THE DEVELOPMENT AND TESTING OF APTAMERS FOR THE DETECTION OF PEANUT ALLERGENS

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Abstract

Peanut allergies are one of the most common food allergies. Peanut contamination in supposedly peanut free food can lead to allergic reactions. Methods to detect allergens (proteins that trigger an allergic reaction) are therefore needed. In this study, we aim to produce aptamers that target peanut allergens and apply them in an aptamer based assay for the detection of peanut allergens. Aptamers are structured single stranded oligonucleotides that bind to a target with high specificity and affinity. We selected DNA aptamers against the peanut allergen Ara h 1 using magnetic bead SELEX. Eight clones (possible aptamers) were selected for testing from sequencing data. The ability of these clones to bind to Ara h 1 was assessed using flow cytometry. Significant binding was seen in two clones, AraH1_R5c17 and AraH1_R5c102, and they are thus aptamers of Ara h 1. The apparent dissociation constants (apparent K_d) of these aptamers were determined by performing titrations to test binding of the aptamers (at various concentrations) to Ara h 1. Preliminary results show the apparent K_d of AraH1_R5c17 is 59.5nM and the apparent K_d of AraH1_R5c102 is 11.2nM. We also examined whether the two aptamers bind at the same epitope by comparing binding of the aptamers to Ara h 1 individually and in combination. We did not observe an additive binding effect, therefore competitive binding was occurring. This suggests the two aptamers bind at the same epitope on Ara h 1. Future experiments will utilize the aptamers in an ELISA type assay in order to test their ability to detect Ara h 1 in food samples.

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- The members of Berezovski Lab for helping me in everyday matters such as troubleshooting malfunctioning instruments and locating reagents

Statement of Contribution

I worked on this project for approximately 7 months, from the beginning of September 2015 to the end of March 2016. Before I began, Dr. Pavel Milman (postdoctoral fellow) and Alex Nantsios (undergraduate student) performed magnetic bead SELEX experiments to select aptamers against Ara h 1. I began working on the project to test possible Ara h 1 aptamers (identified after SELEX) for binding to Ara h 1.

My daily supervisor, Dr. Milman, was responsible for the design of all experiments in this project. Dr. Milman also set up the flow cytometry protocol (gating and voltage). Results in figure 4 were generate by Dr. Milman and I. I generated the remaining results.

I originally wrote this thesis. Some sections were read and edited by Dr. Pavel Milman.

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Introduction

Peanut Allergies and Peanut Detection

Food allergy is defined as an adverse immune response (allergic reaction) to food proteins. The protein that triggers the immune system, causing an allergic reaction, is called an allergen. Peanut allergy is one of the most common food allergies (Sicherer & Sampson, 2006). They affect approximately 0.8% of young children and 0.6% of adults in the United States. The Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies currently recognizes 17 peanut allergens, Ara h 1 through Ara h 17 (WHO/IUIS Allergen Nomenclature Sub-Committee, 2016). Ara h 1 is a peanut seed storage protein that comprises 12-16% of the total protein content of peanuts and is one of the major peanut allergens (Koppelman et al., 2001). It has a molecular weight of 63.5kDa and a pI of 4.55 (Burks et al., 1991). Ara h 2 is also a major peanut allergen and comprises 5.9-9.3% of the total protein content in peanuts (Koppelman et al., 2001). It has a molecular weight of 17kDA and a pI of 5.2 (Burks et al., 1992). For those allergic to peanuts, strict avoidance of peanuts is the only way to prevent severe symptoms (Sampson et. al., 2003). Traces of peanuts may exist in food products labelled peanut-free because of peanut contamination in raw materials (Schäppi et al., 2001) or in production lines (Kiening et al., 2005). Reliable detection methods for peanut proteins, including allergens, are therefore required to determine when traces of peanuts are present in food and thus help avoid allergic reactions.

Currently, various methods are available to detect traces of peanuts in food. Many of these methods require centralized laboratories and highly trained personal. They can be split into two general categories: protein-based methods and DNA-based methods (Wen et al.,

2007). DNA-based methods are less common and are based on PCR amplification of genes specific to peanut allergens (Hird et al., 2003). The protein-based methods detect the presence of proteins, which can be total peanut proteins or a specific peanut allergen. Most of these methods involve the use of an antibody specific to an allergen or to total peanut protein (Wen et al., 2007). These include dip stick assays (Stephan et al., 2002), lateral flow assays (Wen et al., 2005) and enzyme-linked immunosorbent assay (ELISA) (Stephan & Vieths, 2004; Kiening et al., 2005). ELISA is the most commonly used immunoassay in the laboratories of the food industry to detect and quantify hidden allergens in food (Iqbal et al., 2016). Although antibodies are regularly used in these assays, they do have some disadvantages: they are expensive, may be difficult to generate, and may be denatured due to temperature changes (Tran et al., 2013). Aptamers present a possible alternative to antibodies in these methods.

Aptamers

Aptamers are structured single stranded oligonucleotides that are pre-selected to bind to desired targets with high affinity and specificity. They are sometimes referred to as "chemical antibodies" because they share many properties with antibodies, but are produced synthetically (Darmostuk et al., 2015). The use of aptamers instead of antibodies in a protein-based detection method for peanut allergens would be advantageous as aptamers are more stable than antibodies (Tran et al., 2013) and they are produced synthetically, making them easier and less expensive to produce than antibodies.

Aptamers against a specific target are selected using an *in vitro* process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). In SELEX, a pool of oligonucleotides with random sequences is exposed to the target. The oligonucleotides that

bind to the target are separated from those that do not. The separation technique used differs depending on the type of SELEX performed. The bound oligonucleotides are then amplified by PCR to be used in the next selection cycle. This cycle is repeated many times and may be referred to as a "round" of SELEX. After each cycle, an aliquot of the pool, which is enriched in oligonucleotides that bind to the target, is obtained before the pool is used in the next cycle (Darmostuk et al., 2015). Theoretically, after each cycle the number of oligonucleotides in the pool that do not bind to the target is reduced, so the pools obtained in later cycles should contain oligonucleotides with higher affinity for the target. In practice, this is not always the case. A major reason for this discrepancy is PCR bias, wherein certain oligonucleotides are preferentially amplified by PCR because of their sequence and secondary structure (Tsuji et al., 2009). It has been shown that oligonucleotides that are rich in pyrimidines and have highly stable (low minimum free energy) secondary structures are preferentially amplified during the SELEX process (Thiel et al., 2011). These preferentially amplified oligonucleotides may bind weakly to the target, and in this case PCR bias will result in large amounts of weakly binding oligonucleotides in pools obtained after many rounds of SELEX. Consequently, pools from later rounds will not necessarily contain a large number of oligonucleotides that bind with high affinity to the target. To determine the relative enrichment of the pools in oligonucleotides that bind to the target with high affinity, the ability of each pool to bind to the target is assessed (Tran et al., 2013). After this assessment, the oligonucleotides present in each pool are sequenced. By analyzing this sequencing data and considering the affinity of each pool for the target, specific oligonucleotides are chosen as potential aptamers. These potential aptamers are termed "clones" and are tested to determine whether they bind to the target. Most of the

time, only a few of these clones will bind to the target while the other clones have no affinity for the target (Tsuji et al., 2009). If they do bind, the clones are considered aptamers for that target and their affinity for the target is determined.

Aptamer testing methods

Various techniques may be employed to test the affinity of aptamers for their target. These include affinity capillary electrophoresis, fluorescence anisotropy, surface plasmon resonance (Tran et al., 2013), and ELISA (Wang et al., 2014). Flow cytometry was also shown to be an effective method in assessing the ability of DNA ligands to bind to a target (Davis et al., 1996). In the present study, flow cytometry on 1µm beads conjugated with the target was used to assess the ability of oligonucleotides to bind to a target. The oligonucleotides were conjugated to biotin so they could be detected using a streptavidin probe with a fluorescent label. The fluorescent signal given off by individual beads was then detected using flow cytometry. The strength of the fluorescent signal given off by a bead is proportional to the number of oligonucleotides that are bound to the target, which is conjugated to the bead. As the affinity of the oligonucleotides for the target increases, a larger number of oligonucleotides will bind to a target on the bead, and the fluorescent signal given off by the bead will therefore increase.

Dissociation Constant (K_d)

The affinity of a ligand for its target is usually measured using the dissociation constant (K_d), a parameter measured in units of concentration. The K_d of aptamers varies greatly: some aptamers have K_d's in the pM range while others have K_d's in the mM range (Darmostuk et al.,

2015). The dissociation constant is defined as the equilibrium constant for the reaction in which a ligand dissociates from its target, as shown below.

$$RL \rightleftharpoons R_{free} + L_{free}$$

$$K_d = \frac{[R]_{free}[L]_{free}}{[RL]}$$

Where $[R]_{free}$ is the concentration of free receptor (the target), $[L]_{free}$ is the concentration of free ligand (the binding entity), and [RL] is the concentration of the complex formed when the ligand and receptor bind. An alternate derivation of K_d is the concentration of free ligand when 50% of the target is bound (when $[R]_{free}$=[RL]). This relationship is derived from the definition of K_d, as shown below.

$K_d = \frac{[R]_{free}[L]_{free}}{[RL]}$, if $[R]_{free}$ = [RL], then $K_d = [L]_{free}$

When there is 1:1 binding of a ligand to a receptor (there is one ligand binding site per molecule of receptor), the free receptor concentration can be represented in terms of the total receptor concentration and the concentration of ligand-receptor complex:

$$[R]_{free} = [R]_{total} - [RL]$$

By inputting this into the K_d equation and performing algebraic manipulation, the following equation can be obtained:

$$[RL] = \frac{[R]_{total}\,[L]_{free}}{K_d + [L]_{free}} \quad (1)$$

This equation can be applied to perform curve fitting and determine the apparent K_d of a ligand for its receptor (Sanders, 2010).

Determining K_d

One of the principal goals of this project is to develop aptamers capable of binding the peanut allergen Ara h 1. Several clones were tested to determine which ones are aptamers for Ara h 1, and the K_d of the aptamers were then be determined using a modification of equation (1). In this case, the receptor (R) is Ara h 1 protein and the ligand (L) is the aptamer for Ara h 1. The affinity of the aptamers for Ara h 1 will be assessed by using flow cytometry as described above. However, in order to apply equation (1) to the acquired data to obtain an apparent K_d, several assumptions must be made. It is very difficult to determine the free ligand concentration ($[L]_{free}$), but when the concentration of target ([R]) is much smaller than the K_d, a very small proportion of the total ligand ($[L]_{total}$) will bind to the target. The amount of ligand that binds to the target becomes insignificant, and the assumption that $[L]_{free}$ is equal to $[L]_{total}$ can be made. In this case, the target has been conjugated to a magnetic bead so only an estimation of its concentration is known. This concentration was used to determine the approximate moles of protein conjugated to each bead, which allowed the determination of the molar concentration of the protein in a solution. However, a very small amount of these beads cannot be used as this will prevent efficient detection by flow cytometry, and the protein concentration used was therefore not ideal. Additionally, the effect of having the target bound to a bead (a small mobile surface in solution) on binding kinetics is unknown. These factors made it difficult to ensure that the concentration of target is smaller than the K_d, but it will nonetheless be assumed this is the case and $[L]_{free}$ will be approximated as $[L]_{total}$ (the initial aptamer concentration). Another assumption made concerns measuring the concentration of ligand receptor complex ([RL]). With this experimental design, there are many protein

molecules bound per bead and the fluorescent signal is measured per bead. Each aptamer molecule bound to a protein molecule on a bead will contribute to the fluorescence of that bead. The fluorescent signal given off by a bead is therefore proportional to the concentration of aptamer bound to the target and consequently the concentration of receptor ligand complex ([RL]). [RL] in this equation will thus be approximated as the median fluorescent signal per bead (in relative fluorescent units, or RFU). Lastly, in order to use equation (1), the assumption was made that there is 1:1 binding. This binding ratio also means that $[R]_{total}$ will be proportional to the theoretical maximum amount of receptor-ligand complexes that can form per bead (when all protein molecules on a bead are bound by the ligand). This means that on a graph of fluorescent signal per bead versus ligand concentration, $[R]_{total}$ is proportional to the horizontal asymptote approached as the concentration of ligand increases (B_{max}, also in RFU).

With these three assumptions in mind, equation (1) becomes:

$$y = \frac{B_{max} + x}{K_d + x} \quad (2)$$

Where *y* is the fluorescent signal, B_{max} is the horizontal asymptote approached as x increases, and *x* is the concentration of ligand. This equation will be used to perform curve fitting to determine values for the constants K_d and B_{max} at which the y values calculated with this equation will be as near as possible to experimentally obtained y values. The K_d value obtained will be an apparent K_d, as it is an approximation made using data gathered with this specific method.

Research Goals

Originally, this project aimed to develop aptamers for peanut total protein extract, the peanut allergen Ara h 1 and the peanut allergen Ara h 2. An aptamer against Ara h 1 has

previously been developed by another research group using capillary electrophoresis (CE)-SELEX (Tran et al., 2013). We nonetheless developed aptamers for Ara h 1 for several reasons. Firstly, aptamers with improved affinity for Ara h 1 were desired. Three aptamers were developed by Tran et al., with K_d's of 450 ± 60nM, 1770 ± 140nM and 1210 ± 60nM, respectively (Tran et al., 2013). This project attempted to produce aptamers with K_d's in the low nanomolar (nM) range. Furthermore, multiple aptamers with these improved affinities for Ara h 1 were desired, as these could be used to develop a "sandwich" ELISA assay (Toh et al., 2015). In these assays, one aptamer would be used to bind to the target in solution ("capture" the target), then be immobilized on a surface, at which point the second, labelled aptamer would bind to the target to allow detection (Toh et al., 2015). To create such an assay, two aptamers that bind at different epitopes on the target are required. Additionally, this project did not aim to solely produce aptamers for Ara h 1, but also looked to generate aptamers for Ara h 2 and peanut total protein extract. The selection process for Ara h 2 was unsuccessful, but selection against peanut total protein extract was successful. However, my project focused on testing clones selected for Ara h 1 and this will be the aspect of the project I focus on in this thesis.

The clones I tested for binding to Ara h 1 were obtained after several experiments performed by Dr. Pavel Milman. This included 10 rounds of magnetic bead SELEX against Ara h 1, testing of the pools obtained for binding to Ara h 1 and sequencing. From the sequencing results, 8 clones were ordered. The goal of my project is to test this group of clones and identify Ara h 1 aptamers. After identifying aptamers, I will determine their affinity for Ara h 1.

The apatmers will subsequently be used to develop an aptamer based assay for the detection of the peanut allergen Ara h 1 in food.

Materials and Methods

Preparation of Protein Conjugated Magnetic Beads – PureProteome Carboxy FlexiBind Magnetic Beads (1µm beads in a 10mg/mL slurry) from EMD Millipore were used. Two-step coupling with EDC was performed according to the manufacturer's protocol (EMD Millipore, 2012). Slight modifications were made to the protocol and are described here, with the step they affect in parenthesis. Two separate coupling experiments were performed, one to conjugate Ara h 1 to 1mg of beads and another to conjugate Ara h 2 to 1mg of beads. For Ara h 1 coupling, the protein solution was prepared (step 1) by mixing 70µL of purified Ara h 1 protein solution from ProteinLabs (1.094 mg/mL Ara h 1 in 20 mM Na-phosphate pH 8, 150 mM NaCl and 10% glycerol) with 70µL of Activation/coupling buffer (50mM MES, pH 6.0, and 0.01% Triton X-100 in double distilled water (ddH_20)). For Ara h 2 coupling, the protein solution was prepared by mixing 70µL of a purified Ara h 2 protein solution from ProteinLabs (1 mg/mL Ara h 2 in 20 mM Na-phosphate pH 8 and 10% glycerol) with 70µL of Activation/coupling. A 30µL aliquot of both these protein solutions was taken before coupling for use in the Bradford Assay. In both cases, 100µL of protein solution was used in coupling (step 12) and the bead and protein mixture were incubated for 4 hours with shaking (step 13). The unbound ligand fraction was saved for the Bradford Assay (step 14). The beads were incubated with quench buffer for 30 minutes with shaking (step 17).

Bradford Assay – Linearized Bradford Assays were performed according to Ernst and Zor (Ernst & Zor, 2010) to determine the protein concentration in the protein solutions before and after bead conjugation. All solutions were prepared in triplicates in the wells of a clear 96-well

plate (CoStar). Bovine serum albumin (BSA) solutions were used in a standard curve. A 1µg/µL stock solution of BSA in ddH_20 was prepared from solid BSA (Fisher Bioreagents). A serial dilution of this 1µg/µL stock solution was done in the wells of the 96 well plate to obtain 100µL solutions containing 6 µg, 3 µg, 1.5µg, 0.75 µg and 0 µg of BSA. Three serial dilutions (to produce triplicates) were performed on each of the protein sample solutions (aliquot taken of Ara h 1 solution before conjugation and aliquot of unbound protein fraction). This was done by adding 30µL of the solution to 30µL of ddH_2O in a well of the plate, then adding 30µL of this solution to 30µL of ddH_2O in another well, and repeating this process for a total of 5 solutions. This produced 5 30µL solutions, each a 1 in 2 dilution of the last, for each protein sample solution. These were then completed to 100uL with ddH_2O. 100µL of a 1 in 2.5 dilution (1mL in 1.5mL ddH_2O) of Bradford reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) was added to each well. The solutions were mixed by pipetting up and down. The plate was incubated at room temperature for 5 minutes. A microplate reader (Molecular Devices, Spectra Max M5) was used to obtain absorbance readings at 595nm and 450nm for each each solution. This process was repeated in the same manner in a separate 96 well plate to determine the concentration of the aliquots taken during Ara h 2 conjugation.

Bead Verification with Antibodies – To ensure that protein binding to the carboxy magnetic beads was successful, each set of beads was tested using two primary antibodies and flow cytometry. Purified polyclonal rabbit anti Ara h 1 antibody from Indoor Biotechnologies (anti-Arah1) and purified polyclonal rabbit anti Ara h 2 antibody from Indoor Biotechnologies (anti Ara h 2) were used as primary antibody (1°Ab). Goat F(ab')2 fragment anti-rabbit IgG (H+L) - FITC conjugated (Beckman Coulter Immunotech) was used as secondary antibody (2°Ab).

10µg of beads were added to 100µL of protein binding buffer (1% w/v BSA in DPBS without Ca^{2+} and Mg^{2+}) in a 1.5mL microtube. To separate the supernatant from the beads, the microtube was placed in the magnetic stand (PureProteome), the beads were allowed to migrate to the magnet and the supernatant was removed. 100µL of a 1/500 or 1/250 dilution of the 1° antibody in protein binding buffer was added to the beads and this mixture was incubated at room temperature for 1 hour with shaking. The supernatant was removed. In order to wash the beads, 100µL of protein binding buffer was added to the beads, they were incubated at room temperature for 3 minutes and the supernatant was removed. This wash was repeated 5 times. After washing, 100µL of a 1/300 dilution of the 2° antibody was added to the beads and this mixture was incubated at room temperature for 30 minutes. The supernatant was removed, and another 5 washes were performed as above. 500µL of protein binding buffer was added to the beads, and this solution was transferred to a flow cytometry tube. Analysis with a flow cytometer (Beckman Coulter Gallios) was performed to detect the fluorescent signal from 10 000 beads (events) with excitation at 488nm and detection at 529nm.

Clone preparation – Magnetic bead SELEX against Ara h 1, beginning with an 80 nucleotide DNA library, was performed by Dr. Pavel Milman. The aptamer pools were tested for binding to Ara h 1 and the pools were sent for sequencing. From the sequencing results, 8 oligonucleotides were chosen as possible aptamers against Ara h 1. The 8 oligonucleotides, conjugated to biotin, were ordered from Integrated DNA Technologies. The oligonucleotides were received as a lypholized powder and dissolved in TE buffer (10mM Tris pH 8.0, 0.1mM EDTA, filtered 0.2µm) to create 100 µM stock solutions.

Measurement of Clone Binding to Ara h 1 by Flow Cytometry – The capability of the biotinylated clones to bind to Ara h 1 was tested using the Ara h 1 conjugated magnetic beads (Cb-H1), Ara h 2 conjugated magnetic beads (Cb-H2) and flow cytometry. Solutions with various concentrations of the clones were prepared in 100µL of DNA binding buffer (0.01% w/v triton X-100 DPBS with Ca^{2+} and Mg^{2+}). These solutions were incubated at 95°C for 5 minutes (heat denaturation) and then incubated on ice for 5 minutes (snap cool). 5µg of beads (Cb-H1 or Cb-H2) was mixed with 100 µL of DNA binding buffer in a microtube. The tube was placed in the magnetic stand and the supernatant was removed. The 100µL solutions containing the clones were then mixed with the beads. This mixture was incubated at room temperature with intermittent vortexing for 2 hours. The supernatant was removed, 100µL of streptavidin binding buffer (1% w/v BSA in DPBS with Ca^{2+} and Mg^{2+}) was added to the beads and the solution was incubated at room temperature with shaking for 10 minutes. The supernatant was removed and 100µL of a probe solution (5µg/mL streptavidin conjugated to DTAF (Beckman Coulter) in streptavidin binding buffer) was added to the beads. The mixture was incubated at room temperature for 20 minutes with shaking. The probe solution was removed and the beads were suspended in 500µL of streptaviding binding buffer. Flow cytometry was performed on these solutions to detect fluorescence information on 10 000 beads (events) with excitation at 488nm and emission detected at 529nm.

Measurement of AraH1_R5c17 and AraH1_R5c102 Binding at Various Concentrations - A method similar to the method used to test clone binding was used to create a titration curve for two aptamers, AraH1_R5c17 and AraH1_R5c102. Solutions with various concentrations of one of the aptamers or with no DNA (negative control) were prepared in 100µL of DNA binding

buffer (0.01% w/v triton X-100 in DPBS with Ca^{2+} and Mg^{2+}). These solutions were incubated at 95°C for 5 minutes (heat denaturation) and then incubated on ice for 5 minutes (snap cool). In later experiments, heat denaturation and snap cooling were performed on one high concentration solution of the aptamer. This solution was then used to create the 100µL solutions containing aptamer at various concentrations in DNA binding buffer. 5µg, or in later experiments 2.5µg, of bead solution (Cb-H1) was mixed with 100 µL of DNA binding buffer in a microtube. The tube was placed in the magnetic stand and the supernatant was removed. The 100µL solutions containing the aptamers were then mixed with the beads. This mixture was incubated at room temperature with shaking for time periods ranging from 30 minutes to 3 hours, depending on the experiment. The supernatant was removed, 100µL of streptavidin binding buffer (1% w/v BSA DPBS with Ca^{2+} and Mg^{2+}) was added to the beads and the solution was incubated at room temperature with shaking for 10 minutes. The supernatant was removed and 100µL of a probe solution (5µg/mL streptavidin conjugated to DTAF (Beckman Coulter) in streptavidin binding buffer containing) was added to the beads. The mixture was incubated at room temperature for 20 minutes with shaking. The probe solution was removed. The beads were rinsed by adding 500µL of streptavidin binding buffer to the beads, vortexing, and removing the supernatant. A wash was then performed by adding 100µL of streptavidin binding buffer to the beads and incubating the mixture at room temperature for 10 minutes with shaking. The streptaviding binding buffer was removed, and in some experiments the samples were rinsed and washed a second time. The beads were suspended in 500µL of streptavidin binding buffer and these solutions were transferred to flow cytometry tubes. Flow

cytometry was performed on these solutions to detect fluorescence information on 10 000 beads (events) with excitation at 488nm and emission detected at 529nm.

Titration Curve Analysis – Data obtained for the binding of AraH1_R5c17 and AraH1_R5c102 at various concentrations was used to determine the apparent K_d of the two aptamers. Binding at a given concentration of ligand (x) was measured with the median fluorescence per bead (y_{exp}) as determined by flow cytometry. These values were plotted on Microsoft Excel. A theoretical median fluorescence (y_{calc}) at a given aptamer concentration was calculated in Excel using the equation $y = \frac{B_{max} + x}{K_d + x}$. Values for B_{max} and K_d were randomly assigned at 1. A Chi-square (chi^2) value for each point was calculated by taking the square error between the experimental and theoretical y values ($(y_{exp}-y_{calc})^2$). The sum of these chi^2 values was then determined. The solver function in Microsoft Excel was then used to minimize the sum of chi^2 value by changing the constants B_{max} and K_d in the equation. This yielded a "fitted" equation which gave y_{calc} values near the corresponding y_{exp} values and from which the apparent K_d and B_{max} for the aptamer were determined. The new sum of chi^2 value was divided by the number of data points to give an average chi^2 value per data point.

Verifying Whether AraH1_R5c17 and AraH1_R5c102 bind at the same epitope – To determine whether AraH1_R5c17 and AraH1_R5c102 bind to the same epitope on Ara h 1, another binding experiment was done. The protocol was carried out in the same fashion as the aptamer titrations above, except that some of the 100µL aptamer solutions contained 1nM or 10nM of both AraH1_R5c17 and AraH1_R5c102 in DNA binding buffer.

Results

Estimating amount of Ara h 1 and Ara h 2 conjugated to beads

In order to estimate the amount of protein that had been conjugated to the carboxy magnetic beads, a Bradford Assay was performed. Standard curves were created using solutions containing a known amount of BSA (appendix, figure 9 and 10). Absorbance readings were determined for several dilutions of the two protein solutions: an aliquot taken before binding and an aliquot taken after binding. These values were plotted against the amount of protein solution in the dilution to create a linear plot. The trendline was determined and the slope of this line, along with the slope of the line in the standard curve, was used to determine the concentration of protein in the solution. For testing the beads conjugated to Ara h 1, the standard curve had a slope of 0.3565/µg (appendix, figure 9), the slope of the line for the protein solution before conjugation was 0.5199/µL and the slope of the line for the protein solution after conjugation was 0.4786/µL (figure 1). By dividing the slope of the line representing the solution obtained before conjugation by the slope of the standard curve, the concentration of Ara h 1 in this solution was found to be 1.4583 µg/µL. By performing the same division for the slope of the line of representing the solution of unbound protein, the concentration of Ara h 1 in this solution was found to be 1.3425µg/µL. Considering there was 100µL of each solution, 145.8µg of Ara h 1 was present before conjugation and 134.3µg of Ara h 1 was present after conjugation, meaning about 11.5µg of Ara h 1 became conjugated to the beads. The Ara h 1 concentration is therefore 11.5µg/mg of bead or 11.5 ng/µg of bead. Ara h 1 has a molecular weight of 63500 Da. By dividing the mass per bead by this molecular weight, the number of moles of Ara h 1 per bead was determined to be 0.18pmol/µg of bead.

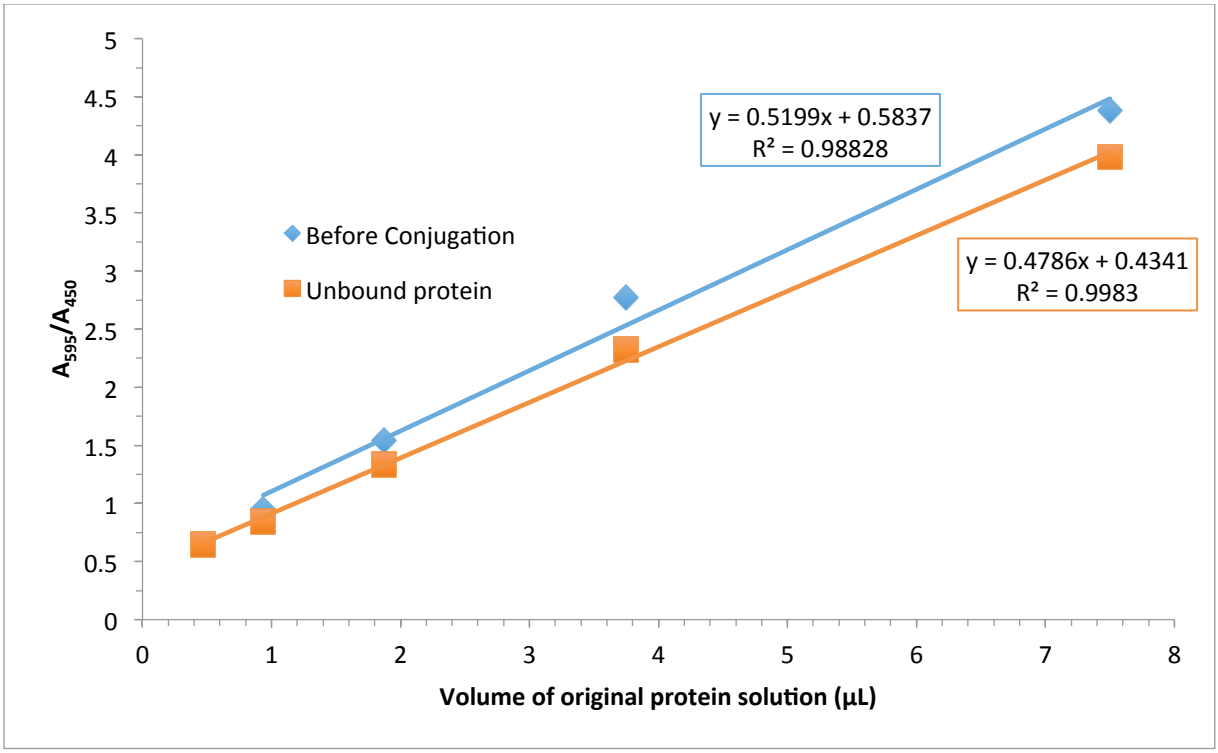

Figure 1. 11.5µg of Ara h 1 was conjugated to 1mg of carboxy beads according to a Bradford Assay. Purified Ara h 1 protein (ProteinLabs) was conjugated to PureProteome Carboxy FlexiBind Magnetic Beads (1µm beads) according to the manufacturer's protocol. Aliquots of the protein solution before conjugation and of the unbound protein fraction after conjugation were taken. Serial dilutions were performed to obtain solutions that contained varying volumes of these aliquots. Absorbance readings at 595nm and 450nm were obtained for these solutions and the BSA standards (results shown in appendix, figure 9) using the Spectra Max M5 plate reader (Molecular Devices). The volume of original protein solution in the dilution is presented on the x axis (in µL), and the quotient obtained by dividing the absorbance readings at 595nm (A_{595}) by those at 450nm (A_{450}) is presented on the y axis (no units). For the protein solution before conjugation, the trendline equation is y = 0.5199x + 0.5837, with a linear regression of R^2 = 0.98828. For the unbound protein solution, the trendline equation is y = 0.4786x + 0.4341, with a linear regression of R^2 = 0.9983. Using this data and the standard curve, the Ara h 1 concentration per bead was determined to be 11.5 ng/µg of bead.

When testing the Ara h 2 protein solutions, the standard curve had a slope of 0.4044/µg (appendix, figure 10), the slope of the line for the protein solution before conjugation was 0.1015/µL and the slope of the line for the protein solution after conjugation was 0.0976/µL (figure 2). By dividing the slope of the line before conjugation by the slope of the standard curve, the concentration of Ara h 2 in the solution before conjugation was found to be 0.2510µg/µL. By performing the same division on the slope of the line of the unbound protein, the concentration of Ara h 2 in the unbound protein solution was found to be 0.2413µg/µL. Considering there was 100µL of each solution, 25.10µg of Ara h 2 was present before conjugation, and 24.13µg of Ara h 2 was present after conjugation, meaning about 0.97µg of Ara h 2 became conjugated to the beads. The Ara h 2 concentration is therefore 0.97µg/mg of bead or 0.97ng/µg of bead. The molecular weight of Ara h 2 is 17 000Da, and using this value the number of moles of Ara h 2 per bead was determined to be 0.057pmol/µg of bead.

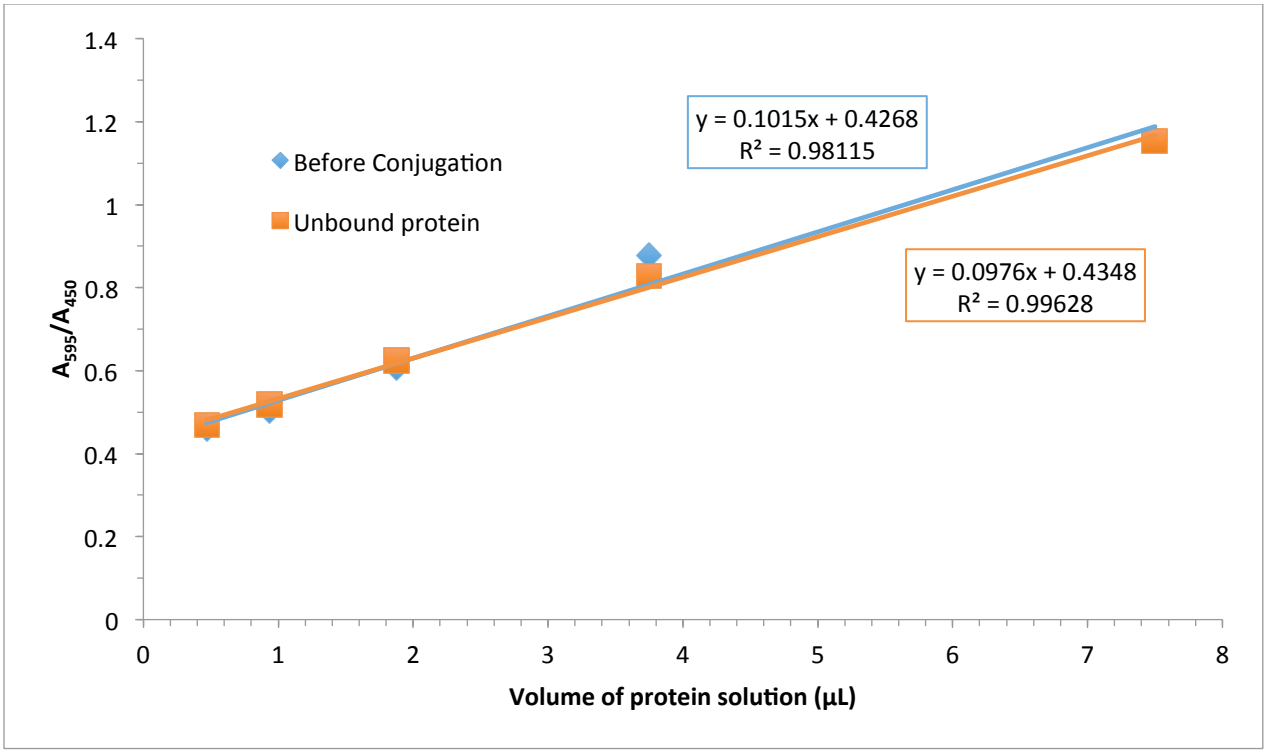

Figure 2. 0.96µg of Ara h 2 conjugated to 1mg of carboxy beads according to Bradford Assay. Purified Ara h 2 protein (ProteinLabs) was conjugated to PureProteome Carboxy FlexiBind Magnetic Beads (1µm beads) according to the manufacturer's protocol. Aliquots of the protein solution before conjugation and of the unbound protein fraction after conjugation were taken. Serial dilutions were performed to obtain solutions that contained varying volumes of these aliquots. Absorbance readings at 595nm and 450nm were obtained for these solutions and the BSA standards (results shown in appendix, figure 10) using the Spectra Max M5 plate reader (Molecular Devices). The volume of original protein solution in the dilution is presented on the x axis (in µL), and the quotient obtained by dividing the absorbance readings at 595nm (A_{595}) by those at 450nm (A_{450}) is presented on the y axis (no units). For the protein solution before conjugation, the trendline equation is y = 0.1015x + 0.4268, with a linear regression of R^2 = 0.98115. For the unbound protein solution, the trendline equation is y = 0.0976x + 0.4348, with a linear regression of R^2 = 0.99628. Using this data and the standard curve, the Ara h 2 concentration per bead was determined to be 0.97ng/µg of bead.

Verifying conjugation of Ara h 1 and Ara h 2 to beads

To ensure that Ara h 1 and Ara h 2 were successfully conjugated to the carboxy magnetic beads, the beads were tested using anti-Arah1 and anti-Arah2 antibodies. The beads were tested with rabbit anti-Arah1 and rabbit anti-Arah2 antibodies, using goat anti-rabbit conjugated to FITC as secondary antibody. Flow cytometry results (figure 3) indicate that conjugation of the proteins to the beads was successful. The anti-Arah1 antibodies showed binding to the beads conjugated with Ara h 1 (CB-H1), indicating that Ara h 1 was present on these beads, while the anti-Arah2 antibodies, in this case used as a negative control, did not bind to these beads. The anti-Arah2 antibodies showed binding to the beads conjugated with Ara h 2 (CB-H2), indicating that Ara h 2 was present on the beads, while the anti-Arah1 antibodies, in this case used as a negative control, did not bind to these beads. A much larger signal was seen for anti-Arah1 antibodies binding to CB-H1 beads than for anti-Arah2 antibodies binding to CB-H2 beads, suggesting more Ara h 1 protein was successfully conjugated to the beads than Ara h 2 protein.

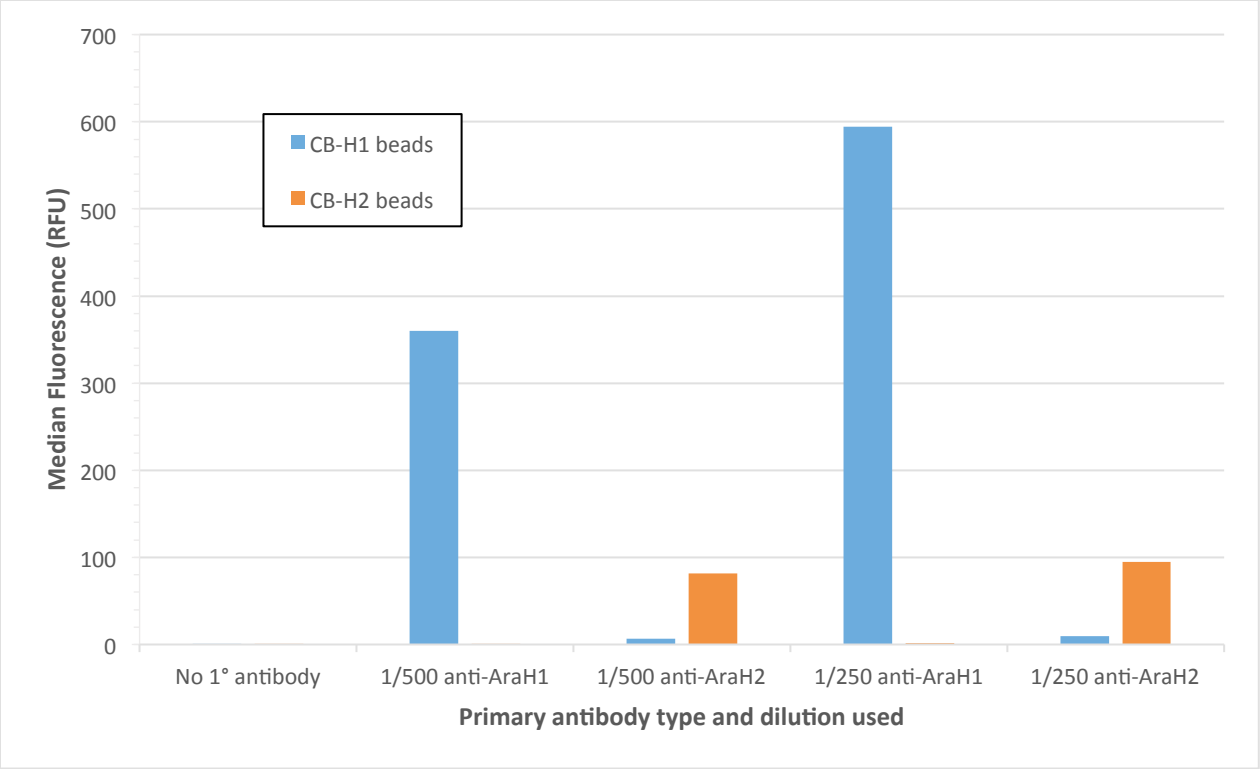

Figure 3. Testing with antibodies and flow cytometry show conjugation of Ara h 1 and Ara h 2 to carboxy beads was successful. Purified Ara h 1 and Ara h 2 protein were conjugated to separate sets of PureProteome Carboxy FlexiBind Magnetic Beads (1µm beads) according to the manufacturer's protocol. Anti-Ara h 1 antibody (purified polyclonal rabbit anti Ara h 1 from Indoor Biotechnologies) and anti-Ara h 2 antibody (purified polyclonal rabbit anti Ara h 2 from Indoor Biotechnologies) were used as primary antibodies (1°Ab) to test whether conjugation of the proteins to the bead was successful. A fluorescently labelled anti-rabbit antibody (Goat anti-rabbit IgG conjugated to FITC, from Beckman Coulter Immunotech) was used as secondary antibody (2°Ab). 10µg of beads were used. 1/500 or 1/250 dilutions of the 1° antibodies in protein binding buffer (1% w/v BSA in DPBS without Ca^{2+} and Mg^{2+}) were used and a 1/300 dilution of the 2° antibody in protein binding buffer was used. Flow cytometry was performed to detect fluorescence information on 10 000 beads (events) with excitation at 488nm and emission detected at 529nm. The median bead fluorescence at 529nm in relative fluorescence units (RFU) is presented on the y axis and the type and dilution of primary antibody used is presented on the x axis. Results obtained for the Ara h 1 conjugated beads (CB-H1, blue) and Ara h 2 conjugated beads (CB-H2, orange) are presented.

Testing binding of 8 clones to Ara h 1

The ability of the 8 clones (see appendix for sequences), obtained after *in vitro* selection with magnetic bead SELEX, to bind to Ara h 1 was assessed using CB-H1 beads. The clones were also mixed with CB-H2 beads as a negative control and to ensure specificity. Flow cytometry results (figure 4) indicate that two clones, AraH1_R5c17 and AraH1_R5c102, bind to Ara h 1. These clones display higher binding to Ara h 1 than to Ara h 2. The other clones to not bind significantly to Ara h 1, as they showed approximately the same amount of binding to Ara h 1 and Ara h 2. All of the clones showed about the same amount of binding to the Ara h 2 beads.

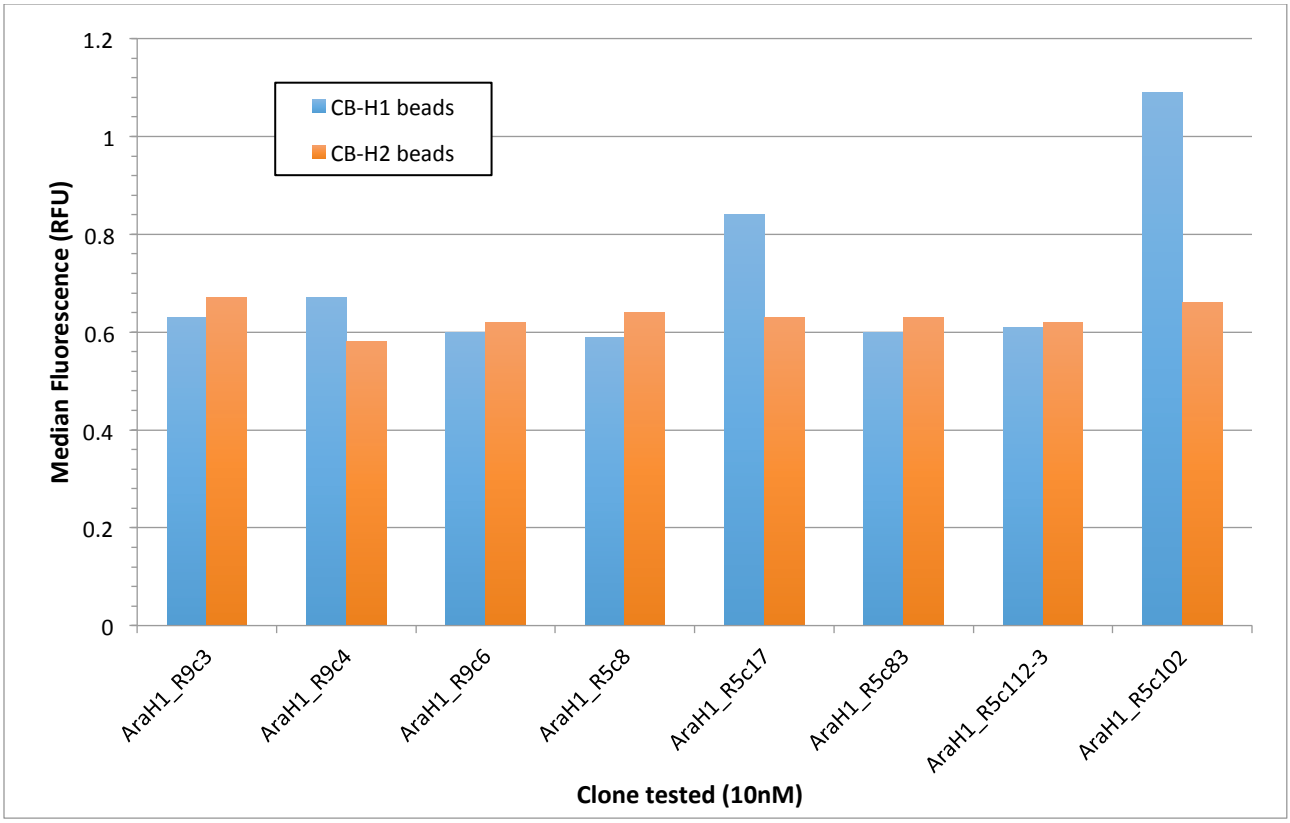

Figure 4. Testing binding of clones to Ara h 1 and Ara h 2 shows that AraH1_R5c17 and AraH1_R5c102 bind to Ara h 1. Two 100µL solutions were prepared for each of the 8 biotinylated clones (see appendix for sequences). These solutions contained 10nM of the clone in DNA binding buffer (DPBS with Ca^{2+} and Mg^{2+}, 0.01% triton X-100). The solutions were heat denatured (95°C for 5 minutes) then snap cooled (5 minutes on ice). For all clones, one 100µL solution was mixed with 5µg of Ara h 1 conjugated beads (CB-H1 beads) and the other 100µL solution was mixed with 5µg of Ara h 2 conjugated beads (CB-H2 beads). These mixtures were incubated for 2 hours. A probe solution consisting of 5µg/mL of fluorescently labelled streptavidin in streptavidin binding buffer (1% w/v BSA in DPBS with Ca^{2+} and Mg^{2+}) was used to conjugate a fluorescent label to the clones. After the probe solution as removed, the beads were suspended in 500µL of streptavidin binding buffer. Flow cytometry was performed on these solutions to detect fluorescence information on 10 000 beads (events) with excitation at 488nm and emission detected at 529nm. The median bead fluorescence at 529nm in relative fluorescence units (RFU) is presented on the y axis and the clone tested is presented on the x axis.

Determining affinity of aptamers for Ara h 1

After AraH1_R5c17 and AraH1_R5c102 were identified as aptamers, their affinity for Ara h 1 was determine by performing a titration. Curve fitting was then performed to determine the apparent K_d of the aptamers and thus estimate their affinity for Ara h 1. In the first of these titrations, the binding of each aptamer to Ara h 1 at concentrations ranging from 0.1nM to 500nM was assessed (figure 5 and figure 6). After curve fitting was performed using the titration curve for AraH1_R5c17 (figure 5), the apparent K_d of AraH1_R5c17 was found to be 59.5nM, the B_{max} was found to be 1.64 RFU, and the average chi^2 value per point was 0.0058. Curve fitting was also performed using the titration curve for AraH1_R5c102 (figure 6). The apparent K_d of AraH1_R5c102 was found to be 5.31nM, the B_{max} was found to be 1.88 RFU, and the average chi^2 value per point was 0.0065. AraH1_R5c102 therefore has a higher affinity for Ara h 1 than AraH1_R5c17, but both have K_d's in the nanomolar range.

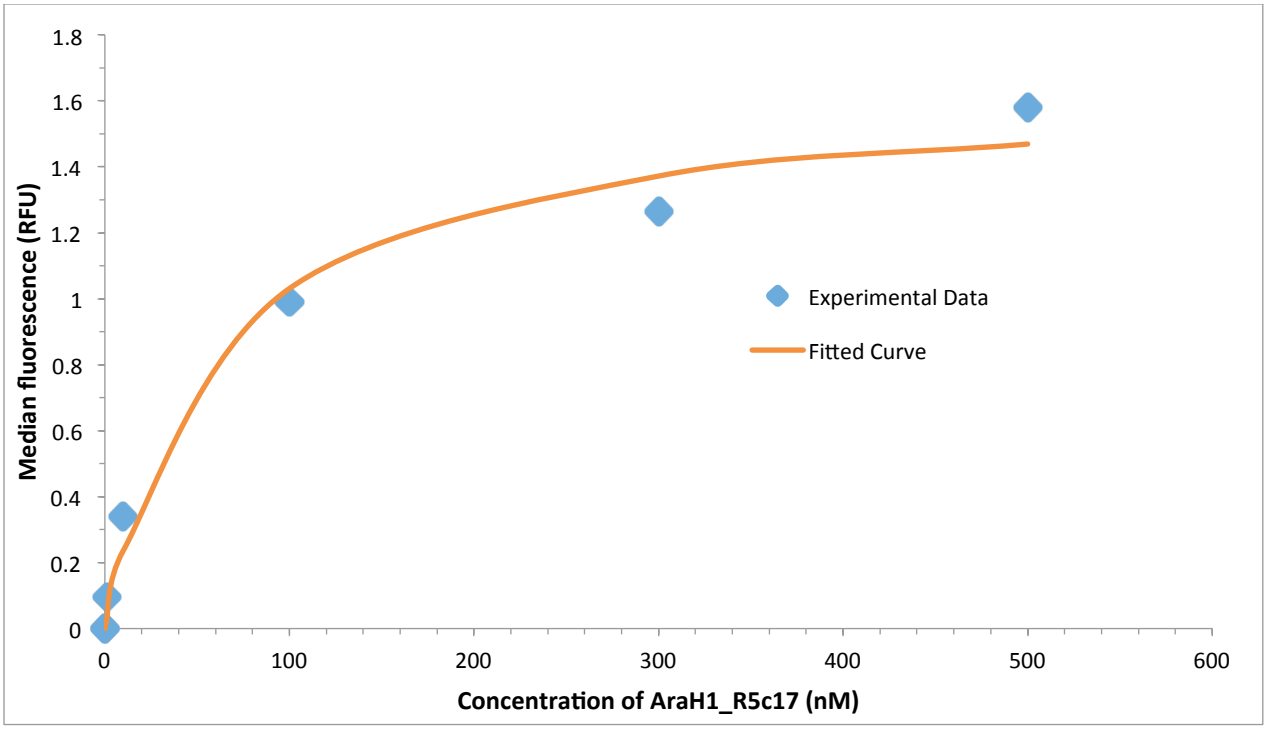

Figure 5. Titration curve of AraH1_R5c17 shows it binds to Ara h 1 with an apparent K_d of 59.5nM. Solutions of the biotinylated aptamer AraH1_R5c17 at various concentrations in DNA binding buffer (DPBS with Ca^{2+} and Mg^{2+}, 0.01% triton X-100) were prepared, as well as a negative control consisting of DNA binding buffer with no DNA. The solutions were heat denatured (95°C for 5 minutes) then snap cooled (5 minutes on ice). The aptamer solutions were added to 5µg of Ara h 1 conjugated beads (CB-H1 beads) and the mixture was incubated for 20 minutes. A probe solution consisting of 5µg/mL of fluorescently labelled streptavidin in streptavidin binding buffer (1% w/v BSA in DPBS with Ca^{2+} and Mg^{2+}) was used to conjugate a fluorescent label to the aptamers. After the probe solution was removed, the beads were washed twice with streptavidin binding buffer, then suspended in 500µL of streptavidin binding buffer. Flow cytometry was performed on these solutions to detect fluorescence information on 10 000 beads (events) with excitation at 488nm and emission detected at 529nm. The median bead fluorescence at 529nm in relative fluorescence units (RFU) is presented on the y axis and the concentration of AraH1_R5c17 is presented on the x axis. The experimental data (in blue) was normalized by subtracting the median fluorescence for each sample by the median fluorescence for the control sample (no DNA present). Curve fitting was performed using this data and the resultant curve is displayed in orange. As determined by curve fitting, for AraH1_R5c17 the apparent K_d is 59.5nM and the B_{max} is 1.64 RFU, with an average chi^2 of 0.0058 per point.

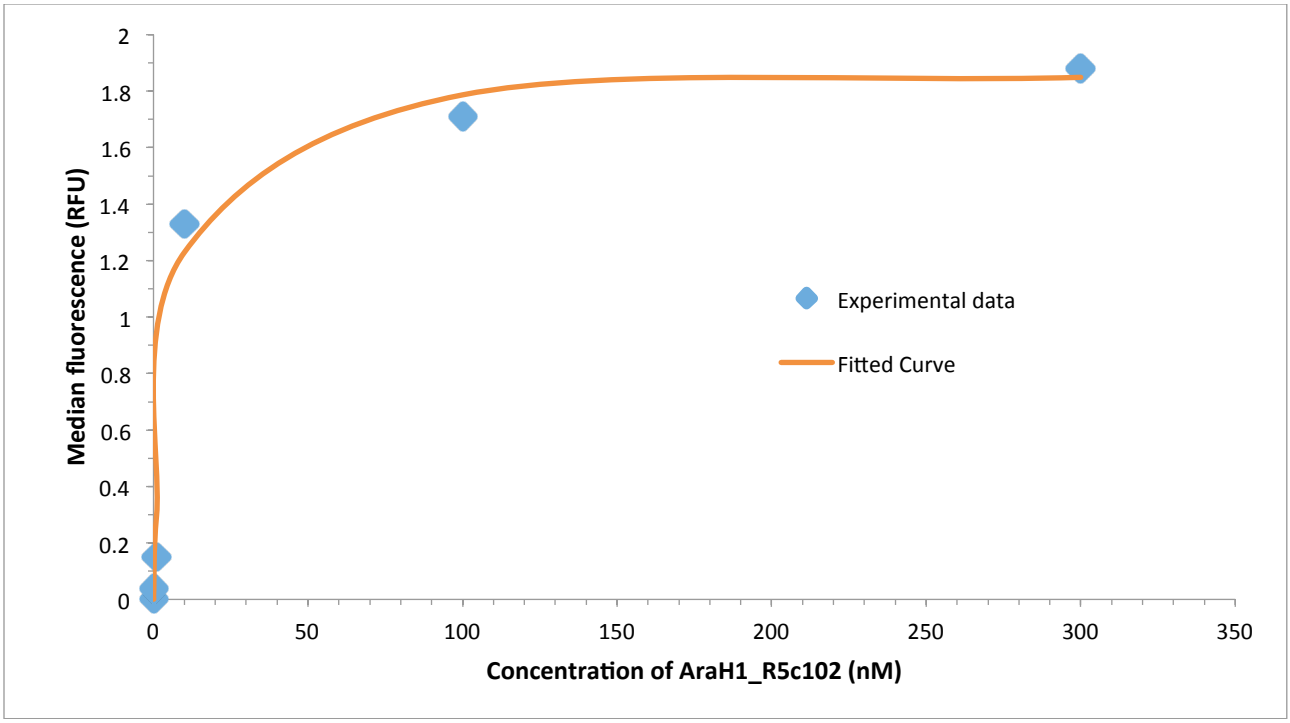

Figure 6. Titration curve of AraH1_R5c102 shows it binds to Ara h 1 with an apparent K_d of 5.31nM. Solutions of the biotinylated aptamer AraH1_R5c102 at various concentrations in DNA binding buffer (DPBS with Ca^{2+} and Mg^{2+}, 0.01% triton X-100) were prepared, as well as a negative control consisting of DNA binding buffer with no DNA. The solutions were heat denatured (95°C for 5 minutes) then snap cooled (5 minutes on ice). The aptamer solutions were added to 5µg of Ara h 1 conjugated beads (CB-H1 beads) and the mixture was incubated for 20 minutes. A probe solution consisting of 5µg/mL of fluorescently labelled streptavidin in streptavidin binding buffer (1% w/v BSA in DPBS with Ca^{2+} and Mg^{2+}) was used to conjugate a fluorescent label to the aptamers. After the probe solution was removed, the beads were washed once with streptavidin binding buffer, then suspended in 500µL of streptavidin binding buffer. Flow cytometry was performed on these solutions to detect fluorescence information on 10 000 beads (events) with excitation at 488nm and emission detected at 529nm. The median bead fluorescence at 529nm in relative fluorescence units (RFU) is presented on the y axis and the concentration of AraH1_R5c102 is presented on the x axis. The experimental data (in blue) was normalized by subtracting the median fluorescence for each sample by the median fluorescence for the control sample (no DNA present). Curve fitting was performed using this data and the resultant curve is displayed in orange. As determined by curve fitting, for AraH1_R5c102 the apparent K_d is 5.31nM and the B_{max} is 1.88 RFU, with an average chi^2 of 0.0065 per point.

After these initial titrations, an addition titration was performed using AraH1_R5c102 at concentrations near its estimated K_d. For this titration, the concentrations of AraH1_R5c102 ranged from 0.9766nM to 31.25nM (figure 7). Using curve fitting, the apparent K_d of AraH1_R5c17 was found to be 11.2nM and the B_{max} was found to be 1.64 RFU, with an average chi^2 value of 8.0×10^{-5} per point. Curve fitting for this titration curve resulted in a lower chi^2 value than was obtained with previous titration curves. This titration also indicates that the affinity of AraH1_R5c102 for Ara h 1 is slightly lower than was estimated with the titration shown in figure 6.

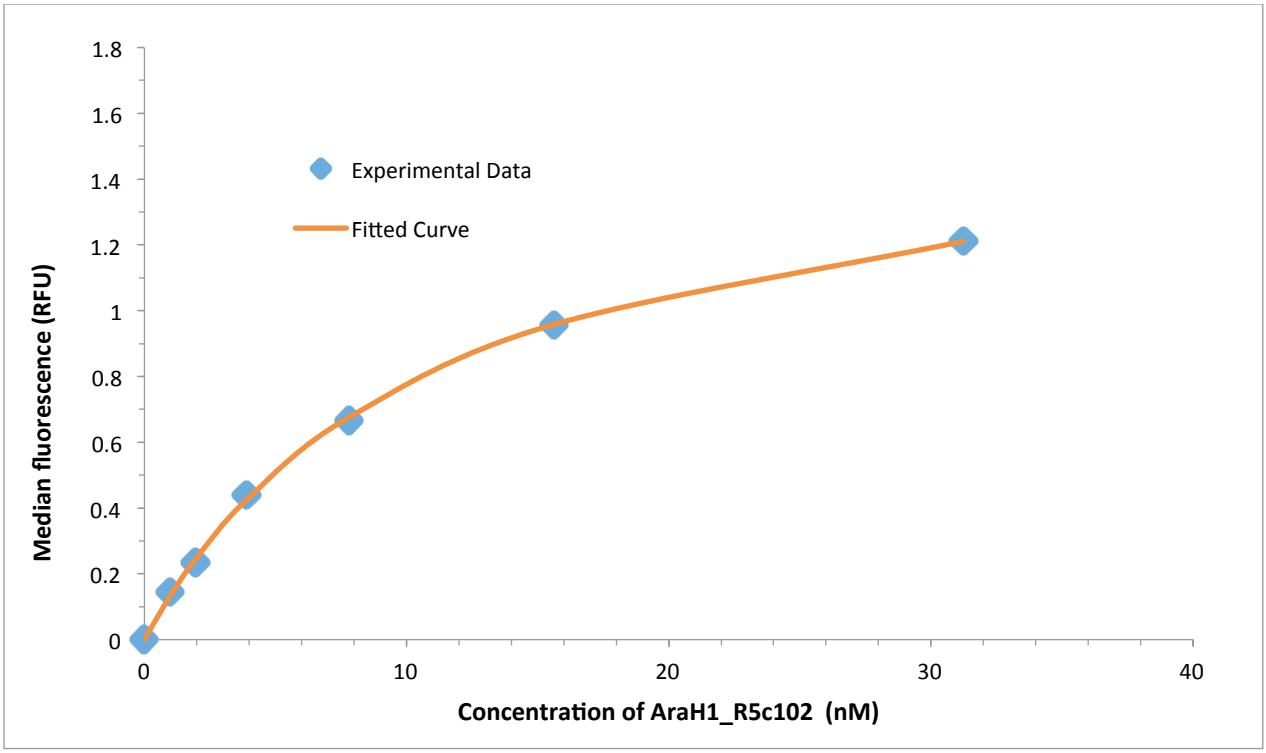

Figure 7. Titration curve of AraH1_R5c102 over a small concentration range shows it binds to Ara h 1 with an apparent K_d of 11.2nM. A 500nM solution of the biotinylated aptamer AraH1_R5c102 in DNA binding buffer (DPBS with Ca^{2+} and Mg^{2+}, 0.01% triton X-100) was prepared, as well as a negative control consisting of DNA binding buffer with no DNA. These two solutions were heat denatured (95°C for 5 minutes) then snap cooled (5 minutes on ice). The 500nM solution of aptamer was diluted to create aptamer solutions with various concentrations. The aptamer solutions were added to 2.5µg of Ara h 1 conjugated beads (CB-H1 beads) and the mixture was incubated for 3 hours. A probe solution consisting of 5µg/mL of fluorescently labelled streptavidin in streptavidin binding buffer (1% w/v BSA in DPBS with Ca^{2+} and Mg^{2+}) was used to conjugate a fluorescent label to the aptamers. After the probe solution was removed, the beads were washed once with streptavidin binding buffer, then suspended in 500µL of streptavidin binding buffer. Flow cytometry was performed on these solutions to detect fluorescence information on 10000 beads (events) with excitation at 488nm and emission detected at 529nm. The median bead fluorescence at 529nm relative fluorescence units (RFU) is presented on the y axis and the concentration of AraH1_R5c102 is presented on the x axis. The experimental data (in blue) was normalized by subtracting the median fluorescence for each sample by the median fluorescence for the control sample (no DNA present). Curve fitting was performed using this data and the resultant curve is displayed in orange. As determined by curve fitting, for AraH1_R5c102 the apparent K_d is 11.2nM and the B_{max} is 1.64 RFU, with an average chi^2 value of 8.0×10^{-5} per point.

Determining whether the aptamers bind at the same epitope on Ara h 1

In order to verify whether AraH1_R5c17 and AraH1_R5c102 could be used together in a "sandwich" ELISA assay, the two aptamers were tested to determine whether they bind at the same epitope on Ara h 1. Binding to Ara h 1 for AraH1_R5c17 alone, AraH1_R5c102 alone and both aptamers together was assessed (figure 8). At both 1nM and 10nM, the highest fluorescent signal was observed with AraH1_R5c102 alone. Additive binding was not observed, as a slightly smaller median fluorescence was observed when both aptamers were present in solution compared to when only AraH1_R5c102 was present. This result suggests that the two aptamers are unable to simultaneously bind to the same molecule of Ara h 1 and thus bind to the same epitope on Ara h 1.

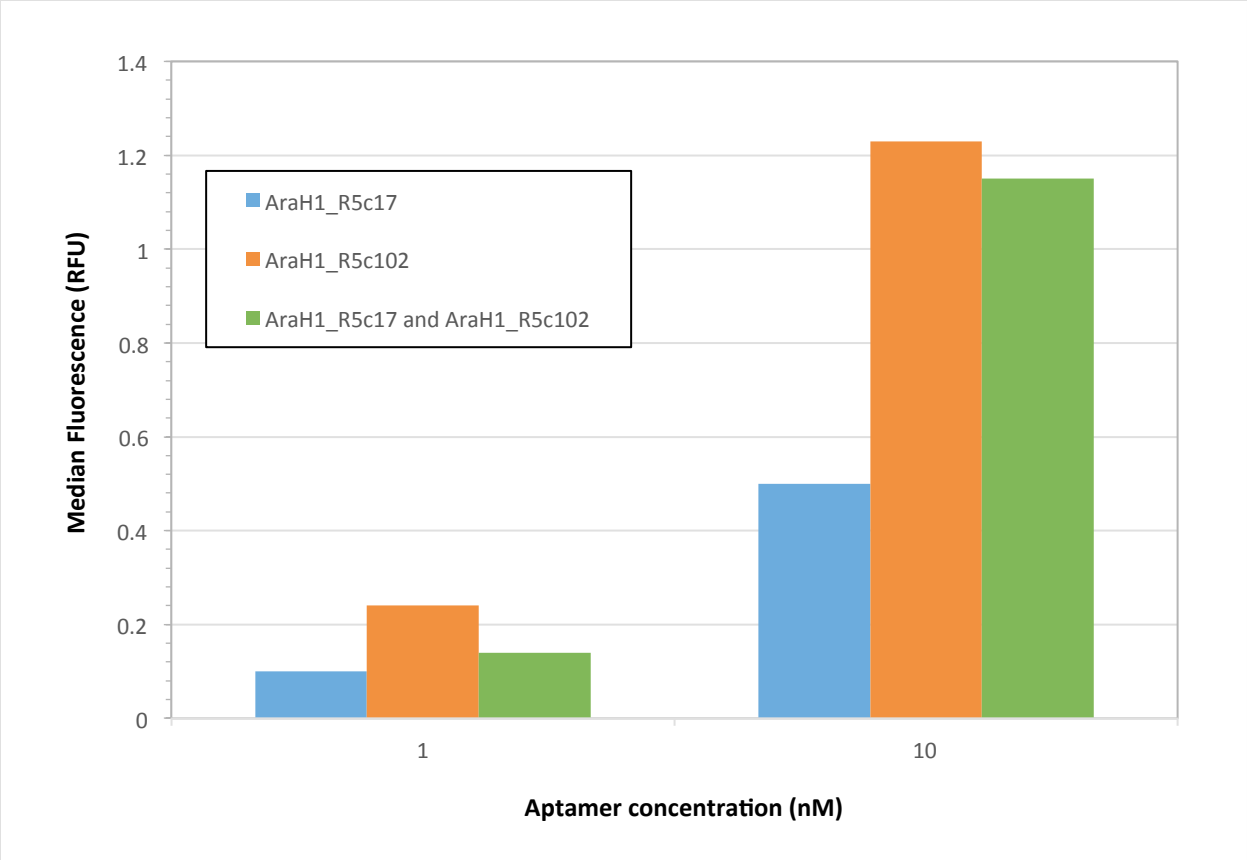

Figure 8. AraH1_R5c17 and AraH1_R5c102 bind to the same epitope on Ara h 1. 1nM and 10nM solutions of the biotinylated aptamers AraH1_R5c17 and AraH1_R5c102, alone and in combination, in DNA binding buffer (DPBS with Ca^{2+} and Mg^{2+}, 0.01% triton X-100) were prepared. A negative control consisting of DNA binding buffer with no DNA was also prepared. The solutions were heat denatured (95°C for 5 minutes) then snap cooled (5 minutes on ice). The aptamer solutions were added to 5µg of Ara h 1 conjugated beads (CB-H1 beads) and the mixture was incubated for 20 minutes. A probe solution consisting of 5µg/mL of fluorescently labelled streptavidin in streptavidin binding buffer (1% w/v BSA in DPBS with Ca^{2+} and Mg^{2+}) was used to conjugate a fluorescent label to the aptamers. After the probe solution was removed, the beads were washed once with streptavidin binding buffer, then suspended in 500µL of streptavidin binding buffer. Flow cytometry was performed on these solutions to detect fluorescence information on 10 000 beads (events) with excitation at 488nm and emission detected at 529nm. The median bead fluorescence at 529nm in relative fluorescence units (RFU) is presented on the y axis and the concentration of aptamer is presented on the x axis. The data was normalized by subtracting the median fluorescence for each sample by the median fluorescence for the control sample (no DNA present). The median fluorescence for AraH1_R517 alone (blue), AraH1_R5102 alone (orange), and the two aptamers in combination, both at the indicated concentration (green), is presented. Additive binding does not occur when both aptamers are present in solution, therefore competitive binding is most likely occurring.

Discussion

The present study aimed to test 8 clones for binding to Ara h 1 in order to determine if any were aptamers for Ara h 1. It also aimed to determine the affinity of any identified aptamers for Ara h 1. The first step of the study involved conjugating magnetic beads to both Ara h 1 and Ara h 2. Using these beads, the ability of the clones to bind to Ara h 1 was assessed. After conjugation, two different methods were used to analyze the beads: one to determine the amount of protein bound to the beads, and the other to confirm that the correct proteins were conjugated to the beads. First, a Bradford Assay was used to determine the amount of protein bound to the beads. Using the Bradford Assay, the protein content in the protein solution before it was mixed with the beads and the protein solution after it was mixed with the beads was determined. By subtracting the protein content in the protein solution before binding by the protein content in that solution after binding, the total amount of protein that was conjugated to the beads could be determined. This value was used to obtain an estimation of the average amount of protein bound per bead. The Bradford assay indicated that about 11.5µg (0.18nmol) of Ara h 1 protein became bound to 1mg of beads, and 0.97µg (0.057nmol) of Ara h 2 protein became bound to 1mg of beads. Although the same protocol was carried out for both conjugation experiments, the Ara h 1 beads contain more protein per bead than the Ara h 2 beads. This may be because, according to the Bradford Assay, the initial protein solution of Ara h 2 contained less protein (about 0.25µL/µL) than the initial Ara h 1 protein solution (1.46µg/µL). These initial protein solutions were both ½ dilutions of 1µg/µL purified protein solutions obtained from a manufacturer, so their expected concentrations are about 0.5µg/µL. However, the Bradford Assays indicated that this was not the case. This

discrepancy may have been caused by inaccuracies in the Bradford Assay, inaccurate concentration values for the purified protein from the manufacturer, or the denaturation of protein during storage.

The second test performed on the beads employed antibodies against Ara h 1 and Ara h 2 to ensure that the correct protein was conjugated to the beads. For the Ara h 1 conjugated beads (CB-H1 beads), the anti-Arah1 antibodies showed binding, while the anti-Arah2 antibodies did not bind (figure 3). For the Ara h 2 conjugated beads (CB-H2 beads), the anti-Arah1 antibodies did not bind, while the anti-Arah2 antibodies showed binding (figure 3). These results indicate that the desired protein successfully became bound to the magnetic beads. They also support the Bradford Assay results that suggest a larger amount of Ara h 1 (11.5µg) became bound to the beads than did Ara h 2 (0.97µg) since the test showed that there is a much weaker signal generated by the anti-Arah2 antibodies binding to the CB-H2 beads than by the anti-Arah1 antibodies binding to the CB-H1 beads.

After the target was successfully conjugated to the magnetic beads, these beads were used to test the ability of various clones to bind to Ara h 1. The 8 biotinylated clones were incubated separately with both CB-H1 beads and CB-H2 beads, then the beads were probed with fluorescently conjugated streptavidin and analyzed by flow cytometry (figure 4). The fluorescent signal given off by the beads is indicative of the amount of clone that became bound to the protein on the beads. All 8 clones showed some binding to the CB-H2 beads. The clones were selected against Ara h 1, so it is unlikely that they are binding to the Ara h 2 protein at this concentration. Instead, the signal seen with CB-H2 beads is most likely due to non-specific binding of the clones to the surface of the beads. This signal is therefore set as the

"baseline" signal, the signal seen even when no binding to protein is occurring. For 6 of the clones, the fluorescent signal seen with the CB-H1 beads was at the baseline fluorescent signal seen with the CB-H2 beads. This indicates that these clones did not bind Ara h 1. This was not surprising, as it was expected that the majority of the clones selected for testing would not bind to the target, as this has been the case in previous aptamer selections (Tsuji et al., 2009). Two of the clones showed a higher fluorescent signal with CB-H1 beads compared to the signal seen with CB-H2 beads. These results suggest that these two clones, AraH1_R5c17 and AraH1_R5c102, bind to Ara h 1. These two aptamers were subjected to further analysis to determine their affinity for Ara h 1.

Titration curves were created for both AraH1_R5c17 and AraH1_R5c102 by testing binding to Ara h 1 at various concentrations of the aptamers. Curve fitting was then performed on these curves, using equation (2), to determine the apparent K_d of the aptamers. Using 7 concentrations ranging from 0 to 500nM, a titration curve for AraH1_R5c17 was created (figure 5). By performing curve fitting on this data, the apparent K_d was found to be 59.5nM. Curve fitting also allowed determination of the B_{max} value. B_{max} is representative of the horizontal asymptote approached as the concentration of aptamer increases. This horizontal asymptote is indicative of the maximum fluorescence per bead that can be observed, and is thus proportional to the amount of protein bound to each bead. For AraH1_R5c17, B_{max} was 1.64 RFU. The average chi^2 value per data point is indicative of how well the fitted curve (theoretical data) conforms with the experimental data. The chi^2 value is calculated by finding the square error between an experimentally obtained data point and a data point calculated with equation (2). The lower the chi^2 value, the more accurately the fitted titration curve reflects the

experimentally obtained curve. In this instance, the average chi^2 per point was 0.0058. The apparent K_d of 59.5nM determined for AraH1_R5c102 indicates that this aptamer has a high affinity for Ara h 1, and the aptamer would be appropriate for use in an ELISA assay.

Using 6 concentrations ranging from 0 to 300nM, a titration curve for AraH1_R5c102 was created (figure 6). Using curve fitting, the apparent K_d was determined to be 5.31nM and the B_{max} was determined to be 1.88 RFU. The average chi^2 per point was 0.0065. This indicates that this aptamer has an extremely high affinity for Ara h 1, even higher than that of AraH1_R5c17, and is able to bind to the protein at very low concentrations. It could thus be used in an ELISA assay. To obtain an improve measurement of the apparent K_d of AraH1_R5c102, another titration of this aptamer was performed using 7 concentrations in the range of 0 to 31nM (figure 7). These concentrations are near the apparent K_d estimated for this aptamer using the data shown in figure 6. By performing curve fitting using this newly acquired data, a more accurate apparent K_d was obtained. The apparent K_d determined using this data, 11.2nM, was slightly higher than the previously determined apparent K_d. The B_{max} was slightly lower at 1.64 RFU. The average chi^2 value per point was much smaller at 8.0×10^{-5}. This average chi^2 value is much lower than the average chi^2 values obtained in the two previous titrations, and indicates that the fitted curve conforms very well with the experimental data. The fitted curve and, by extension, the apparent K_d, are therefore accurate reflections of the data obtained. The B_{max} obtained from this curve is the same as the B_{max} obtained with the titration for AraH1_R5c17. This is expected, as B_{max} is indicative of the maximum possible fluorescent signal per bead and thus the maximum number of targets the aptamer can bind to

per bead. The similar B_{max} values suggest there is the same amount of protein per bead in both cases.

It is important to note that there were differences between the early titration curve experiments, whose results are shown in figures 5 and 6, and the later titration curve experiment whose results are shown in figure 7. In the first experiments, the beads and aptamer solution were incubated together for 20 minutes, while in the later experiment they were incubated for 3 hours. The incubation time was lengthened to ensure that equilibrium was reached for binding of the aptamer to the protein target. In order for an accurate K_d value measurement, equilibrium must be reached, as K_d is the equilibrium constant for the dissociation of the ligand from its target (Sanders, 2010). The data obtained with a 3 hour incubation was better able to fit equation (1), as curve fitting yielded a smaller average chi^2 value per point compared to curve fitting with the data obtained with a 20 minute incubation. This suggests that a 3 hour incubation will allow equilibrium to be reached. In all future repetitions of the titration experiments, a 3 hour incubation will be used.

The second difference between the early and late titration experiments is the amount of beads used. In the first experiment, 5µg of CB-H1 beads was used, while in the second experiment 2.5µg of CB-H1 beads was used. Initially, 5µg was used to ensure there was a large enough amount of beads in solution for detection by flow cytometry. 2.5µg of beads was then tested and this amount of beads was also detectible with flow cytometry. A small amount of beads was desired as this would mean a small amount of protein was being used. To make the assumption that $[L]_{free}$ is equal to $[L]_{total}$ in equation (1), the concentration of protein (the target) should be smaller than the K_d. There are 0.18pmol of Ara h 1 per µg of bead. At 5µg, 0.9pmol

of protein is present in a 100µL solution, meaning 9nM of protein is present. At 2.5µg, 0.45pmol of protein is present in a 100µL solution, so 4.5nM of protein is present. With both 5µg and 2.5µg of beads, the concentration of protein is in the same range as the apparent K_d's (59.5nM and 11.2nM). This is not ideal, and could contribute to inaccuracies in measuring K_d. To remedy this, the experiments could be repeated with a smaller amount of beads, but if too few beads are present in solution there is the risk that the beads will not be accurately detected by flow cytometry. Another option would be to repeat the conjugation of Ara h 1 to the beads using less Ara h 1 in the initial solution, and thus obtain beads conjugated to a smaller amount of Ara h 1. However, due to time limitations these changes were not performed, and the K_d's obtained using the assumption that $[L]_{free}$ is equal to $[L]_{total}$ were deemed adequate.

The difference in the amount of beads between experiments may also affect our ability to compare results from the early titrations with results from the later titrations. With 2.5µg of beads, there are less beads present in solution and thus less target for the aptamer to bind to than with 5µg of beads, so the aptamer may bind to more protein per bead. An increase in the median fluorescence per bead would therefore be expected when a smaller amount of beads is used. However, this expected increase in median fluorescence is not seen when comparing the titrations of AraH1_R5c102 in figures 6 and 7. Other factors, such as the B_{max}, will not be affected by a reduced amount of beads. B_{max} is representative of the amount of protein bound to each bead. The amount of protein bound to each bead does not change when a smaller amount of beads is used, so B_{max} will not change.

These changes in protocol make it difficult to compare results between experiments, but a general comparison can still be made. More conclusive results could be obtained if more

repetitions of these experiments were performed, but due to time restraints this was not possible. The results presented were obtained while the experiments were still being optimized and are not ideal. Though these are preliminary results and not definitive, they still allow for an approximation of the K_d of the aptamers.

In order to determine if these two aptamers could be used together in a "sandwich" ELISA assay, a test was performed to determine if the two aptamers targeted the same epitope on Ara h 1. Binding to Ara h 1 was tested in a solution containing 1nM of both aptamers and a solution containing 10nM of both aptamers. These binding results were compared to the binding results obtained with 1nM and 10nM solutions of only one aptamer (figure 8). If the atpamers bound at a different epitope on Ara h 1, the median fluorescent signal seen with both aptamers in solution should be about equal to the sum of the signals produced by the aptamers alone. However, this additive effect was not seen. The median fluorescence seen when both aptamers were present in solution was slightly lower than the median fluorescence seen with AraH1_R5c102 alone. This suggests the two aptamers are competing to bind to the same epitope on Ara h 1. Since this is the case, the two aptamers cannot be used in a sandwich ELISA assay.

Several experimental modifications and additional experiments will be performed as this project continues. In the experiment to test clone binding (figure 4), non specific binding was seen as the clones all showed some binding to CB-H2 beads. This could be due to the oligonucleotides binding to the surface of the beads, and is most likely also occurring during the titration experiments. It was recently suggested that a blocking agent be employed to mitigate this problem. RNA will be used as this blocking agent. The RNA, which shares some similarities

in molecular composition to the oligonucleotides, will bind to sites on the beads were non-specific binding is occurring, but will not be labelled with biotin and thus will not produce a fluorescent signal. The amount of RNA blocking agent to use will be optimized so that efficient blocking is achieved but the RNA does not interference with binding of the aptamers. The blocking agent will be used for clone testing and aptamer titrations. Other future experiments will involve repeating the titration for AraH1_R5c17 with a smaller concentration range, using many concentrations near its estimated K_d of 59.5nM. Additionally, this titration and the titration with AraH1_R5c102 will be repeated multiple times to ensure reproducible results are obtained. Following this, the aptamers will be used in an ELISA type assay to test their ability to detect Ara h 1 in food samples.

My project successfully identified two clones capable of binding to Ara h 1, AraH1_R5c17 and AraH1_R5c102. The affinity of these aptamers for Ara h 1 was then determined, and preliminary results indicate that the apparent K_d of AraH1_R5c17 is 59.5nM and the apparent K_d AraH1_R5c102 is 11.2nM. After further testing, the Berezovski Lab hopes to employ these aptamers in an assay for the detection of Ara h 1 in food.

References

Burks, A. W., Williams, L. W., Connaughton, C., Cockrell, G., O'Brien, T. J., & Helm, R. M. (1992). Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *The Journal of Allergy and Clinical Immunology*, *90*(6 Pt 1), 962–969.

Burks, A. W., Williams, L. W., Helm, R. M., Connaughton, C., Cockrell, G., & O'Brien, T. (1991). Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *The Journal of Allergy and Clinical Immunology*, *88*(2), 172–179.

Darmostuk, M., Rimpelova, S., Gbelcova, H., & Ruml, T. (2015). Current approaches in SELEX: An update to aptamer selection technology. *Biotechnology Advances*, *33*(6 Pt 2), 1141–1161.

Davis, K. A., Abrams, B., Lin, Y., & Jayasena, S. D. (1996). Use of a high affinity DNA ligand in flow cytometry. *Nucleic Acids Research*, *24*(4), 702–706.

EMD Millipore (2012). User Guide: PureProteome Carboxy Flexibind Magnetic Beads. Retrieved from http://www.emdmillipore.com/CA/en/product/PureProteome™-Magnetic-Beads,MM_NF-C77625#documentation.

Ernst, O., & Zor, T. (2010). Linearization of the bradford protein assay. *Journal of Visualized Experiments: JoVE*, (38).

Hird, H., Lloyd, J., Goodier, R., Brown, J., & Reece, P. (2003). Detection of peanut using real-time polymerase chain reaction. *European Food Research and Technology*, *217*(3), 265–268.

Iqbal, A., Shah, F., Hamayun, M., Ahmad, A., Hussain, A., Waqas, M., … Lee, I.-J. (2016). Allergens of Arachis hypogaea and the effect of processing on their detection by ELISA. *Food & Nutrition Research*, *60*(0).

Kiening, M., Niessner, R., Drs, E., Baumgartner, S., Krska, R., Bremer, M., … Weller, M. G. (2005). Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *Journal of Agricultural and Food Chemistry*, *53*(9), 3321–3327.

Koppelman, S. J., Vlooswijk, R. A., Knippels, L. M., Hessing, M., Knol, E. F., van Reijsen, F. C., & Bruijnzeel-Koomen, C. A. (2001). Quantification of major peanut allergens Ara h 1 and Ara h 2 in the peanut varieties Runner, Spanish, Virginia, and Valencia, bred in different parts of the world. *Allergy*, *56*(2), 132–137.

Sampson, H. A., Srivastava, K., Li, X.-M., & Burks, A. W. (2003). New perspectives for the treatment of food allergy (peanut). *Arbeiten Aus Dem Paul-Ehrlich-Institut (Bundesamt Für Sera Und Impfstoffe) Zu Frankfurt a.M*, (94), 236–244.

Sanders, C. R. (2010) Biomolecular Ligand-Receptor Binding Studies: Theory, Practice, and Analysis, pp 1−42, Vanderbilt University, Nashville, TN, structbio.vanderbilt.edu/sanders/Binding_ Principles_2010.pdf.

Schäppi, G. F., Konrad, V., Imhof, D., Etter, R., & Wüthrich, B. (2001). Hidden peanut allergens detected in various foods: findings and legal measures. *Allergy*, *56*(12), 1216–1220.

Sicherer, S. H., & Sampson, H. A. (2006). Food allergy. *Journal of Allergy and Clinical Immunology*, *117*(2), S470–S475.

Stephan, O., Möller, N., Lehmann, S., Holzhauser, T., & Vieths, S. (2002). Development and validation of two dipstick type immunoassays for determination of trace amounts of peanut and hazelnut in processed foods. *European Food Research and Technology*, *215*(5), 431–436.

Stephan, O., & Vieths, S. (2004). Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (Arachis hypogaea) in processed foods. *Journal of Agricultural and Food Chemistry*, *52*(12), 3754–3760.

Thiel, W. H., Bair, T., Thiel, K. W., Dassie, J. P., Rockey, W. M., Howell, C. A., … Giangrande, P. H. (2011). Nucleotide Bias Observed with a Short SELEX RNA Aptamer Library. *Nucleic Acid Therapeutics*, *21*(4), 253–263.

Toh, S. Y., Citartan, M., Gopinath, S. C. B., & Tang, T.-H. (2015). Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay. *Biosensors & Bioelectronics*, *64*, 392–403.

Tran, D. T., Knez, K., Janssen, K. P., Pollet, J., Spasic, D., & Lammertyn, J. (2013). Selection of aptamers against Ara h 1 protein for FO-SPR biosensing of peanut allergens in food matrices. *Biosensors and Bioelectronics*, *43*, 245–251.

Tsuji, S., Hirabayashi, N., Kato, S., Akitomi, J., Egashira, H., Tanaka, T., … Ohtsu, T. (2009). Effective isolation of RNA aptamer through suppression of PCR bias. *Biochemical and Biophysical Research Communications*, *386*(1), 223–226.

Wang, Z.-W., Wu, H.-B., Mao, Z.-F., Hu, X.-P., Zhang, H., Hu, Z.-P., & Ren, Z.-L. (2014). In vitro selection and identification of ssDNA aptamers recognizing the Ras protein. *Molecular Medicine Reports*, *10*(3), 1481–1488.

Wen, H.-W., Borejsza-Wysocki, W., DeCory, T. R., Baeumner, A. J., & Durst, R. A. (2005). A novel extraction method for peanut allergenic proteins in chocolate and their detection by a liposome-based lateral flow assay. *European Food Research and Technology*, *221*(3-4), 564–569.

Wen, H.-W., Borejsza-Wysocki, W., DeCory, T. R., & Durst, R. A. (2007). Peanut Allergy, Peanut Allergens, and Methods for the Detection of Peanut Contamination in Food Products. *Comprehensive Reviews in Food Science and Food Safety*, *6*(2), 47–58.

WHO/IUIS Allergen Nomenclature Sub-committee (2016). Allergen Nomenclature Database. Retrieved from http://www.allergen.org/search.php?allergensource=Arachis+hypogaea.

Zhou, Y., Wang, J., Yang, X., Lin, D., Gao, Y., Su, Y., … Zheng, J. (2013). Peanut Allergy, Allergen Composition, and Methods of Reducing Allergenicity: A Review. *International Journal of Food Science*, *2013*, 1-8.

Appendix

Figures

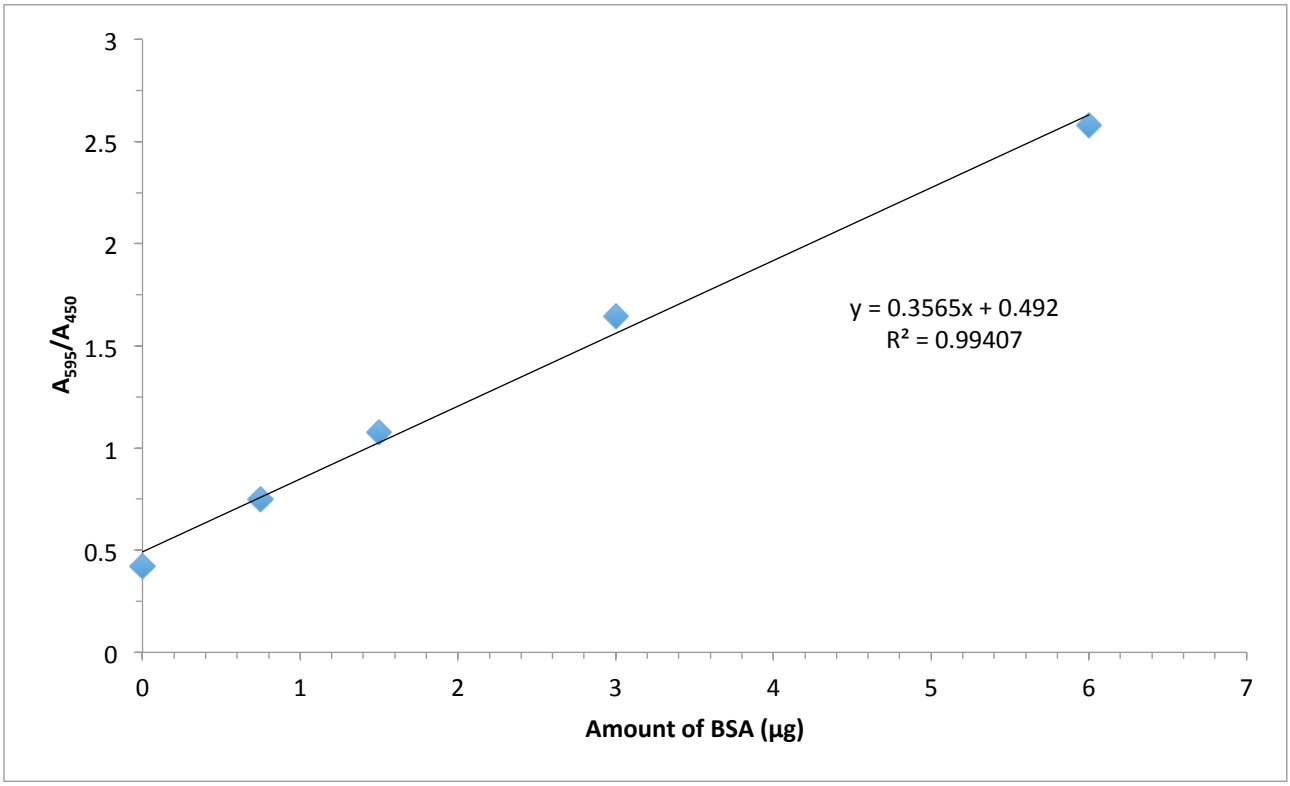

Figure 9. Standard Curve for Bradford Assay to determine amount of Ara h 1 conjugated to carboxy beads. A 1µg/µL stock solution of BSA was used in a serial dilution to create triplicate solutions containing 0.75 µg, 1.5 µg, 3 µg and 6 µg of BSA in the wells of a 96 well plate (Costar). The solutions were completed to 100µL with ddH_20 and then 100µL of a 2.5x dilution of Protein Assay Dye Reagent Concentrate (Bio-Rad) was added to each well. The plate was incubated for 5 minutes at room temperature. Absorbance readings at 595nm and 450nm were obtained for these solutions and samples (results shown in figure 1) using the Spectra Max M5 plate reader (Molecular Devices). The amount of BSA in solution is presented on the x axis, and the quotient obtained by dividing the absorbance readings at 595nm (A_{595}) by those at 450nm (A_{450}) is presented on the y axis. The trendline equation is y = 0.3565x + 0.492, with a linear regression of R^2 = 0.99407.

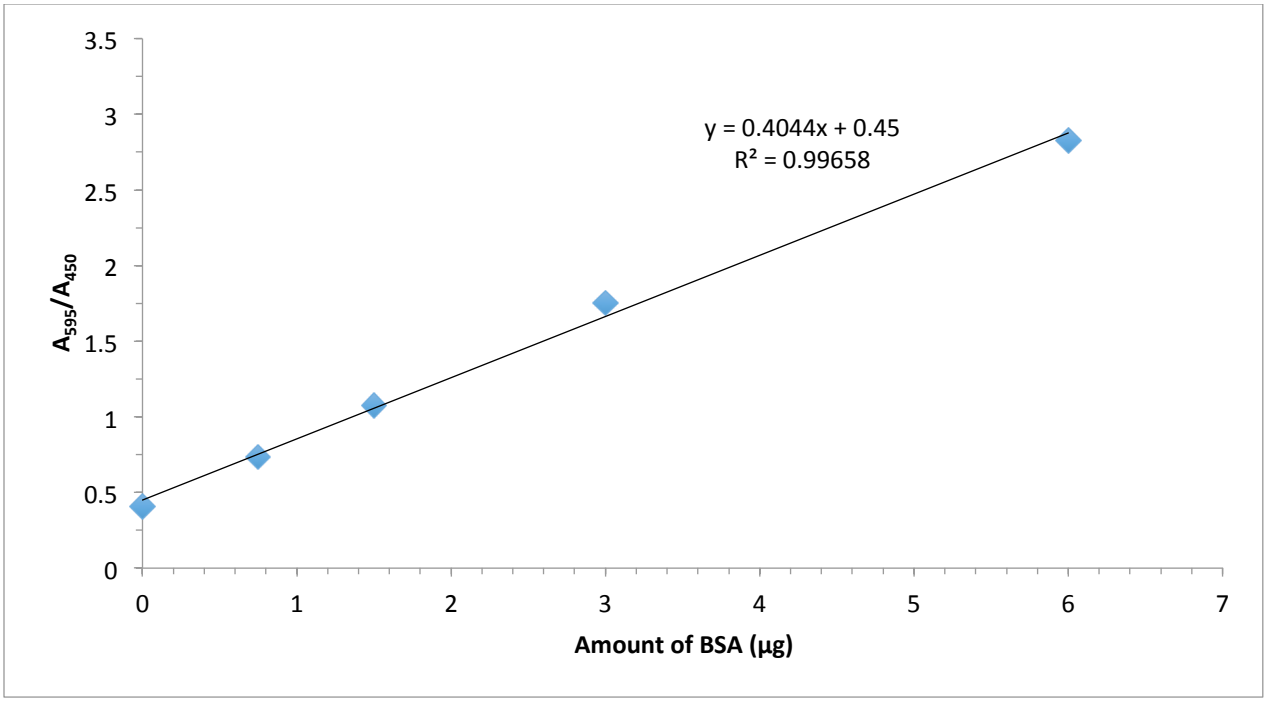

Figure 10. Standard Curve for Bradford Assay to determine amount of Ara h 2 conjugated to carboxy beads. A 1µg/µL stock solution of BSA was used in a serial dilution to create triplicate solutions containing 0.75 µg, 1.5 µg, 3 µg and 6 µg of BSA in the wells of a 96 well plate (Costar). The solutions were completed to 100µL with ddH_20 and then 100µL of a 2.5x dilution of Protein Assay Dye Reagent Concentrate (Bio-Rad) was added to each well. The plate was incubated for 5 minutes at room temperature. Absorbance readings at 595nm and 450nm were obtained for these solutions and samples (results shown in figure 2) using the Spectra Max M5 plate reader (Molecular Devices). The amount of BSA in solution is presented on the x axis, and the quotient obtained by dividing the absorbance readings at 595nm (A_{595}) by those at 450nm (A_{450}) is presented on the y axis. The trendline equation is y = 0.4044x + 0.45, with a linear regression of $R^2 = 0.99658$.

Sequences

The sequences of the 80 nucleotide clones are presented below. The first 20 nucleotides and last 20 nucleotides are complimentary to the primers used in PCR during SELEX. The middle 40 nucleotides (**bolded**) represent the variable region.
The clones are named according to the rounds of SELEX after which they were obtained (so R9 clones were obtained after 9 rounds of SELEX) and their prominence in that round (R9c3 was the 3rd most prominent sequence in round 9).

AraH1_R9c3
CTCCTCTGACTGTAACCACG**ATTCACCTCTGACACTTCGACGGGATTGGTTTGGAAAGTG**GCATAGGTAGTCCAGAAGCC

AraH1_R9c4
CTCCTCTGACTGTAACCACG**TTCTGCTTGAACCCAAAACGTACCGTACGAGATAGTGTGC**GCATAGGTAGTCCAGAAGCC

AraH1_R9c6
CTCCTCTGACTGTAACCACG**TCTTTAATATCGTTAGACTACCGGGGGCTTGGGGTGGTTG**GCATAGGTAGTCCAGAAGCC

AraH1_R5c8
CTCCTCTGACTGTAACCACG**TTGAGGCTTGCGTTTATGGTACGCTGGACTTTGTAGGATA**GCATAGGTAGTCCAGAAGCC

AraH1_R5c17
CTCCTCTGACTGTAACCACG**TAGGGGGTTTGGGGGACCCTTCCCCCAATTTATTCATCGA**GCATAGGTAGTCCAGAAGCC

AraH1_R5c83
CTCCTCTGACTGTAACCACG**GCGCAAGCCGGGGTGTACGCGTTATATGTGCGTGTATCGA**GCATAGGTAGTCCAGAAGCC

AraH1_R5c112-3
CTCCTCTGACTGTAACCACG**AAGTGGTTGCCGTGTACACATACTACGTACACATGTCGAT**GCATAGGTAGTCCAGAAGCC

AraH1-R5c102
CTCCTCTGACTGTAACCACG**GGGGGTATCGGGGGGGTTTCCCCTCCGCCAATAGTCTGTC**GCATAGGTAGTCCAGAAGCC