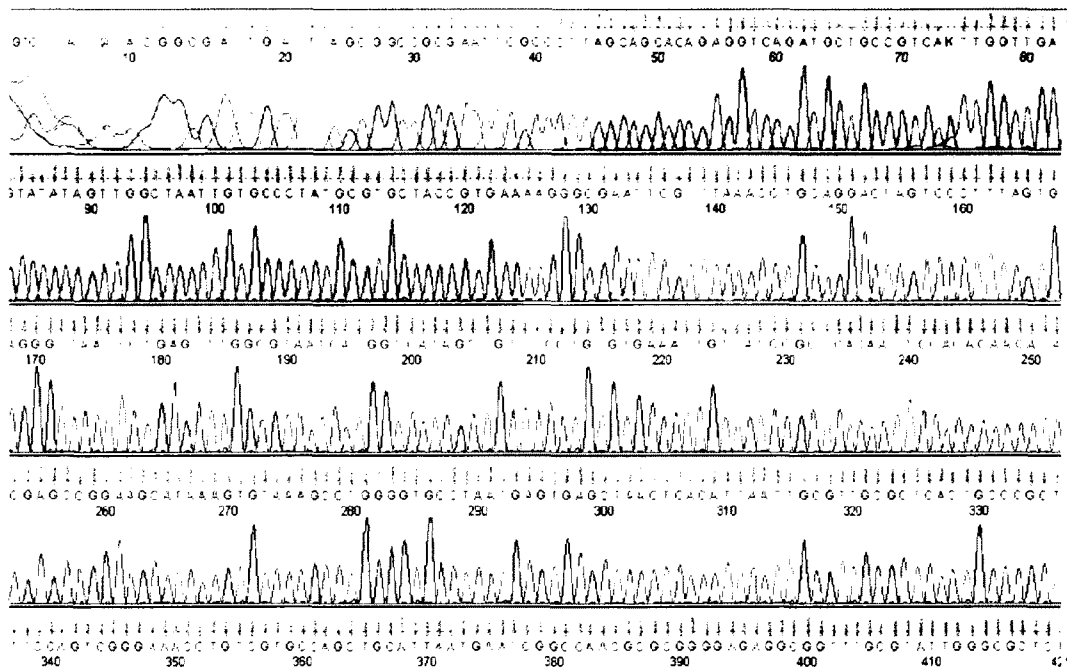


Figure 3.5.3. Electropherogram of a sample DNA aptamer sequence within the cloning plasmid. The 80nt aptamer insert region is highlighted above.

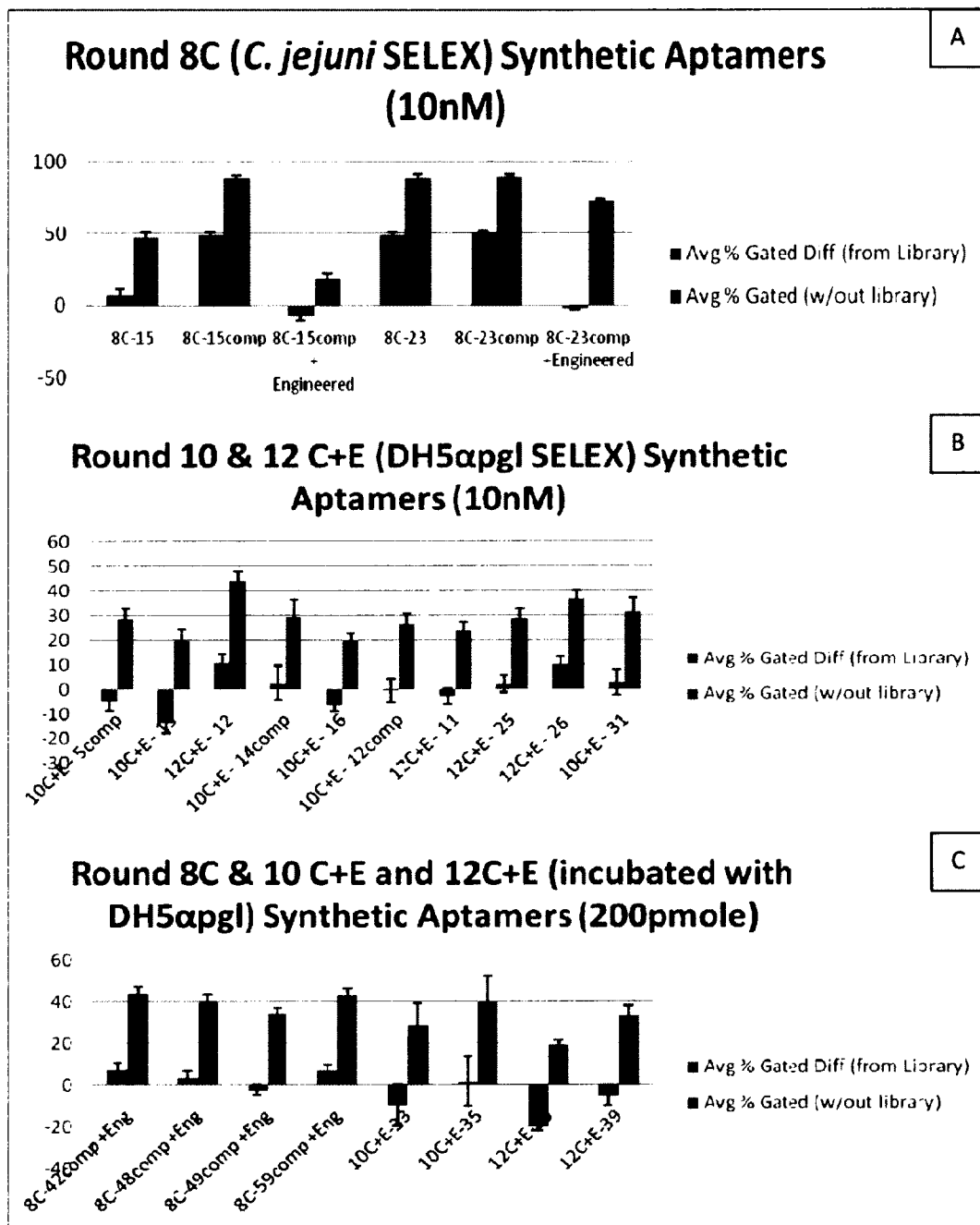


Figure 3.6.1. Results of flow cytometry analysis of synthetic aptamers from the *C. jejuni* SELEX round 8C and from the *E. coli* DH5α*agl* SELEX rounds 10C+E and 12C+E. Most aptamers from the *C. jejuni* SELEX round 8C were incubated with *C. jejuni* (A) unless indicated as a “+Engineered” or “Eng”, meaning the 8C aptamer was incubated with DH5α*agl* cells. All aptamers obtained from the DH5α*agl* SELEX rounds 10C+E and 12C+E were incubated with DH5α*agl* cells. % gated difference in fluorescence intensity is along the y-axis.

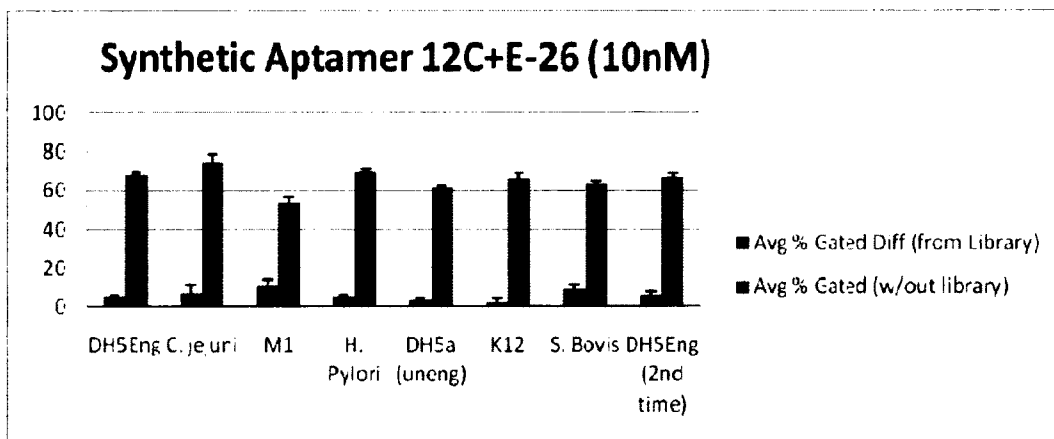


Figure 3.6.2. Results of flow cytometry analysis using fluorescently labelled aptamer sequence 12C+E-26 to incubate with various bacterial cells. Ten nM aptamer 12C+E-26 was separately incubated with 10^7 cells each type for 50 minutes followed by flow cytometry analysis. DH5Eng = *E. coli* DH5apl. % gated difference in fluorescence intensity is along the y-axis.

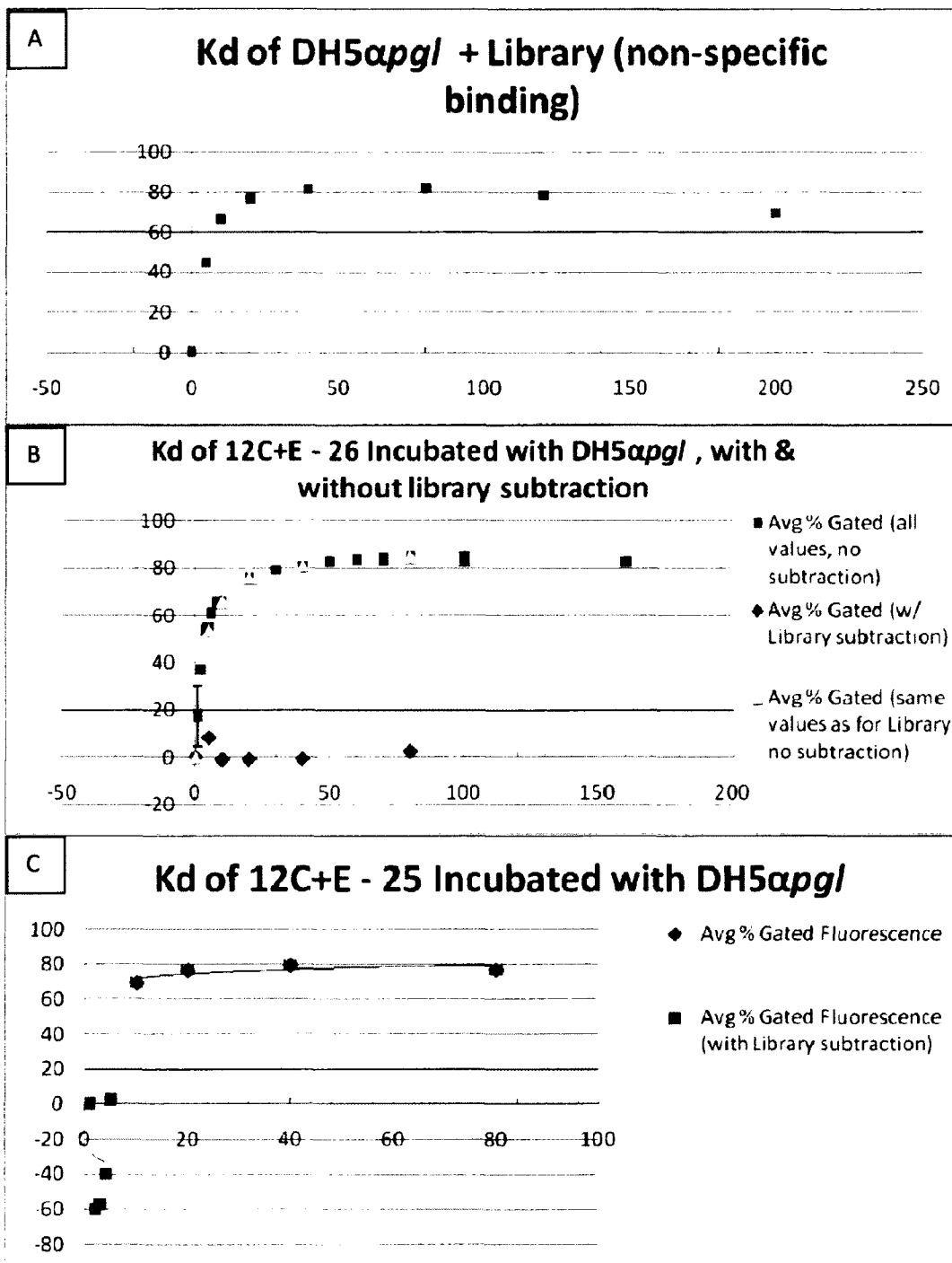


Figure 3.6.3. Aptamer binding to DH5αpgl cells with increasing aptamer concentration of random DNA oligonucleotide library (A), and synthetic aptamers 12C+E-26 (B) and 12C+E-25 (C) obtained from the DH5αpgl SELEX round 12. Fluorescence intensity is plotted on the y-axis against aptamer concentration (in nM) on the x-axis, to be used for estimation of Kd ranges.

Chapter 4. Summary, Conclusions, and Future Work

4.1 Summary and Conclusions

Two sets of SELEX were performed on different but related bacterial targets. One on *C. jejuni* ATCC33560, and one on *E. coli* DH5 α gl, which expresses a *C. jejuni* surface carbohydrate. Part-way through each set, the target of one was switched with the target of the other in an attempt to enhance specificity and affinity of aptamers to both targets. Twelve rounds of selections were performed for the *E. coli* DH5 α gl set and 8 rounds were performed for the *C. jejuni* set. Flow cytometry assessment of aptamer-bacteria binding was performed on aptamer pools for most rounds. Aptamer pools from later rounds with highest fluorescence intensity were selected for cloning and sequence analysis, as these represent the greatest aptamer binding to target. These pools were 8C of the *C. jejuni* set, and 12C+E and 10C+E of the *E. coli* DH5 α gl set.

After cloning, 140 plasmids were extracted and digested. Of these, 83 inserts were sent for sequencing. Only 80 nt sequences were considered useable (sequences returned permitted synthesis of an 80 nt aptamer), unless a deletion occurred in the primer region, which could be corrected upon synthesis. Seventy six of the sequences returned were useable. Twenty six of these 76 sequences were synthesized for testing of binding affinity and cross-reactivity. These were selected based on ΔG values and predicted secondary structures. Two sequences (12C+E-30 and 10C+E-31) were obtained twice, out of the 21 sequences from each of the 12C+E and 10C+E set returned (9.5% recurrence each), or out of the 42 useable sequences from the 10C+E and 12C+E sets combined (4.8% recurrence each). 12C+E-30 was not seen in the sequences returned from the 10C+E set of the 10th round of selection for the same target cell. Nor was the 10C+E-31 sequence seen in those of the 12C+E set. They were also not seen in the 8C set for the other target bacterium. This is not considered to be unusual,

however, as the number of inserts sent for sequencing was quite low relative to the number of colonies yielded during cloning (typically 100-200 colonies per plate, with only 30-60 colonies picked for extraction per plate). Therefore, the potential number of aptamer sequences in each pool is even greater than represented in sequencing results.

Based on previous work (30, 31, 32, 47, 51), the most promising aptamers are likely to be 12C+E-30 and 10C+E-31, which were the only sequences seen more than once. These aptamers, and complement sequences, should also be subjected to flow cytometry analysis of fluorescence shifts with target and non-target bacteria, to assess specificity and relative binding affinity. K_d analysis should also be performed to quantify binding affinity. The association of sequence repetition and high binding affinity was corroborated by the work of Hamula et al and McKeague et al (30, 31, 32, 51).

Washing steps to separate bound and unbound aptamers are not 100% effective. The results of synthetic aptamers from *C. jejuni* round 8C (unbound fraction after target-switch step that should only bind *C. jejuni*) incubated with both *C. jejuni* and *E. coli* DH5 α agl, suggest that some of the bound aptamers may be removed in the wash steps as well. Figure 3.6.1 A and C demonstrate that, although most of the synthetic aptamers assessed show high shifts in fluorescence intensity with *C. jejuni*, some also show cross-reactivity with *E. coli* DH5 α agl. This suggests that some bound aptamers were transferred along with the unbound fraction during the wash steps of the target-switch step. Also, the results from synthetic aptamer 12C+E-26, of the *E. coli* DH5 α agl set, suggest that counter-selection could be improved as well. Figure 3.6.2 illustrates that even though *E. coli* DH5 α was used in the counter-selection step, aptamers in later rounds are still capable of binding to it. Therefore, a significant amount of unbound sequences may be left behind after wash steps, and the counter-selection and/or wash steps may require improvement.

The target-switch step however, appears to be effective, based on preliminary results. Figure 3.6.2 also shows that the synthetic aptamer from *E. coli* DH5 α round 12C+E, is capable of binding both *E. coli* DH5 α and *C. jejuni*. This suggests that the target-switch step may have been a successful step toward generation of aptamers against 2 bacterial targets. Further testing would be required to make more definitive conclusions about the effectiveness of the target-switch and counter-selection steps, however, as these are important features of this method.

After flow cytometry analysis of aptamer-bacteria binding for the 26 synthetic aptamers, K_d analysis was performed on aptamer sequences 12C+E-25 and 12C+E-26. K_d ranges (when incubated with *E. coli* DH5 α) were roughly estimated to be in the low nanomolar range for 12C+E-26, 12C+E-25, and the library. Synthetic 12C+E-26 was tested via flow cytometry for cross-reactivity and binding to *S. pyogenes* M1, *S. bovis*, *E. coli* K12, *E. coli* DH5 α , *H. pylori* A158, *C. jejuni* ATCC 33560, and *E. coli* DH5 α . Highest fluorescence intensity (without library subtraction) was with *C. jejuni* ATCC 33560, *H. pylori* A158, and *E. coli* DH5 α , respectively (68.2% -74.6%). Although lower, fluorescence intensities were also similarly high with the other bacteria (53.9% - 66.5%), demonstrating cross-reactivity for bacteria unrelated to the target organism.

Flow cytometry results were divided to show fluorescence intensity with and without library subtraction. This was done to show the variability created in results after library subtraction, due to batch variability between random DNA libraries. Random DNA oligonucleotide library may not be suitable for use as a control with cells, as cells have many potential binding sites that a library could bind non-specifically to. Perhaps another control, other than the DNA library, should be used for subtraction of non-specific binding with cells. Non-target cells

could be used with the aptamers being measured, instead of the library with target cells. This would be more representative of the non-specific binding ability of each aptamer with another cell. Another method of measuring fluorescence intensity or binding affinity is also needed before conclusions can be made regarding the reliability of flow cytometry in aptamer binding studies.

In summary, although initial objectives remain to be proven, promising headway has been made towards generation of DNA aptamers against *C. jejuni*. With only 26 cloned aptamer sequences being synthesized and tested, several with high shifts in fluorescence above background, many other sequences could still be synthesized and assessed via flow cytometry or another method of binding analysis. Based on the literature (31, 32, 47, 51), the most promising aptamers in need of further analysis are likely to be 12C+E-30 and 10C+E-31, and complement sequences, due to their recurrence (sequences obtained more than once).

4.2 Future Work

As part of future work, one would narrow down the K_d ranges of 12C+E-25 by incubating the target cells with aptamers of various concentrations within the K_d range. Since 10-80 nM has already been measured, and the K_d range is between 1 and 10 nM, one would incubate *E. coli* DH5α and/or *C. jejuni* cells with 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 nM of 12C+E-25. 100 nM would also need to be measured, to ensure the plateau is reached with a sufficient number of readings, as the highest concentration previously measured for this aptamer was 80 nM. Shift of fluorescence intensity above background would then be measured.

Fluorescence intensity would then be plotted against the aptamer concentrations to determine the K_d values, as done in section 3.6. Yet aptamers should first be incubated with several target and non-target bacteria, to gauge specificity of the aptamers.. Then aptamers with desirable specificity can be subjected to K_d

analysis. Otherwise, assessing K_d values of aptamers that may not be very specific could be a waste of resources.

Efficacy of both counter-selection and target-switch steps should be further assessed. This could be done for counter-selection by comparing shifts in fluorescence intensity in round pools before and after the step, when incubated with the bacteria used in counter-selection. For the *E. coli* DH5 α agl set, this would involve comparing fluorescence intensity of round pool 3 with *E. coli* DH5 α agl and with unengineered *E. coli* DH5 α , to that of round pool 4 with each of the two bacteria. For the *C. jejuni* set, pools from round 3 and 4 would be incubated with *C. jejuni* and *E. coli* ATCC 25922. Lower fluorescence intensities for the non-target cells in round 4 would be suggestive of a more effective counter-selection step.

To assess efficacy of the target-switch step, shifts in fluorescence intensity would be compared for rounds before and after the target-switch step. Target cells were switched after round 4 for the *E. coli* DH5 α agl set (for 2 rounds), and after round 5 (for 2 rounds) for the *C. jejuni* set. For the *C. jejuni* set, rounds 5, 6, and 7 should be assessed with both *E. coli* DH5 α agl and *C. jejuni*. For the *E. coli* DH5 α agl set, rounds 4, 5, and 6 should be assessed with both targets. An increase in fluorescence intensity shifts after the target-switch step, for the other target, would suggest that the step had been effective. Shifts in intensity from the “bound” fraction (C+E and E+C sets) of the target switch and preceding rounds, could also be compared to shifts from the “unbound” fraction (C and E sets) of the same rounds. Increased shifts in intensity for both bacteria in “bound” fractions, but not “unbound” fractions, would further support the efficacy of the target-switch step.

Slide binding assays could also be done as a secondary measure of aptamer specificity. Target and non-target bacteria would be fixed to slides, then fluorescently labelled aptamers would be added, and slides would be washed to

remove unbound aptamers. Slides would be examined for fluorescence above background (slides with no cells, and slides with cells and random fluorescent library added). However, it should be noted that the method of fixing bacteria to the slides, such as heat-fixing, may kill the bacteria. Therefore, since the aptamers were generated against live, whole bacterial cells, they may no longer recognize the dead or lysed cells.

Alternatively, biotinylated aptamers could be fixed to streptavidin-coated slides, target and non-target bacteria could be added to slides, then washed to remove unbound cells. Then fluorescently labelled aptamers (same sequence as the biotinylated aptamers, but tagged differently) could be added, then washed to remove unbound aptamers. Slides could then be examined for fluorescence above background (slides with no cells, but fluorescent aptamers added, and slides with cells and fluorescent random library added in place of fluorescent aptamers) to assess binding of aptamers to target and non-target bacteria.

C. jejuni is sensitive to stress in the environment, and may alter its surface expression during such conditions (22, 54). Thus, it would be useful if, in addition to recognizing live *C. jejuni* cells, aptamers could also recognize these cells under stress. Environmental conditions that stress the bacterium include the presence of oxygen, as well as heat, and drying conditions. Binding affinity and K_d s would need to be tested under these conditions as well, if the aptamers were to be applied in environmental assays.

Also, it is unknown as to the exact target that aptamers have been generated against. The SELEX procedure (including the target switch step) was designed to enhance the yield of aptamers that bound both *C. jejuni* and the *C. jejuni* surface carbohydrate expressed on the *E. coli* DH5 α ggl cells. To test whether an aptamer is binding to the DH5 α ggl surface carbohydrate, one could run the DH5 α ggl carbohydrate extracts on a Western gel, as done previously to confirm expression.

Then a biotinylated aptamer could be used in place of the primary antibody, and retain the anti-biotin antibody as the secondary antibody. The membrane could then be imaged in the same manner as detailed in Chapter 2.4. Yet an aptamer specific for a carbohydrate or protein on a membrane surface may no longer bind that target once it has been denatured or altered in conformation, when dissociated from the membrane. This would still be an informative first step, however, in determining whether certain aptamers from the *DH5apgl* (and even *C. jejuni*, if desired) selections are actually binding their intended target.

If aptamers with desirable properties are not found from the group synthesized, more aptamers could be synthesized and tested. Also, more of the aptamer pools can be cloned, picked, and sequenced. More sequencing could be done as well, to look for more repeat sequences. Any repeat sequences could then be synthesized and tested. This may be more cost effective, as sequencing is cheaper than synthesis.

4.3 Potential Uses for Aptamers

Aptamers found to be highly selective with high affinity could potentially be used for detection and diagnostic purposes in a number of ways. Sandwich assays could be used with aptamers in place of antibodies. For example, the aptamer of choice could be synthesized with a biotin tag (assuming it does not alter binding affinity of the aptamer), and bound to a streptavidin-coated slide (or multi-well plate). A second batch of the aptamer could be synthesized with a fluorescent tag. A sample which may or may not contain the organism of interest could then be added to the slide, along with the 2nd batch of the fluorescent aptamer, and allowed to incubate for a period of time. The slide would then be washed a few times to remove any unbound target molecules and fluorescent aptamer. The fluorescent aptamer will only remain on the slide if target molecule is present and bound to the fixed biotinylated aptamer. This is assuming the biotin and

fluorescent modifications do not alter aptamer binding. The slide could then be subjected to fluorescence microscopy. A certain level of fluorescence on the slide, above background, would be indicative of target presence.

Ferreira et al (26) demonstrated the use of aptamers to MUC1 protein in sandwich assays, for the early detection of epithelial tumours. Biotinylated aptamers were fixed to 96 well plates (coated with extravidin), then MUC1 protein was added, followed by addition of mouse anti-MUC1 antibody. Then rabbit anti-mouse antibody conjugated to alkaline phosphatase was added for visualization of a colorimetric signal, which was read using an ELISA plate reader. This group also used Surface Plasmon Resonance (SPR) to assess K_d values, which may be a potentially useful method for non-fluorescent K_d analysis of *C. jejuni*/*E. coli* DH5 α *agl* aptamers. Here aptamers were affixed to a chip, target was added, and the chip was bombarded with energy. Bound versus unbound aptamers produce different SPR signals, and the differences are measured and interpreted. To measure K_d , varying concentrations of aptamer would be fixed to the chip and target allowed to bind. SPR signals would increase with increasing amount of target bound. From this, a binding curve could be generated and K_d calculated.

Another possible application of aptamers is concentration of target bacterial cells in water samples. Water testing requires the processing of large volumes of water which may only contain a few contaminating cells. Therefore, a method of concentration of cells could be useful. The aptamer of choice could be synthesized with a biotin tag, and bound to streptavidin-coated magnetic beads. Here, magnetic beads could be added to a large volume of water to be tested, stirred, and left to incubate. A magnet could then be used to attract the beads to one area, while the large volume of water is decanted. If the target bacterium has bound to the beads, the cells can be removed from the beads, or the beads can be plated or placed in liquid media to grow, if they have been found to not interfere with cell growth.

Even if these aptamers aren't useful for identifying certain bacteria specifically, such as *Campylobacter*, or binding a specific target, such as a surface carbohydrate, some aptamers may still serve some purpose. For example, 12C+E-26 may be useful as a probe in Western Blotting or similar technique where one may wish to label a bacterium non-specifically. Since this aptamer showed strong fluorescence with multiple bacteria, it could be used as a non-specific probe for *S. pyogenes* M1, *S. bovis*, *E. coli* K12, *E. coli* DH5 α , and *H. pylori* A158, *C. jejuni* ATCC33560, and *E. coli* DH5 α agl, which had all shown strong fluorescence when bound to the aptamer. To test the aptamers utility as a Western Blot probe, one could biotinylate the aptamers of interest, rather than adding a fluorescent tag, and run whole cell extracts of various bacteria on a Western gel. Once the gel contents are transferred to a membrane, the biotinylated aptamers can be used in place of the primary antibody, and anti-biotin antibody as the secondary antibody, as described above.

On a related note to this work, Dwivedi et al (22) recently generated DNA aptamers against *C. jejuni* strain A9a using whole-cell SELEX in 2010. One aptamer, ONS-23, had high binding affinity (K_d was in the high nM range) and specificity for several other *C. jejuni* strains tested (including *C. jejuni* ATCC 33560). The aptamer was found to have low affinity for non-*C. jejuni* strains. The exact cell surface molecule with which the aptamer interacts is not known, and was not investigated. The group performed 2 rounds of counter-selection after the 10th round of selections, and used a mixed culture of several non-target bacteria (including one or more species of *Pseudomonas*, *Shigella*, *E. coli*, *Bacillus*, *Staphylococcus*, *Listeria*, *Salmonella*, *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*). Interestingly, the predicted structure of ONS-23 was composed of 3 hairpin loops, which is quite similar in structure to the aptamers from round 8C, found to produce the highest shifts in fluorescence

with *C. jejuni* ATCC 33560. This provides hope that DNA aptamers may be used for the detection of *C. jejuni* in the near future.

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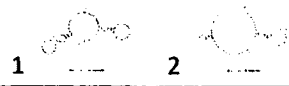
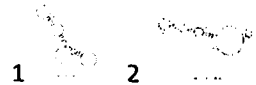



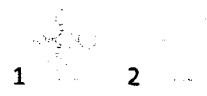

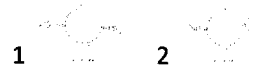
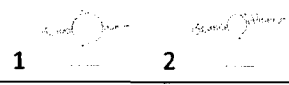
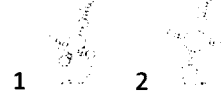
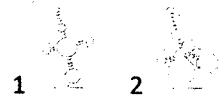

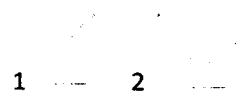



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Appendix

Table A.1. Summary of predicted secondary structures and Gibbs Free Energy (kcal/mol) values (ΔG) of aptamers sent for sequencing with favourable ΔG values, for round pool 8C of *C. jejuni* selections. Comp = complementary reverse sequence to the forward sequence obtained from the sequencing facility. * = sequence was sent for synthesis. nt = nucleotide. bp = base pair. 79 nt sequences were only included if the deletion was in the primer region, and could be replaced during synthesis, where the 79 vs 80 nt structure did not result in a change in predicted secondary structure or ΔG values.

Sequence	$\Delta G1(-)$	$\Delta G2(-)$	description	
8C-15	6.05			*
8C-15comp	6.04	5.84	1 2	*
8C-17	4.97			
8C-22	8.92	6.58	1 2	*
8C-22comp	8.37	7.27	1 2	*
8C-23	8.2			*
8C-23comp	7.63	7.32	1 2	*
8C-19 (79bp – missing 1 st nt of F primer).	6.41	5.88	1 2	
8C-19comp (79bp – missing last nt of F primer).	5.77	5.61	1 2	
8C-28 (78bp – missing last 2 nt's of R primer).	4.3			

8C-28comp (78bp – missing 1 st 2 nt's of F primer).	4.1			
8C-31	4.76	4.4	1 2	
8C-31comp	5.41	5.33	1 2	
8C-32	5.21	4.45	1 2	
8C-34	5.89	5.83	1 2	
8C-34comp	6.47	5.95	1 2	
8C-35	5.14	4.21	1 2	
8C-35comp	4.47			
8C-36comp	4.4			
8C-37	4.49	4.12	1 2	
8C-37comp	5.53	4.26	1 2	
8C-41	5.84	5.74	1 2	
8C-41comp	6.98	5.11	1 2	
8C-42	9.94	9.73	1 2	
8C-42comp	11.65	11.28	1 2	*
8C-43	6.21	4.55	1 2	

8C-43comp	5.68	4.5		
8C-44	4.96	4.89		
8C-44comp	4.75	4.07		
8C-45	5.37	4.12		
8C-45comp	5.93	5.49		
8C-47	5.42	4.56		
8C-47comp	4.59	4.51		
8C-48	9.37	7.84		
8C-48comp	12.59	10.37		*
8C-49	10.12	10.07		
8C-49comp	10.93	9.43		*
8C-51	6.48			
8C-51comp	5.1	4.57		
8C-52	4.06	3.67		
8C-52comp	4.26			*
8C-53	5.6	4.11		







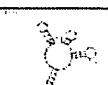
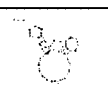
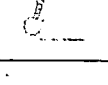



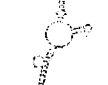



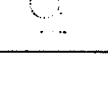




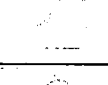

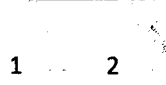



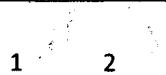

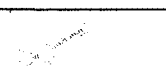
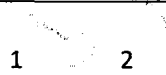


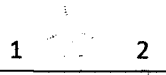


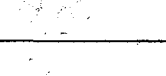
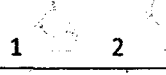
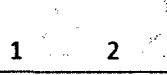
8C-53comp	6.06	5.9	1  2 	
8C-54	6.53	6.03	1  2 	
8C-54comp	5.05	4.52	1  2 	
8C-55comp	6.44	6.18	1  2 	
8C-56	4.77	4.22	1  2 	
8C-56comp	6.17	5.59	1  2 	
8C-58	7.1	6.17	1  2 	
8C-58comp	6.81	6.45	1  2 	
8C-59	9.6	9.18	1  2 	
8C-59comp	9.61	9.38	1  2 	
8C-60	4.67			
8C-60comp	5.0	4.0	1  2 	

Table A.2. Summary of predicted secondary structures and Gibbs Free Energy (kcal/mol) values (ΔG) of aptamers sent for sequencing with favourable ΔG values, for round pool 10C+E of DH5 α pgl selections. Comp = complementary reverse sequence to the forward sequence obtained from the sequencing facility. * = sequence was sent for synthesis.

Sequence	$\Delta G1=(-)$	$\Delta G2=(-)$	description	
10C+E-1	5.48	4.95	1  2	
10C+E-2comp	4.06			
10C+E-5	7.87	7.21	1  2	*
10C+E-5comp	9.24	8.78	1  2	*
10C+E-12	6.14	5.74	1  2	
10C+E-12comp	7.61			*
10C+E-13	9.15			*
10C+E-13comp	6.11	5.91	1  2	
10C+E-14	6.46	5.93	1  2	
10C+E-14comp	7.52	7.11	1  2	*
10C+E-16	8.14	7.51	1  2	*
10C+E-16comp	6.99	6.76	1  2	
10C+E-18	4.98			
10C+E-18comp	5.09			
10C+E-20	6.65	6.19	1  2	
10C+E-20comp	6.76	6.59	1  2	


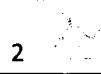
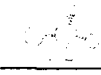


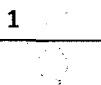
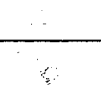
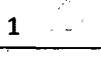


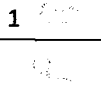
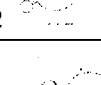
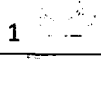
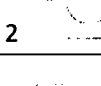
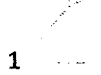


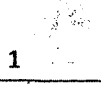
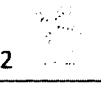



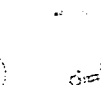
10C+E-26	6.93	6.82	1  2 	
10C+E-26comp	5.97			
10C+E-28 & 10C+E-30 (same sequence)	6.83	5.63	1  2 	
10C+E-31	4.98	4.86	1  2 	*
10C+E-31comp	4.28			
10C+E-33	4.83	4.35	1  2 	*
10C+E-33comp	4.74	4.59	1  2 	
10C+E-35	9.01	8.34	1  2 	*
10C+E-35comp	5.75			
10C+E-36	7.63	7.58	1  2 	
10C+E-36comp	8.75	6.58	1  2 	
10C+E-40	5.55	5.43	1  2 	
10C+E-40comp	4.71	4.15	1  2 	

Table A.3. Summary of predicted secondary structures and Gibbs Free Energy (kcal/mol) values (ΔG) of aptamers sent for sequencing with favourable ΔG values, for round pool 12C+E of DH5 α pgl selections. Comp = complementary reverse sequence to the forward sequence obtained from the sequencing facility. * = sequence was sent for synthesis.

Sequence	$\Delta G1=(-)$	$\Delta G2=(-)$	description	
12C+E-2	4.87			
12C+E-8	7.37	6.58	1 2	
12C+E-8comp	8.61			*
12C+E-11	8.29	7.45	1 2	*
12C+E-11comp	5.2	4.67	1 2	
12C+E-12	5.97			*
12C+E-13	5.31	4.91	1 2	
12C+E-13comp	5.13			
12C+E-17	4.55			
12C+E-19comp	4.44			
12C+E-22comp	4.37			
12C+E-25	5.61	5.42	1 2	*
12C+E-25comp	5.97	5.79	1 2	
12C+E-26	6.25	5.33	1 2	*







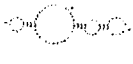




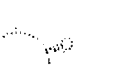






12C+E-26comp	5.81	5.01	1  2 	
12C+E-29	4.72	4.25	1  2 	
12C+E-29comp	4.89	4.53	1  2 	
12C+E-27 & 12C+E-30 (same sequence)	4.93	4.93	1  2 	*
12C+E-33	4.65			
12C+E-33comp	4.62			
12C+E-34	6.04	5.99	1  2 	
12C+E-34comp	5.85	5.28	1  2 	
12C+E-39	8.55	7.58	1  2 	*
12C+E-40	4.74	4.54	1  2 	

Table A.4. Sequences of aptamers selected for synthesis from round 8C of the *C. jejuni* SELEX set, and rounds 10C+E and 12C+E of the *E. coli* DH5apgl SELEX set. Primer sequences (20nt each) are underlined. The variable 40nt aptamer sequence between the 2 primers is not underlined. Comp = complementary reverse sequence to the forward sequence obtained from the sequencing facility.

Aptamer	Sequence
8C-15	<u>AGCAGCACAGAGGTCAGATGCACAGTATATACGTACCAAAGACGCTAACTGTT</u> <u>CGCAGCGCCTATGCGTGCTACCGTGAA</u>
8C-15comp	<u>TTCACGGTAGCACGCATAGGCGCTGCGAACAGTTAGCGTCTTTGGTACGTATA</u> <u>TACTGTGCATCTGACCTCTGTGCTGCT</u>
8C-22	<u>TTCACGGTAGCACGCATAGGGACGAACTAATGCGAGCTATATATGGGCCTCAA</u> <u>ACCATTGCATCTGACCTCTGTGCTGCT</u>
8C-22comp	<u>AGCAGCACAGAGGTCAGATGCAATGGTTTGAGGCCATATATAGCTCGCATT</u> <u>GTTTCGTCCTATGCGTGCTACCGTGAA</u>
8C-23	<u>TTCACGGTAGCACGCATAGGGCCTTGATAATATCTTGCCCTACACAGCTAGCAC</u> <u>TCACCCCATCTGACCTCTGTGCTGCT</u>
8C-23comp	<u>AGCAGCACAGAGGTCAGATGGGGTGAGTGCTAGCTGTGTAGGGCAAGATATT</u> <u>ATCAAGGCCCTATGCGTGCTACCGTGAA</u>
8C-42comp	<u>TTCACGGTAGCACGCATAGGGCGATAGCTAGTCGAGGAATCGATGGTCAGCGT</u> <u>TGGAACCCATCTGACCTCTGTGCTGCT</u>
8C-48comp	<u>TTCACGGTAGCACGCATAGGGCCCTGCTAGCGAAGAGTACAACGGTTTACAGG</u> <u>TCAGCCGCATCTGACCTCTGTGCTGCT</u>
8C-49comp	<u>AGCAGCACAGAGGTCAGATGGACAACCCATCCAACCTAGTTGGCGGGGACAG</u> <u>GCTCGGTGCCTATGCGTGCTACCGTGAA</u>
8C-52comp	<u>TTCACGGTAGCACGCATAGGGGAACGCCTTGAACGAGTCACATAAACTCTGAT</u> <u>CCGGCCGCATCTGACCTCTGTGCTGCT</u>
10C+E-5	<u>TTCACGGTAGCACGCATAGGGCGCATCCGTA</u> <u>CTTGTCTGTTATCTTACCAGGGG</u> <u>TAGGCACATCTGACCTCTGTGCTGCT</u>
10C+E-5comp	<u>AGCAGCACAGAGGTCAGATGTGCCTACCCCTGGTAAGATAACAGACAAGTACG</u> <u>GATGCGCCCTATGCGTGCTACCGTGAA</u>
10C+E-12comp	<u>AGCAGCACAGAGGTCAGATGCACGTAGGAGGGGGTTTTGTACGTGAAATAAG</u> <u>ATGCTGCCCTATGCGTGCTACCGTGAA</u>
10C+E-13	<u>AGCAGCACAGAGGTCAGATGGGGAGTGGGTAAGAGCGCGCTTGTAGGGTTG</u> <u>AACTGGGACCCTATGCGTGCTACCGTGAA</u>
10C+E-14comp	<u>TTCACGGTAGCACGCATAGGGCGCTGGTTGGGTGTCTAGTATGGAGGAGGAC</u> <u>AGGGAGTCCATCTGACCTCTGTGCTGCT</u>
10C+E-16	<u>AGCAGCACAGAGGTCAGATGTGACGAGGCGTGCTAAAGGGTTAGAAGATTAC</u> <u>CACGGGCCCTATGCGTGCTACCGTGAA</u>
10C+E-28 & 10C+E-31 (same sequence)	<u>TTCACGGTAGCACGCATAGGGCTATTCTCTACAACCTACCGTATAATTTA</u> <u>TGCGGGCATCTGACCTCTGTGCTGCT</u>

10C+E-33	<u>AGCAGCACAGAGGTCAGATGCAATTTACACCTTCCCCGCTTTAAACCGCACTC</u> <u>GGGACCCTATGCGTGCTACCGTGAA</u>
10C+E-35	<u>TTCACGGTAGCACGCATAGGCAGGACAGCGAGGGTAGGGGGTCAGGTGGGT</u> <u>ATCTGGGGGCATCTGACCTCTGTGCTGCT</u>
12C+E-8comp	<u>TTCACGGTAGCACGCATAGGGCACAATTAGCCAATATATACTCAACCAACTGA</u> <u>CGGCAGCATCTGACCTCTGTGCTGCT</u>
12C+E-11	<u>TTCACGGTAGCACGCATAGGGCGCTGGTTGCGATGATAGCGTGAAATTATAG</u> <u>ACCATCCCATCTGACCTCTGTGCTGCT</u>
12C+E-12	
12C+E-25	<u>AGCAGCACAGAGGTCAGATGCCGACGCACGACGACCTAAGAAAAGTCTTTGA</u> <u>GGTTCGGGCCTATGCGTGCTACCGTGAA</u>
12C+E-26	<u>TTCACGGTAGCACGCATAGGCAGCCTCCTTGCCACTCTCTCATTATTTGTTTCATT</u> <u>GGTGGCATCTGACCTCTGTGCTGCT</u>
12C+E-27 & 12C+E-30 (same sequence)	<u>AGCAGCACAGAGGTCAGATGCCAGACCACTCCCATCACCGACACGTCTCGC</u> <u>CGACCACCCTATGCGTGCTACCGTGA</u>
12C+E-39	<u>TTCACGGTAGCACGCATAGGCGGAGAGGAGAAGCACTGAAAGCGGGAGGGC</u> <u>GGTGACGACCATCTGACCTCTGTGCTGCT</u>