

Antibody Characterization for use in Clinical Mass Spectrometry

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Abstract

Antibody Characterization for use in Clinical Mass Spectrometry Assays

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Many biomarkers in human serum are in low abundance. Immunoaffinity enrichment via anti-peptide antibody and analysis via liquid chromatography tandem mass spectrometry (LC-MS/MS) is an attractive workflow that depends on the production of new antibody reagents. Although there are different methods available to characterize antibodies such as enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), and LC-MS/MS, using the same platform for screening and assay development is likely beneficial. Antibody apparent dissociation constant (K_D) and on rate and off rate (k_{on} and k_{off}) may be equally as critical as recovery efficiency for screening. A high throughput, manual, in-house antibody LC-MS/MS screen was developed to screen 846 supernatants with antibodies to specific peptides of KLOTTHO and Procollagen type III C-terminal Pro-peptide (P3CP) to find the best 15 antibodies and characterize them for future assay development.

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Chapter 1. INTRODUCTION

Antibodies, proteins also called immunoglobulins, were first discovered in 1890 by von Behring and Kitasato, and since then antibodies have become some of the most versatile biomolecules available [8]. Uses for antibodies range from reagents in experiments and pharmaceuticals to their original purpose, i.e., part of the immune response to foreign materials. Since antibodies are such complex proteins, understanding their structure and function is of utmost importance for redesigning their potential use in laboratory medicine.

An antibody is comprised of four polypeptide chains, two heavy chains and two light chains held together by disulfide bonds [11]. Antibodies are known for their unique structural arrangement containing a constant, crystallizable region (Fc), a hinge region, an antigen binding region (Fab), and a sugar motif [4]. If an antibody is made from a single clone and recognizes only one antigen, then it is called a monoclonal antibody versus a population of antibodies that recognizes more than one epitope, which is referred to as a polyclonal antibody [4]. The number of disulfide bonds, type of sugar motif present, and protein sequence characterize distinct types of immunoglobulins [4,11].

There is a specific classification system available to describe antibodies in mammals. In humans, there are five classes of immunoglobulins: G, A, M, E, and D, which differ in charge, size, solubility, and other properties [4,11]. The immunoglobulin class most commonly used in diagnostic assay development and pharmaceuticals is immunoglobulin gamma (IgG), due partially to its abundance [8]. IgG is about 150 kDa in size and is further classified into 4 isotypes in humans: IgG1, IgG2, IgG3, IgG4 [3]. Alternatively, rabbits have only one isotype of IgG, and there are 5 IgG isotypes in mice: IgG1, IgG2a, IgG2b, IgG2c, and IgG3 [6].

The binding relationship between an antibody and its antigen is complex and dependent on many factors. There are three classes of binding: cavity for hapten antigens, groove for peptide, sugars, or nucleic acid antigens, and planar for protein antigens [19]. Data suggest water molecules fill in the binding cavity when binding is imperfect, and in the case of high affinity binding there is a combination of van der Waals, hydrogen bonds, and coulombic bonds to facilitate binding [19, 20]. Each antibody has its own specific favorable conditions for binding, and changes in matrix complexity, pH, and temperature will all alter the antibody-antigen interaction [20].

There are a few different measurements that can be used to characterize the antibody (Ab) – antigen (Ag) interaction, including the apparent dissociation constant (K_D), the association rate (k_{on}), and the dissociation rate (k_{off}), which are all interrelated. As seen in the equation below, the apparent dissociation constant is equal to the dissociation rate divided by the association rate [23].

$$\text{Equation 1.} \quad K_D = \frac{k_{off}}{k_{on}} = \frac{[Ab][Ag]}{[Ab \cdot Ag]} = \frac{1}{K_A}$$

When you consider total antigen added, at any point in time there will be antigen bound to the antibody or free in solution as seen in the below equation [23].

$$\text{Equation 2.} \quad [Ag]_{Total} = [Ag] + [Ag \cdot Ab]$$

The Langmuir Isotherm is used to find the fraction of the total surface antigen sites bound (θ), and it can be determined by solving the above two equations [23].

$$\text{Equation 3.} \quad \theta = \frac{[Ab]}{K_D + [Ab]}$$

However, these assume only one binding site instead of two found on an antibody. In the case of homogeneous bivalent binding, the total antigen is equivalent to that which is free, bound, and bound to both sites saturating the antibody [23].

$$\text{Equation 4. } [Ag]_{Total} = [Ag] + [Ag \cdot Ab] + [Ag \cdot Ab \cdot Ag]$$

The previous scenario includes both binding sites of an antibody and introduces the idea of valence (λ) which is 0.5 in this scenario due to two binding sites per one antibody as seen below [23].

$$\text{Equation 5. } \lambda = \frac{[Ag \cdot Ab] + [Ag \cdot Ab \cdot Ag]}{[Ag \cdot Ab] + 2[Ag \cdot Ab \cdot Ag]}$$

By combining the scenario of two binding sites with the Langmuir isotherm, the equation can be updated as follows with α as an experimental constant modulating the apparent K_D in any experimental procedure [23].

$$\text{Equation 6. } \theta = \frac{[Ab]\lambda}{\alpha K_D + [Ab]}$$

Usually, antibodies in reagents are coated on a film or bead, and in this case more calculations are necessary. The antibody-binding capacity (ABC) can then be calculated when coating a surface, such as a magnetic bead, with antibody by knowing the number of antigen sites per coating surface (n) as well as the valence and fraction of total surface antibody bound [23].

$$\text{Equation 7. } ABC = n\theta\lambda$$

Once the antibody is bound to a surface as a reagent, such as a paramagnetic bead, it can be more easily manipulated for experimental measurements.

There are several ways to determine the dissociation constant (K_D) mathematically, and two such methods are creating a binding curve or completing a Scatchard analysis. Alternatively, one could determine the on and off rates. Antibody binding curves are prepared by maintaining constant antibody concentration and varying the spiked peptide concentration, and the data is plotted as signal vs. concentration [1]. The K_D is the concentration at half of the maximum binding, B_{max} [1]. Alternatively, the Scatchard plot uses similar data, but plots the ratio of

antibody bound/free versus the amount of antibody bound to produce a linear expression relating K_D and B_{max} [1]. An unextracted sample containing total peptide available is used to determine how much peptide is bound or free in samples.

$$\text{Equation 8.} \quad \frac{Bound}{Free} = \frac{[Ag \cdot Ab]}{[Ag]} = \frac{(B_{max} - Bound)}{K_D}$$

The intercepts of a Scatchard plot contain the most important information with the x-intercept being the maximum binding and the y-intercept being B_{max}/K_D [1]. The apparent K_D found by using a hyperbolic binding curve to reach maximum binding is experimentally equal to the inverse of the y-intercept of the equivalent Scatchard plot [1].

There are a handful of different methods available to measure an antibody's apparent K_D , and some of these methods include ELISA, SPR, and LC-MS/MS. ELISA is relatively cheap and high throughput using absorption of light or percent transmittance as its signal of measurement; however, even under the best conditions ELISA is prone to errors such as limited measurability of high affinity antibodies or large dissociation rates, k_{off} [2,5]. SPR, unlike ELISA, uses refractive index changes to measure concentration of peptide on a sensor containing bound antibody, and Biacore has a commercial SPR system which allows measurement of k_{on} , k_{off} , and K_D [2,5]. A similar commercial system, ForteBio, uses technology like SPR to quantify peptide bound to the sensor via a shift in wavelength due to thickness changes in the film [7]. Unlike ELISA and SPR signals, which are dependent on light for an indirect measure of binding, LC-MS/MS workflows directly measure peptide eluted from antibody following immunoaffinity enrichment [9,10]. LC-MS/MS can approach the sample throughput of ELISAs, but LC-MS/MS use of an antibody in measuring peptides adds layers of specificity to the measurement [10]. Which method to use is an important decision when determining how to measure antibody kinetic constants due to what is being measured and how an antibody will be used in the future.

Due to specificity, LC-MS/MS is an attractive technology for screening antibodies. While kinetic experiments used to assess kinetic constants require many samples to be run, an effective LC-MS/MS antibody screen only needs one sample per hybridoma for comparison of hundreds of antibody containing supernatants using just the amount of signal recovered compared to an unextracted sample [12]. Antibodies that have good recovery at constant supernatant volume can be further studied, whereas those with poor recovery are not attractive for further study, because only hybridomas that secrete well and bind strongly are useful reagents [12]. Previous work has used ELISA and SPR for screening hybridomas for new antibody reagents, but MALDI and ESI mass spectrometry have recently been used to eliminate some of the negative aspects of ELISA screens, such as false positives and false negatives, while maintaining throughput [12,13,14].

Once antibody reagents have been screened and purified, they may be used in multiplex proteomic assays for both research and clinical applications. Some biomarkers of interest are found at low levels in human serum which makes stable isotope standards and capture by anti-peptide antibodies, SISCAPA, an optimal strategy for proteomic assay development using LC-MS/MS [9,10]. Serum is a complex matrix containing proteins that can be digested by enzymes, spiked with a stable isotope standard to account for between-sample variation, enriched using paramagnetic bead support bound with anti-peptide antibodies specific to a peptide of a protein of interest, washed to remove interferences, and concentrated in an eluate to be injected and measured via mass spectrometry [9]. The SISCAPA method of immunoaffinity enrichment provides specificity and lower limits of detection comparable to other platforms available, and it allows for multiplexing the measurement of multiple peptides or proteins in a sample due to the specificity of the antibody reagents used [9,14].

The production of antibody reagents for use in immunoaffinity enrichment is a necessary step, but it is the rate-limiting step of assay development. To create an anti-peptide antibody, peptides must first be assessed by *in silico* digestion to find those unique to the protein of interest, not exceeding 20 amino acids in length, and avoiding amino acid compositions that are prone to post translational modification, hydrolysis, oxidation, or multiple charges [24]. Once peptides are chosen, monoclonal antibody supernatants can be produced by standard methods for screening [22]. Once an antibody is found to be a potential assay reagent, pre-validation testing can be done on a select few antibodies for the same target before starting assay validation [25]. Without proper production, screening, and testing an antibody will not be useful as a reagent.

There are two proteins of interest for which anti-peptide antibodies were developed: KLOTHO and Procollagen type III C-terminal Pro-peptide (P3CP). KLOTHO is a protein known to be decreased in patients with chronic renal failure and in older individuals, and it is found at serum concentrations of 11-181 ng/mL [26, 28]. P3NP is known to be increased in patients with bone and soft tissue growth, and P3NP can be found at concentrations of 1.7-4.2 ng/mL, and it is suspected for P3CP to behave similarly [27, 29]. KLOTHO peptides SSALFYQK, LGYLVAHNLLLAHAK, and GLFYVDFLSQDK, and P3CP peptides GPVGPSGPPGK-OH 81, GPVGPSGPPGK 89, and FTYTVLEDGCTK were chosen for inoculation in mice at the Shared Resource Lab in Eastlake, WA. The Shared Resource Lab preliminarily screened the hybridomas for monoclonal antibodies, and 9 plates, or 846 antibody supernatants, were provided for further LC-MS/MS screening. I established an in-house, 96-well plate-based method of screening antibodies to provide a cost efficient and high-throughput way of finding new antibody reagents to specific proteins of interest. I applied this technique to

screen 9 plates of antibody supernatants produced for peptides from KLOTHO and P3CP, to choose the top antibodies for future pre-validation studies.

Chapter 2. IMMUNOAFFINITY REAGENT AND EQUIPMENT SELECTION

2.1 BASICS OF BEAD-BASED IMMUNOAFFINITY ENRICHMENT

Potential biomarkers of interest are found at low levels in human serum making a functional immunoaffinity enrichment workflow necessary for LC-MS/MS to provide reliable quantification for proteins of interest. Stable isotope standards and capture by anti-peptide antibodies, SISCAPA, solves the problem of low concentrations in proteomic methods using LC-MS/MS [9,10]. Serum is a complex matrix, which can be reduced to peptides by enzymatic digestion and optimized for each protein of interest for LC-MS/MS quantification [9,30]. With the addition of a stable isotope standard into all samples one can account for between-sample variation [9,10,30]. To enrich peptides from a serum digest, a paramagnetic bead support bound with anti-peptide antibodies specific to a peptide of a protein of interest is added to a sample, washed after incubation to remove interferences, and concentrated in an eluate to be injected and measured via mass spectrometry [9,30]. The use of a paramagnetic bead provides a support which can more easily be manipulated with the use of a magnet. With so many commercial options available to help quantify peptides in digested serum, finding the right workflow, reagents, and equipment is essential.

2.2 SERUM DIGESTION AND BEAD PREPARATION

Serum was digested using our current thyroglobulin assay protocol [17]. Unless otherwise listed, chicken serum, a complex matrix lacking the peptide FSPDDSAGASALLR and peptide

VIFDANAPVAVR, was used. All digestions were pooled and vortexed the same day they were digested. Digested serum was stored at -80°C before use.

Digestion was accomplished in a 1.5 mL lo-bind Eppendorf tube. A sample of 400 μL was added with 100 μL denaturant comprised of sodium deoxycholate, tris (2-carboxyethyl) phosphine hydrochloride, and 0.2 M tris (hydroxymethyl) aminomethane (TRIS). The tubes were placed at 40°C to incubate at 1400 RPM for one hour. Samples were placed in a tabletop centrifuge for a touch down briefly in a centrifuge, 100 μL 0.06 M iodoacetamide was added, vortexed, and the tube incubated in the dark for 30 minutes. An addition of 100 μL 0.2 M TRIS was added to the samples, which were then vortexed and touched down. Digestion was accomplished using 100 μL trypsin (10 mg/mL) to incubate for 30 minutes at 37°C at 1400 RPM. At the end of trypsin digestion, 20 μL of 3 mg/mL tosyl-L-lysyl-chloromethane hydrochloride (TLCK), a chemical inhibitor, was added and vortexed. Samples incubated for 20 minutes at 37°C at 1400 RPM. The digestions were then pooled in a 50 mL conical tube for future experiments and aliquoted into 1.5 mL Eppendorf tubes before storage at -80°C .

Two types of paramagnetic beads were used in all immunoaffinity experiments: Dynabead M-280 tosyl-activated beads and Dynabead M-280 Sheep anti-Mouse IgG beads. Sheep anti-mouse IgG beads were only used for the incubation of supernatants in the antibody screen, and all other experiments used tosyl-activated beads. Tosyl-activated beads create a primary amino and sulfhydryl covalent bond to purified antibodies and do not come pre-blocked whereas sheep anti-mouse beads come pre-coated with antibodies which recognize mouse IgG1, IgG2a, IgG2b, but have low affinity to IgG3 [15,16].

Tosyl-activated beads were prepared according to the current thyroglobulin assay protocols [17]. To a 1.5 mL Eppendorf lo-bind tube at 7.5 μL per reaction of beads are added.

These beads were washed three times in 200 μL 100 mM sodium borate. All mixing was done using a pipette to mix or gentle vortexing. Beads were incubated in 100 mM sodium borate at 37°C for 1-3 hours at 2 $\mu\text{g}/\text{mL}$ antibody in a total volume of 12 μL per reaction unless otherwise noted. Beads were then washed with 200 μL PBS/0.1% BSA 4 times. Beads were re-suspended in 400 μL 0.2 M Tris/0.1% BSA for blocking at 37°C for 1-3 hours. Beads were then washed 3 times with PBS/0.1% CHAPS before storage at 4°C in PBS/0.1% CHAPS/0.2% NaN_3 at a volume of 10 μL per reaction. The tube was then covered in parafilm to prevent evaporation.

For the antibody screen, Sheep anti-mouse beads were prepared following alterations from previous work done by Schoenherr, R. and Paulovich, A. [18]. Sheep anti-mouse beads were prepared at 10 μL beads per reaction well. The beads were first washed once in PBS/0.1% CHAPS. Next, in a BioRad hard shell PCR 96 well plate, 25 μL PBS/0.1% CHAPS was added to each well and 25 μL antibody supernatant was added and mixed to its corresponding well using a p200 with filter tip in a biosafety cabinet to prevent contamination. The bead incubation plate was then sealed with foil and put on a Labquake for 1 hour incubation with end-over-end rotation at room temperature (22°C). At the end of the incubation, the plate was touched down and the antibody screen workflow continued.

2.3 VESSEL AND MAGNET PAIRING

A simple yet important aspect of paramagnetic immunoaffinity enrichment is the appropriate pairing of vessel and magnet. Magnets must fit the tube or plate being used to prevent bead loss, and the strength of the magnet should be appropriate. Without proper magnet pairing, beads can be lost or transferred throughout an experiment.

Bead preparation steps and some experiments were done in a 1.5 mL Eppendorf Lo-bind tube. These tubes have several different magnets available including, including the DynaMag-2 which holds 16 tubes and 6 tube magnets. By visual comparison, the DynaMag-2 moves beads more quickly and clears matrix of beads faster than the 6 tube magnet (data not shown). All bead preparation used the DynaMag-2 for this reason as well as the larger number of samples per magnet.

Plate experiments require specific magnets depending on the desired function. Volume and fit to magnet are both limiting factors for experimental workflow development. Current immunoaffinity enrichment uses a volume greater than 700 μL of fluid to provide the necessary amount of signal for sensitivity [17]. Conical plates are better than flat-bottom square-welled plates due to the flat-bottoms causing beads to collect in corners or ledges away from the magnet. It is also preferable to use conical well plates to decrease dead volume. A 2 mL conical plate and a 150 μL PCR plate was first compared with tubes to see if it was possible to move from all-tube-based to all plate-based experiments. Using a 2 mL plate, there is more volume available if necessary to optimize future experiments, and a plate format is necessary for high throughput. The available magnets were the red VP 771BT-LE-A pole magnet, the white VP 771LWAS pole magnet, and the DynalMPC bar magnet. Previous work had been done to confirm that the white magnet fit the 2 mL plate and the red magnet fit for the 150 μL PCR plate in two other workflows [17,31].

2.4 IMMUNOAFFINITY ENRICHMENT OPTIMIZATION

Although the thyroglobulin assay was being used clinically, some optimization steps were done to the workflow further improve signal and decrease error. Antibodies used for these experiments

were Anti-VIF monoclonal rabbit antibody, CPTAC-39d, and Anti-FSP monoclonal rabbit antibody, CPTAC-39c. Method of mixing, elution buffer, and wash time were the variables optimized.

The mixing study was done to determine what type and speed of mixing would be best for immunoaffinity enrichment. Using 1.5mL Eppendorf tubes with the Dynamag-2, a Labquake, and a thermomixer, four mixing types were tested at room temperature. Using three thermomixers, 700, 1000, and 1400 RPM were tested in triplicate, as well as using the Labquake to test end-over-end (EE) mixing. During this experiment were 5 μ L of beads per reaction were used with 2 μ g/mL anti-FSP antibody and anti-VIF antibody. Each tube also contained 610 μ L serum digest and 20 μ L Internal Standard. The tubes were incubated for one hour by each mixing type and then placed on a magnet for digest removal. Using 150 μ L of wash buffer A, the samples were then transferred into 150 μ L PCR plate for washing via plate washer. Wash buffer A was PBS/0.0003% CHAPS and Wash buffer B was 0.1XPBS/0.0003% CHAPS. To the samples, 50 μ L of 5% acetic acid added, the plate was taped, and the samples were incubated at 1200 RPM at room temperature for 5 minutes. Samples were touched down, placed on the red VP 771BT-LE-A magnet, then transferred into 1.5 mL Eppendorf tubes. Tubes were centrifuged for 5 minutes at >13,000 RPM before placement on the tube magnet. The supernatant was then transferred into an autosampler vial for 40 μ L injection on the mass spectrometer.

Figure 2-1 shows the results of the mixing study for the peak corresponding to the best transition for peptide FSP. Mixing at 700 RPM had the largest variance, and as RPM increased the variance decreased and the signal increased. Using response, t-tests were calculated for each comparison of mixing type. End-over-end mixing was statistically different than 700 RPM ($p =$

0.01), but due to low signal and power all other comparisons are not statistically significant at the same level.

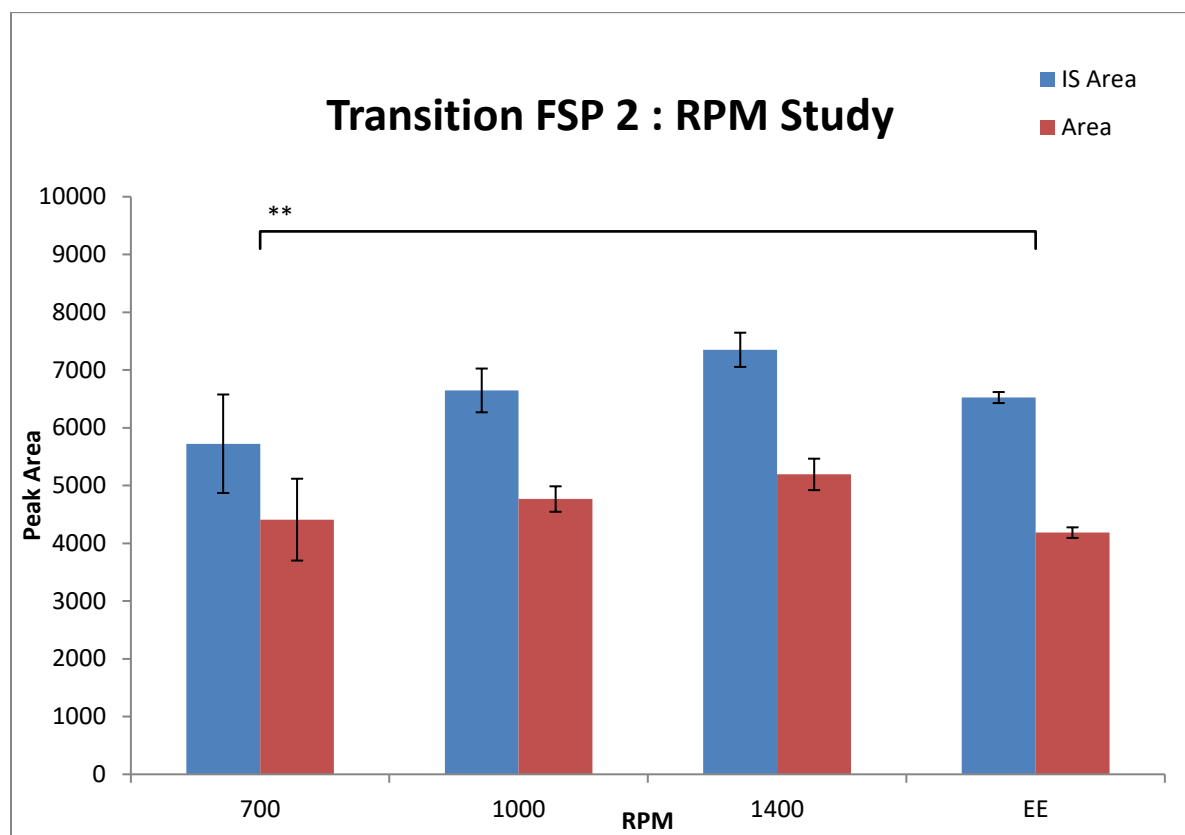


Figure 2-1. Mixing study. Anti-FSP beads and FSP peptide were used with a Labquake and Thermomixers. Error bars show the standard deviation. Smallest variance was seen in end-over-end mixing with increasing variance with slower mixing. (** $p < 0.05$).

Multiple elution buffers have been used for immunoaffinity experiments, so three elution buffers were tested for signal and error. During this experiment were used at 5 μL of beads were used containing 2 $\mu\text{g}/\text{mL}$ anti-FSP and anti-VIF antibodies. Each sample was incubated with 610 μL digested serum, 200 μL 1M Ammonium bicarbonate, 20 μL internal standard, and 5 μL of the beads. The tubes were incubated for 1 hour end-over-end on the Labquake. The tubes were

then touched down, placed on magnet, and the supernatant was removed, and this step was repeated once. The tubes were then washed by hand twice using 500 μL of PBS/CHAPS before the tubes were touched down and all wash buffer was removed. For elution, 50 μL of each elution buffer type was added to the appropriate sample. The elution buffers tested were: 4.85% acetic acid/0.03% CHAPS; 4.85% acetic acid/3% ACN; and 4.85% acetic acid/3% ACN/0.02% CHAPS. After incubation for 5 minutes at 1200 RPM, the tubes were touched down and placed on a magnet. The supernatant was transferred into a fresh 1.5 mL Eppendorf tube for a cleanup step. The tube was centrifuged for 5 minutes at $>13,000$ RPM, placed on a magnet, and the supernatant was transferred into an autosampler vial. The sample was placed on the mass spectrometer for injection of 40 μL per sample.

Figure 2-2 shows the results of the elution buffer comparison. The least variance was seen in the acetic acid with CHAPS condition for peptide FSP. The signal was similar for all three elution buffers; however, the largest variance was seen with a combination of acetic acid, acetonitrile, and CHAPS.

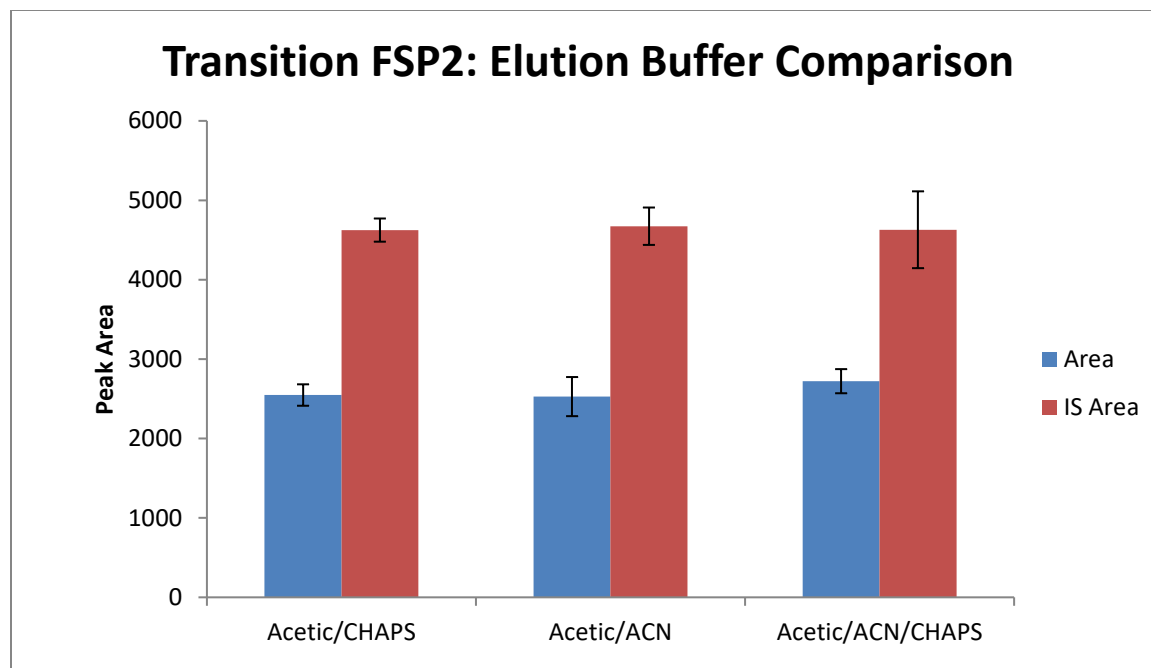


Figure 2-2. Elution buffer comparison. Error bars show the standard deviation. Comparison of select elution buffers using Anti-FSP beads and FSP peptide gave similar signal with differences in variance.

One of the most important steps of immunoaffinity enrichment is the washing of the beads. This step removes potential interferences, prevents the column and mass spectrometer from getting dirty, and allows for later elution of a concentrated analyte of interest. To show proof of principle, three wash times were tested in triplicate via washing by hand. The times were 14 minutes, 24 minutes, and 44 minutes. At the end of the wash, the beads were resuspended in 50 μ L of elution buffer, 5% Acetic Acid/0.1% CHAPS. The plate was then put on a Labquake at room temperature for 5 minutes of end-over-end mixing. After incubation, the plate was touched down and placed on the red magnet for transferring supernatants to tubes. The tubes were centrifuged for 5 minutes at 13000 RPM and then placed on a magnet for transferring the supernatant to fresh clean-up tubes. The new sample tubes were then centrifuged at 13000

RPM and placed back on the tube magnet. The supernatants were transferred into autosampler recovery vials for a 40 μ L injection per sample on the mass spectrometer in random order.

Figure 2-3 shows the results of the wash time study. There was more variance in the 24 minute condition. The highest signal was seen in the shorter wash time with a general downward trend of signal with increased wash time.

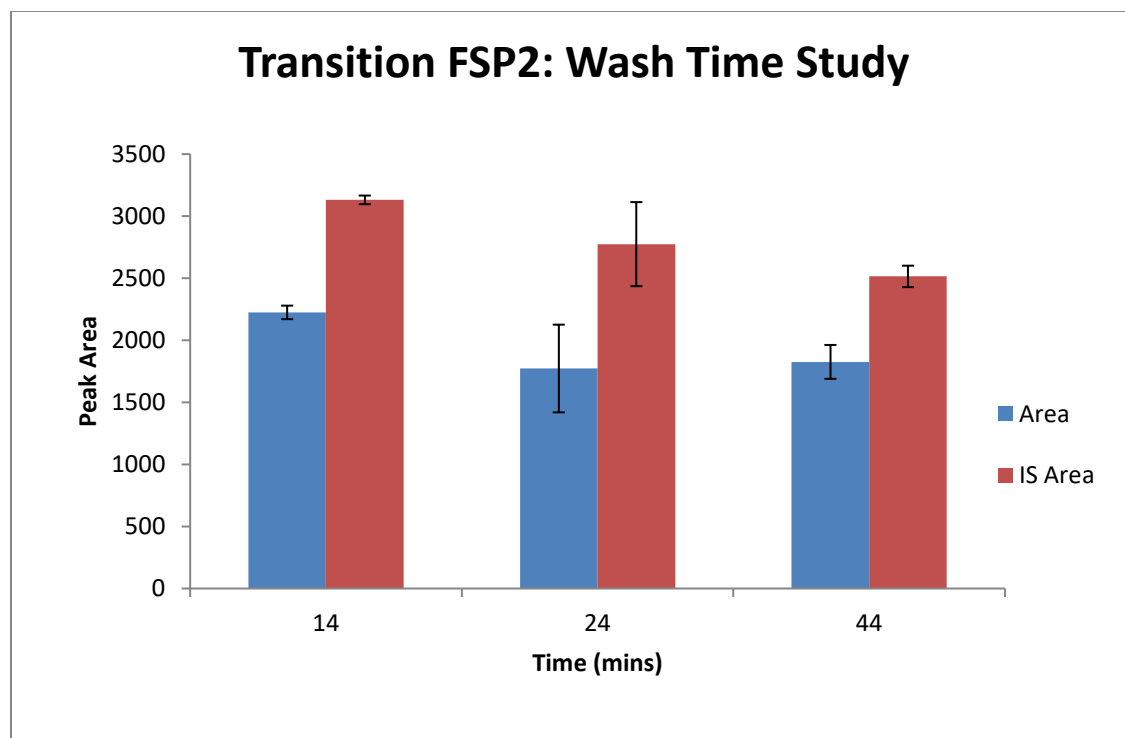


Figure 2-3. Wash time study. Error bars show the standard deviation. Anti-FSP beads and FSP peptide decreased in signal with increased wash time.

With the goal of running an antibody screen, testing in tubes would have too much imprecision, and throughput would be low, with only 32 tubes able to be tested at once. For this screening assay, with how many samples, tubes would require over 26 days of work whereas if a plate method was developed it would only take 9 or 10 days. With this in mind, two plates were tested in tandem with an equivalent volume lo-bind Eppendorf tube.

Table 1 lists all the contents of each tube and plate addition. Each condition was done with 5 replicates using digested chicken serum (CSD) as matrix and Anti-FSP and Anti-VIF beads. The Tube/Biotech sample was a 500 μ L Eppendorf tube to which 136 μ L CSD, 8 μ L peptide mix, and 5 μ L beads were added. The Biotech plate, which holds 150 μ L maximum volumes, contained the same contents as its tube comparison to make a total volume of 149 μ L. The 1.5 mL Eppendorf tube had 820 μ L CSD, 20 μ L peptide mix, and 5 μ L beads added. The 2 mL plate had the same contents as the 1.5 mL Eppendorf tube to make a total volume of 845 μ L. Samples were then sealed, the Biotech plate with foil and the 2 mL plate with pierceable foil, and incubated end-over-end. The Biotech plate and all tubes were incubated using the Labquake; however, due to the size of the 2 mL plate, it was unable to fit on the Labquake and a large end-over-end mixer was used to incubate end-over-end for 1 hour. At the end of incubation, all tubes were touched down in the tabletop mini centrifuge, and the two plates were touched down using the plate centrifuge. All samples were then transferred into a new BioRad PCR plate on the red pole magnet. The plate was then placed on the plate washer. The plate washer method used the red VP 771BT-LE-A magnet and Wash buffer A, PBS/0.0003% CHAPS, and Wash buffer B, 0.1XPBS/0.0003% CHAPS. The plate washer was primed twice before the wash method was run. The wash method included a stationary period, two 150 μ L washes with wash A and shaking between each wash, a single 150 μ L wash with wash B, and aspiration of fluid. At the end of the wash, each sample of beads were resuspended in 50 μ L of elution buffer, 5% Acetic Acid/0.1% CHAPS. The plate was then put on a Labquake at room temperature for 5 minutes of end-over-end mixing. After incubation, the plate was touched down and placed back on the red magnet for transferring samples to tubes. The tubes were centrifuged for 5 minutes at 13000 RPM and then placed on a magnet for transferring the supernatant to fresh clean-up tubes. The

new sample tubes were then centrifuged at 13000 RPM and placed back on the tube magnet. The supernatants were transferred into autosampler recovery vials for a 40 μ L injection per sample on the mass spectrometer in random order.

Table 1. Comparison of tube and plate contents for comparison of signal.

	Tube Biotech	Plate Biotech	Tube 2 mL	Plate 2 mL
Digest (μ L)	136	136	820	820
Peptide (μ L)	8	8	20	20
Beads (μ L)	5	5	5	5
Final Volume (μ L)	149	149	845	845

Figure 2-4 shows the results of comparing the two plates and tubes for all monitored transitions of peptides FSP and VIF. The highest signal was seen in the 1.5 mL Eppendorf tube which was used as a tube comparison for the 2 mL plate; however, tubes had higher variance. The 2 mL plate had smaller variance and the second highest signal for all transitions. Despite the lowest signal, the Biotech plate had the smallest variability of all the conditions and still had peaks able to be integrated in the Mass Lynx software.

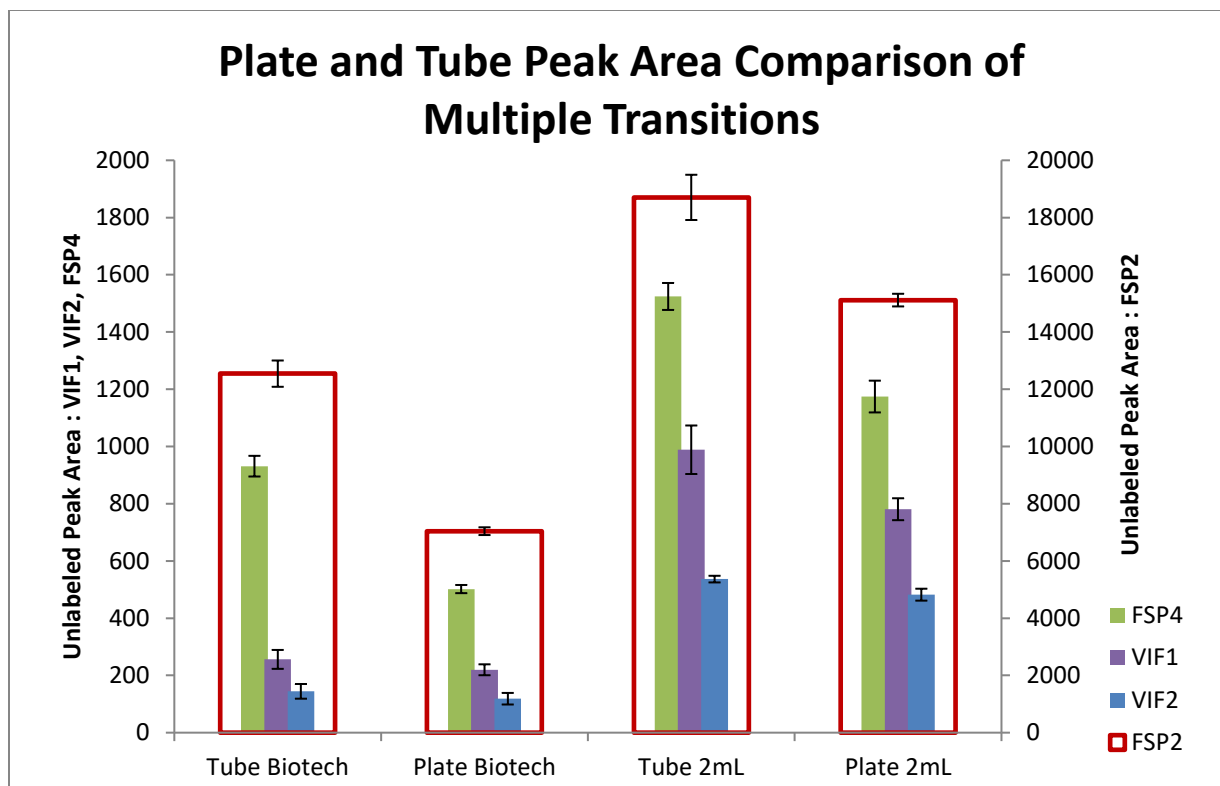


Figure 2-4. Plate and tube comparison. Error bars show the standard deviation. Signal for multiple transitions in two types of tubes and plates were compared.

2.5 BEAD SPECIFICITY

To determine bead specificity, Dynabead M-280 tosyl-activated beads were used due to their potential use in future assay development. Antibodies used for these experiments included Anti-VIF antibody, Anti-FSP antibody, and bovine gamma globulins (BGG). The spiked peptide was 1 pmol/ μ L unlabeled FSP, 1 pmol/ μ L unlabeled VIF, or a mix of both FSP and VIF light peptide. The BioRad hard shell PCR 96 well plate was used with the red VP magnet, and all sample bead-peptide pairings were done in duplicate. Each sample contained 136 μ L CSD, 8 μ L peptide mix, and 5 μ L beads. The pairs are as follows: Anti-FSP bead with VIF peptide, Anti-FSP bead with FSP peptide, Anti-VIF bead with VIF peptide, Anti-VIF bead with FSP peptide,

BGG bead with both FSP and VIF peptides, and a bead without antibody incubated with both FSP and VIF peptides. All beads were bound and blocked separately following the same protocol on the same day [17]. Samples were incubated end-over-end on the Labquake at room temperature for one hour. Samples were then washed via plate washer, eluted in 50 μL of 5% Acetic Acid/0.1% CHAPS, and 40 μL was injected onto the mass spectrometer.

Figure 2-5 and 2-6 show the results of the bead specificity experiment. Figure 2-5 is the FSP peptide transition, and the only signal that can be seen other than noise is found in samples containing Anti-FSP antibody bound to beads. Alternatively, figure 6 shows the VIF peptide transition. The only signal seen in the VIF transition is with the Anti-VIF antibody beads. Interestingly, beads which have been blocked without any antibody bound show only noise suggesting that proper blocking is sufficient to prevent nonspecific binding to the beads.

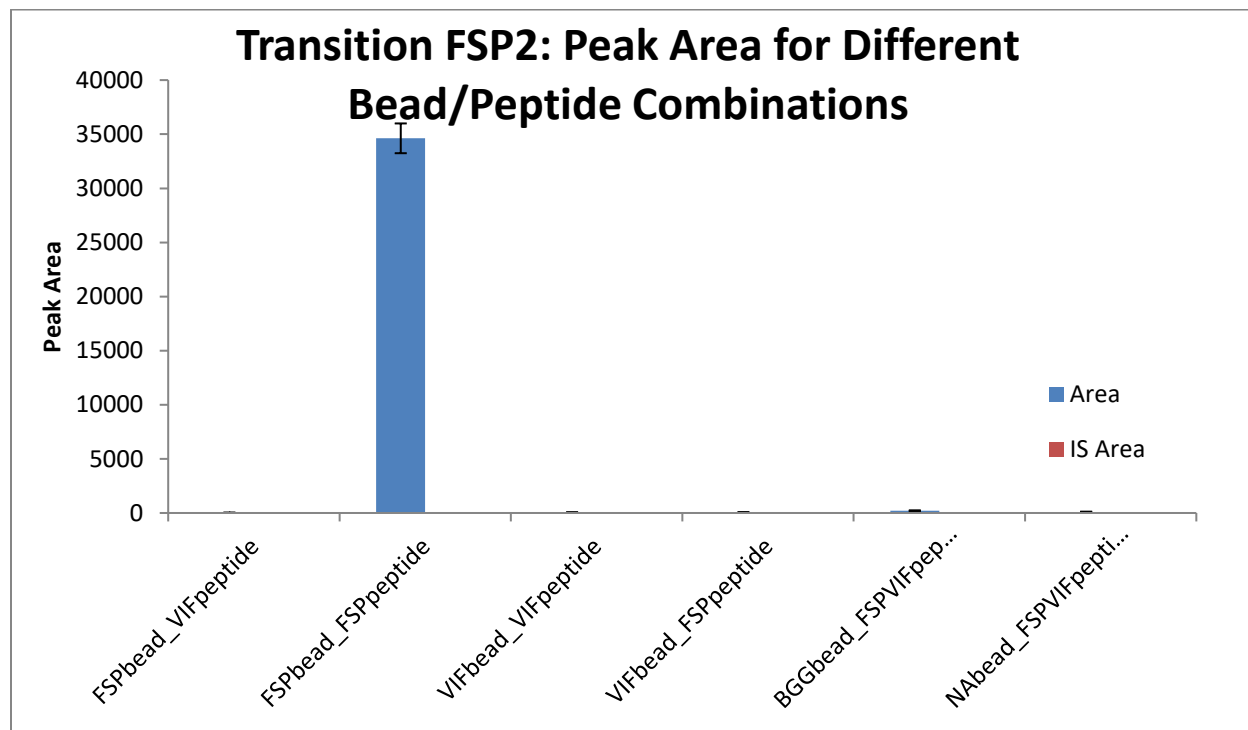


Figure 2-5. Bead specificity for transition FSP2. Error bars show the range of duplicate results.

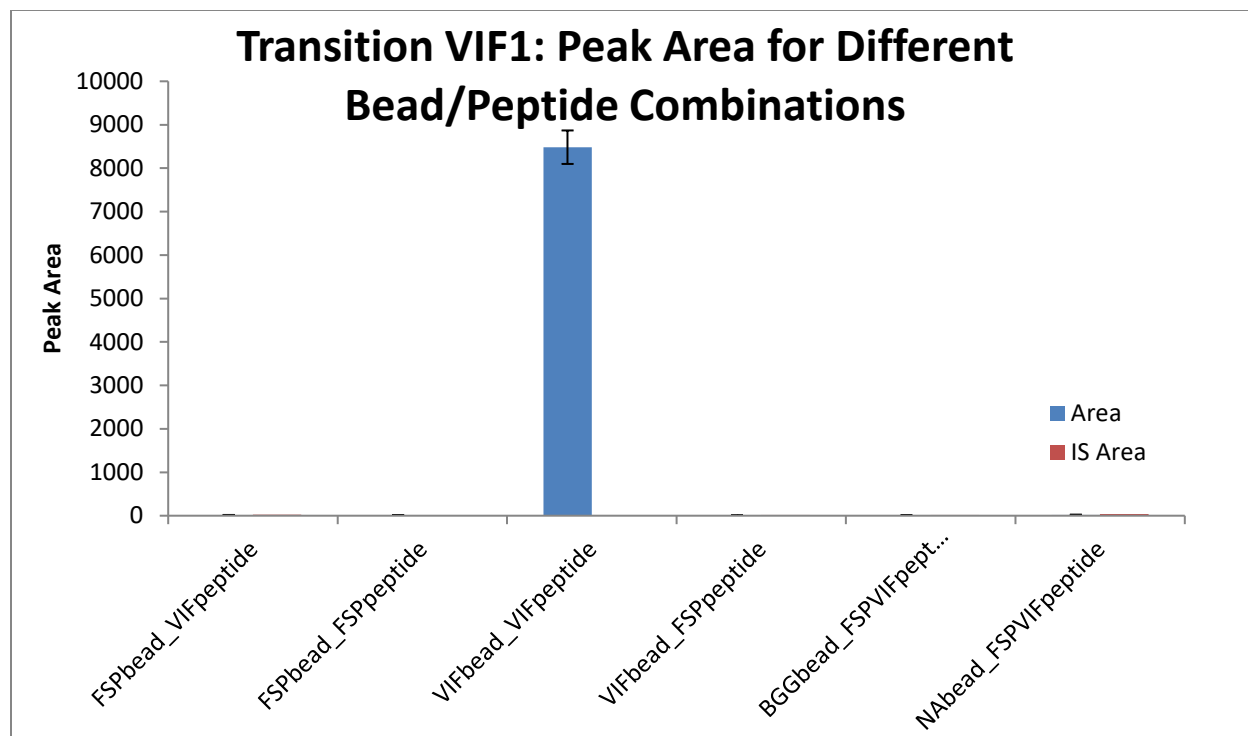


Figure 2-6. Bead specificity for transition VIF1. Error bars show the range of duplicate results.

Due to the high signal of Anti-FSP beads compared to the Anti-VIF beads and given the low background with BGG beads, the FSP beads were diluted and tested. In this experiment, Anti-FSP beads were diluted 1:10 with bovine gamma globulin (BGG) beads. A 1.5 mL Eppendorf tube had 820 μL digested chicken serum, 10 μL peptide, and 5 μL beads added. The peptide spike concentrations were 50 fmol/ μL , 100 fmol/ μL , and 250 fmol/ μL peptide. The beads used were FSP:BGG diluted beads except only for one triplicate which used BGG only beads for comparison at 250 fmol/ μL peptide spike. The tubes were incubated for 1 hour at room temperature, touched down, and placed on the tube magnet. The digest was removed, and each tube was washed by hand using 500 μL of PBS/0.1% CHAPS twice. The beads were then resuspended in 50 μL of 2.5% acetic acid/0.1% CHAPS and placed in a thermomixer for 10 minutes at 1200 RPM. The tubes were then touched down, the supernatant was transferred into

fresh tubes, and a cleanup step was performed. The tubes were centrifuged for 5 minutes at 13,000RPMs, placed on the magnet, and the supernatant was transferred into an autosampler vial for a 20 μ L injection on the mass spectrometer.

Figure 2-7 shows the results of the comparison of FSP:BGG diluted beads to beads conjugated with only BGG. There is almost no signal with the BGG beads, less than 0.02% signal, compared to the FSP beads diluted 1:10 with BGG beads. Also, when looking at the results of multiple concentrations using the FSP:BGG diluted beads, the beads recover well at multiple concentrations with minor variation as seen in figure 2-8.

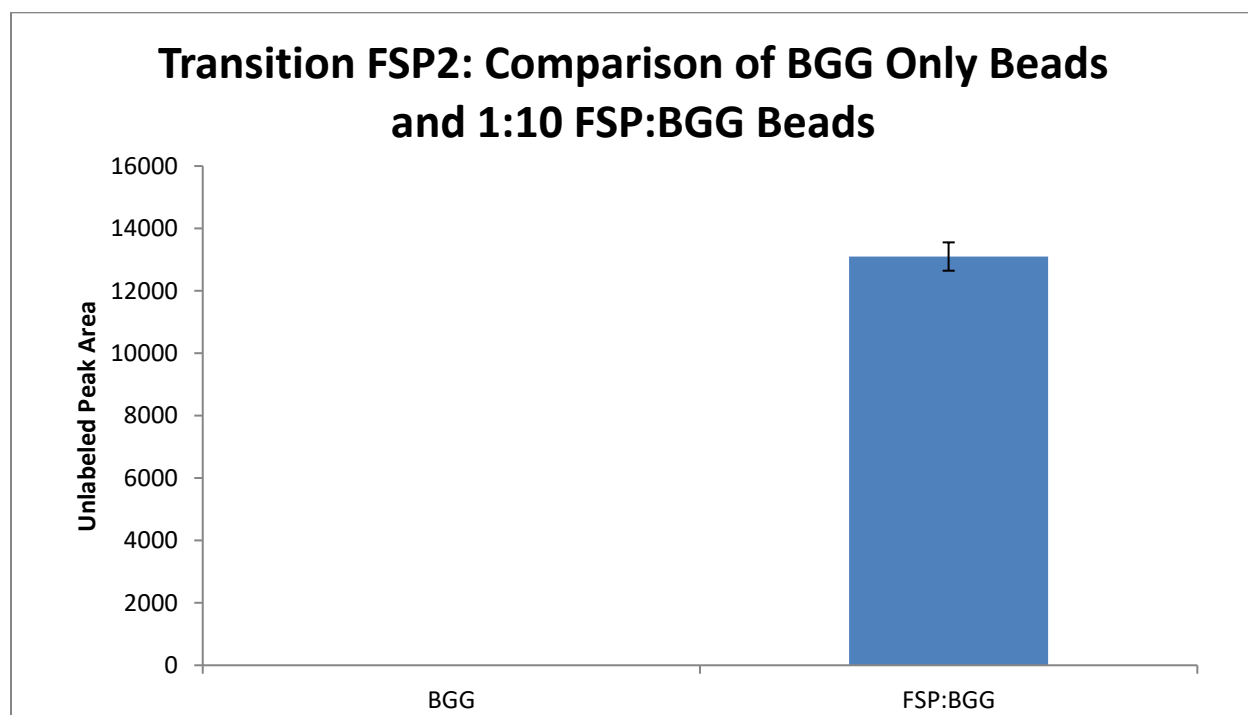


Figure 2-7. Comparison of BGG and FSP beads diluted 1:10 with BGG beads for transition FSP2. Error bars show the standard deviation.

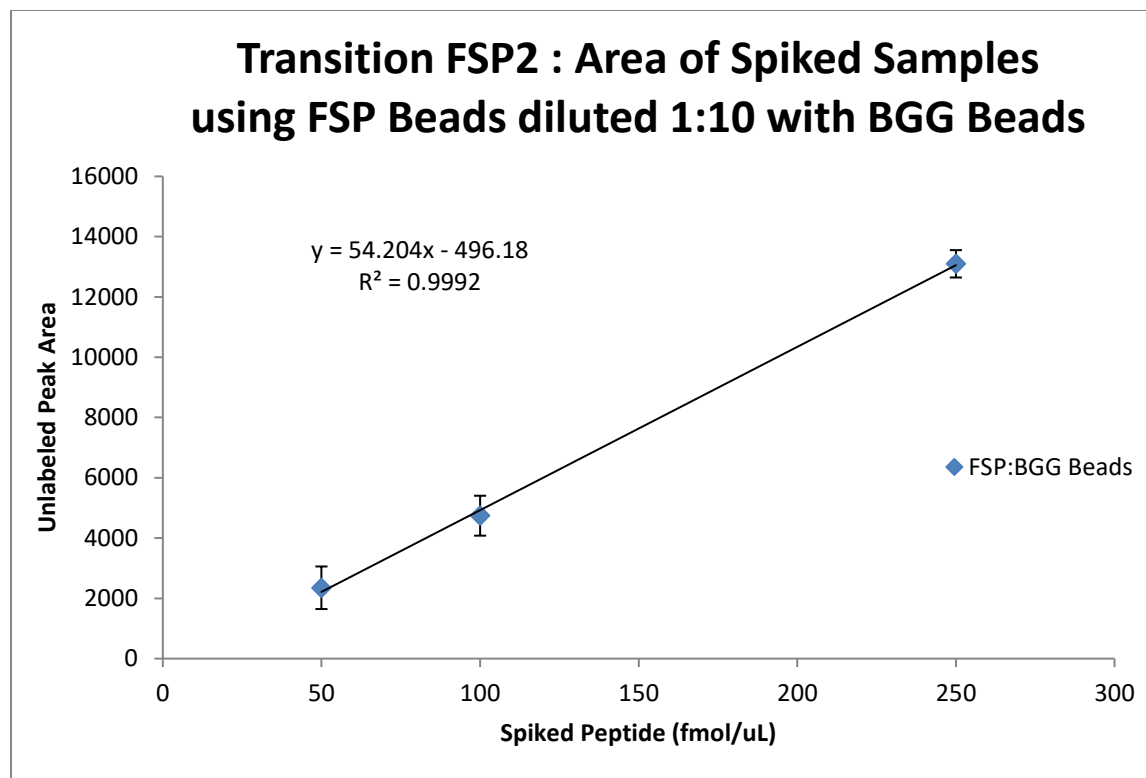


Figure 2-8. Linearity at fmol/ μ L peptide spike concentrations using FSP beads diluted 1:10 with BGG beads. Error bars show the standard deviation.

2.6 MATRIX EFFECTS

Previous work has used a handful of different matrices for screening antibodies with various results in signal and efficacy. To determine how the FSP antibody performed in different matrices, a list was compiled of previously examined matrices and a select few were tested in triplicate [12, 13, 18]. A volume of 85 μ L PBS/CHAPS, 10% serum, 10% BSA, 10% chicken serum digest (CSD), and 85% CSD was added in triplicate to a BioRad PCR plate. To the matrix, 10 μ L of light peptide and 5 μ L of diluted FSP beads were added. The plate was sealed with foil and incubated for 2 hours at room temperature end-over-end on a Labquake. At the end of incubation, the plate was touched down for 10 seconds at 3000 RPM on a centrifuge before the

plate was washed via Biotech plate washer method. The plate washer method used the VP 771BT-LE-A magnet and PBS/0.1% CHAPS as wash buffer, which was primed once before the wash method was run. The wash method included a stationary period, two 150 μ L washes with shaking, and aspiration of fluid. Once the beads were washed, 50 μ L of the elution buffer containing Internal Standard was added to the plate, sealed with foil, and incubated end-over-end at room temperature on the Labquake for 8 minutes. The plate was touched down on a centrifuge for 10 seconds at 3000 RPM and placed on the DynalMPC-96S magnet. The supernatant was transferred to a fresh BioRad PCR plate labeled "cleanup plate 1," centrifuged for 5 minutes at 3000 RPM, and placed back on the DynalMPC-96S magnet. The supernatant was transferred to a fresh BioRad PCR plate labeled "cleanup plate 2," centrifuged for 5 minutes at 3000 RPM, placed back on the DynalMPC-96S magnet. The final supernatant was transferred into the Waters Aquity UPLC 700 μ L round 96-well injection plate paired with VP 771LWAS magnet for injection onto the mass spectrometer.

Figure 2-9 shows the comparison of signal in each of the matrices. The measurement with least variability and highest signal was seen in the PBS/CHAPS matrix whereas the highest variability was seen in the 10% BSA matrix. The least signal was seen in the 85% chicken serum digest matrix with a bit more signal seen in 10% chicken serum digest matrix. The most comparable in signal to the PBS/CHAPS was the 10% chicken serum matrix.

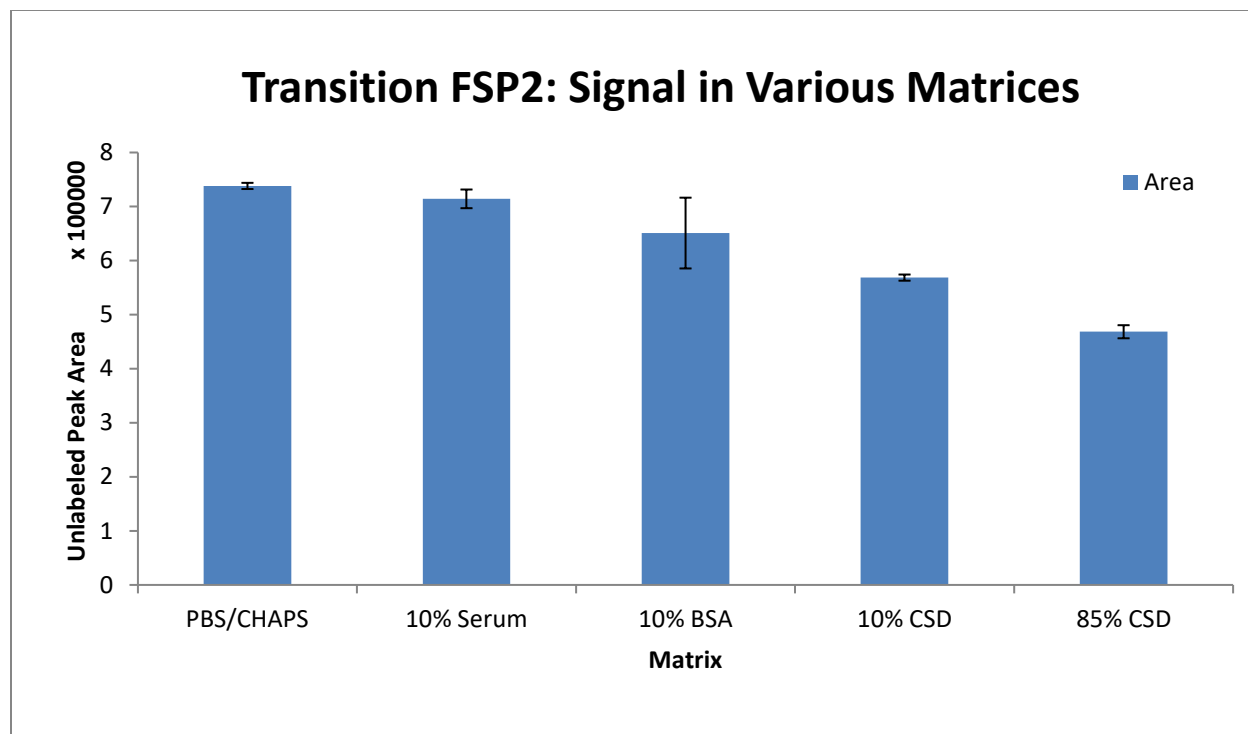


Figure 2-9. Comparison of signal using various matrices in the Biotech plate for transition FSP2. Error bars show the standard deviation.

2.7 IMMUNOAFFINITY WORKFLOW CONCLUSIONS

Selection of the best equipment and reagents for an immunoaffinity enrichment workflow included a handful of experiments such as a mixing study, elution buffer check, wash study, matrix study, and assessment of bead specificity. The best parameters for the current workflow was to do mixing at room temperature end-over-end, to use acetic acid with either CHAPS or ACN added but not both, to wash quickly, and to use PBS/0.1% CHAPS as a matrix whenever possible. The tosyl-activated paramagnetic beads did not display nonspecific binding, and BGG beads were sufficient for diluting the beads to decrease signal if needed.

One of the workflow goals was to find a way to use plates to do the immunoaffinity enrichment, and a comparison between tubes and plates was done. The first choice was to use the

2 mL plate due to its large volume capacity; however, there were a handful of problems that ensued. Sealing a 2 mL plate was the most difficult with multiple options available: blue cap mat, clear cap mat, tape, foil, and heat seal foil. No mat, regular tape, and regular foil proved unusable for end-over-end mixing (data not shown). Heat seal foil was adequate; however, the magnet used with the 2 mL plate did not sufficiently allow for clean separation of supernatant from paramagnetic beads leading to plugging of the injector on the mass spectrometer.

Ultimately, the 150 μ L PCR plate previously used for wash steps was the best option for future experiments due to the low CV. For continued assay development, a larger plate volume will be necessary to get the sensitivity in digested human serum.

Chapter 3. ANTIBODY RECOVERY BASED SCREEN

3.1 PREVIOUS ANTIBODY SCREEN METHODS

A handful of different methods have previously been used for screening antibodies. An effective LC-MS/MS antibody screen only needs one sample per hybridoma for comparison of hundreds of antibody containing supernatants using just the amount of signal recovered [12]. Those which have a good recovery efficiency at constant supernatant volume can be further studied whereas those with poor recovery are not useful for further study because only hybridomas containing antibodies which both bind well and secrete well are those which are attractive for use as a reagent [12]. Previous work used ELISA for screening hybridomas for new antibody reagents; however, MALDI and ESI mass spectrometry has recently been used to eliminate some of the negative aspects of ELISA screens, such as false positives and false negatives, while remaining high throughput [12,13,14]. An automated screening process using a KingFisher magnetic particle processor was developed using eight plates to screen a single plate of antibody supernatants [12]. When comparing LC-MS/MS screening and ELISA screening, one significant difference is that ELISA cannot be multiplexed like LC-MS/MS, which can detect multiple peptides in a single sample [14]. Since LC-MS/MS has previously been successful in screening antibodies and can be multiplexed, I developed an in-house antibody screen to effectively test hundreds of antibody supernatants.

3.2 METHOD DEVELOPMENT AND WORKFLOW IMPROVEMENTS

To ensure the plate method would work well, as seen in the appendix, each step of the method was tested for peptide loss in comparison to an untested standard. The steps tested were

peptide mix contacting the plate once, after the first cleanup step, after the second cleanup step, and at the end of the workflow at final injection. The peptide mix contained the following peptides: FSPDDSAGASALLR, SSALFYQK, GPVGPSGPPGK-OH 81, GPVGPSGPPGK 89, FCHPELK, FTYTVLEDGCTK, LGYLVAHNLLLAHAK, GLFYVDFLSQDK. In other words, this “losses” experiment was an experiment to track peptide loss from elution step to final injection.

Figure 3-1 shows the results of the losses experiment for each step. The two peptides with the most extreme loss were GLFY and LGYL, both of which already had poor chromatography. These two peptides are the most hydrophobic tested. Figure 3-2 shows the percentage of loss for each peptide for easy comparison. Peptide SSA had the least amount of cumulative loss. All other peptides, excluding GLFY and LGYL, had less than 11% loss in signal.

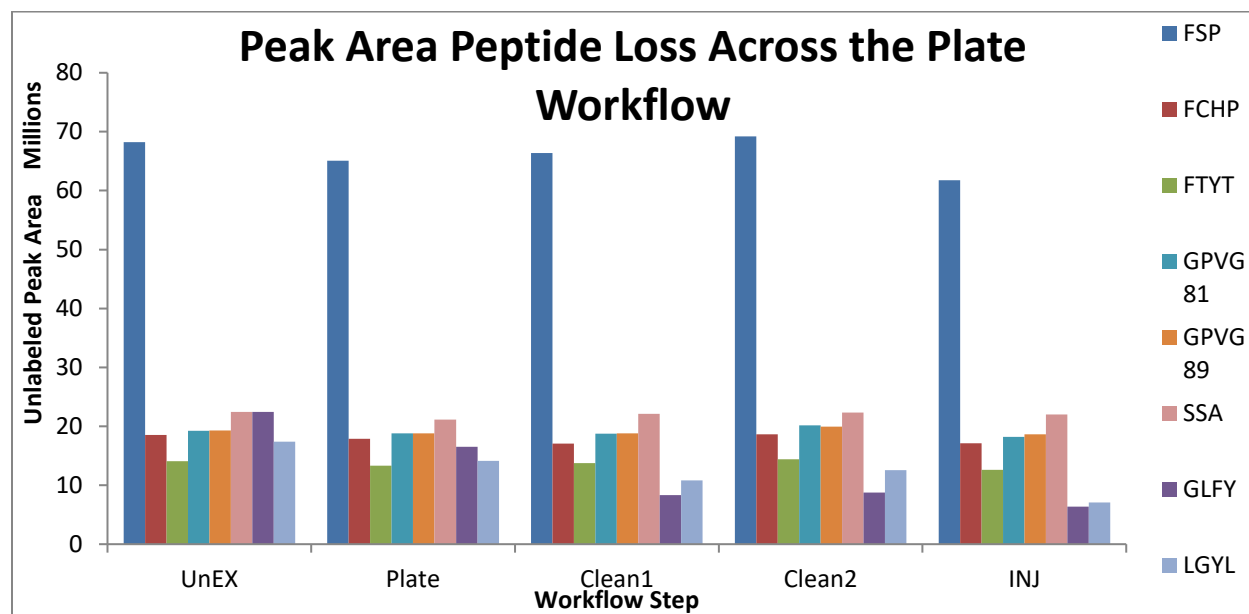


Figure 3-1. Losses experiment using the best transition for all antibody screen peptides: FSP, FCHP, FTYT, GPVG81, GPVG89, SSA, GLFY, and LGYL.

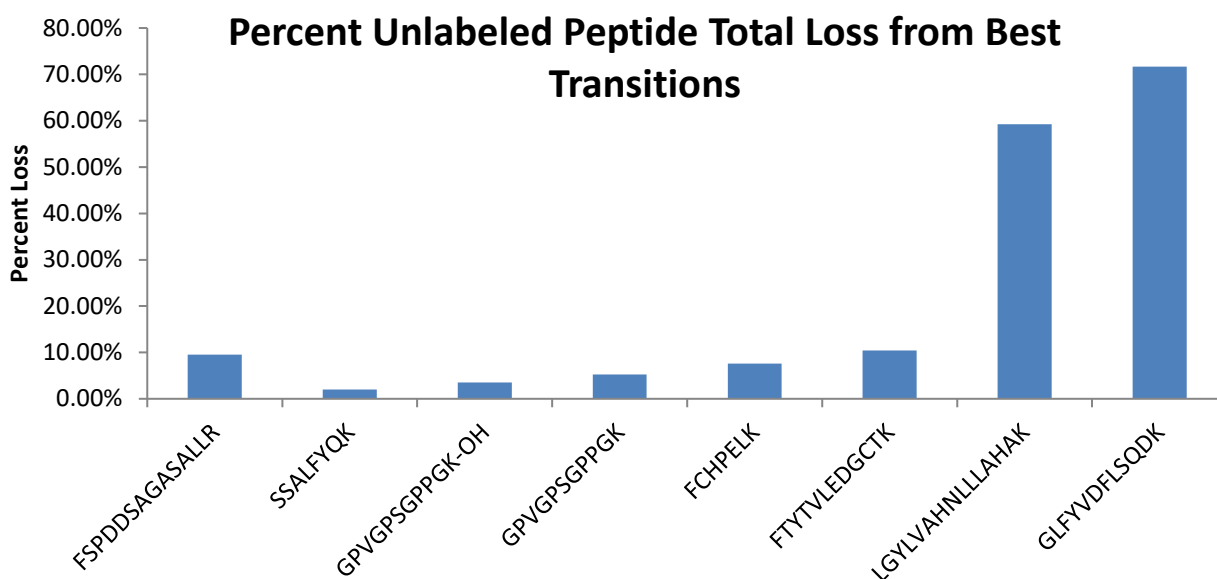


Figure 3-2. Percent unlabeled peptide loss using the best transitions for each peptide.

A plate method was developed to screen 9 plates of supernatants provided by the Shared Resource Lab at the Eastlake Fred Hutch building. The supernatants were produced in a serum growth media stored individually in sealed tubes at 4°C. The antibodies were produced in mice using inoculation of the following peptides: LGYL, GLFY, SSA, FTYT, and GPVG. Although antibody concentrations were unknown, constant antibody volume was used to incubate with sheep anti-mouse IgG paramagnetic beads following the outlined bead preparation in section 2.2. As a positive control, Anti-FSP beads were made using tosyl-activated paramagnetic beads following the bead preparation in section 2.2, but 20 µL of Anti-FSP beads were diluted in 9 mL of PBS/0.1% CHAPS directly before use.

Peptide mixes were aliquoted after initial creation to avoid freeze/thaw degradation between experiments. The endogenous peptide mix was created to ensure a large enough peak area able to be seen even if an antibody recovered at less than 5% the initial concentration. The

endogenous peptide mix contained the following peptide concentrations: FTYT at 5 μM , FSP at 5 μM , GPVG at 13 μM , GPVG-OH at 35 μM , FCHP at 7 μM , GLFY at 6 μM , LGYL at 6 μM and SSA at 5 μM . The Internal standard mix was made at the following approximate concentrations to ensure good peak areas: FTYT at 1.286 μM , FSP at 0.05 μM , GPVG at 1.704 μM , GPVG-OH at 1.854 μM , FCHP at 1.993 μM , GLFY at 1.422 μM , LGYL at 0.828 μM and SSA at 1.953 μM . Internal standard was added to the elution buffer, 5% Acetic Acid/3% Acetonitrile, at 50 μL Internal Standard peptide mix in 5 mL of elution buffer right before use.

After their synthesis, beads were aliquoted into the 96 well-plate. PBS/0.1% CHAPS was added at a 90 μL volume followed by an addition of 10 μL of endogenous peptide into each well. The plate was sealed with foil and incubated for 2 hours at room temperature end-over-end on a Labquake. At the end of incubation, the plate was touched down for 10 seconds at 3000 RPM on a centrifuge before the plate was washed via the Biotech plate washer method. The plate washer method used the VP 771BT-LE-A magnet and PBS/0.1% CHAPS as wash buffer, which was primed once before the wash method was run. The wash method included a stationary period, two 150 μL washes with shaking, and aspiration of fluid. Once the beads were washed, 50 μL of the elution buffer containing Internal Standard was added to the plate, sealed with foil, and incubated end-over-end at room temperature on the Labquake for 8 minutes. The plate was touched down on a centrifuge for 10 seconds at 3000 RPM and placed on the DynalMPC-96S magnet. The supernatant was transferred to a fresh BioRad PCR plate labeled "cleanup plate 1," centrifuged for 5 minutes at 3000 RPM, placed back on the DynalMPC-96S magnet. The supernatant was transferred to a fresh BioRad PCR plate labeled "cleanup plate 2," centrifuged for 5 minutes at 3000 RPM, placed back on the DynalMPC-96S magnet. The final supernatant

was transferred into the Waters Aquity UPLC 700 μ L round 96-well injection plate paired with VP 771LWAS magnet for injection onto the mass spectrometer.

Peptides were auto-tuned on the Waters TQSmicro. Acquisition was scheduled to allow 4-5 transitions per peptide using a C18 T3 column with mobile phases 98% Optima water/2% DMSO/0.1% Formic Acid and 98% Methanol/2% DMSO/0.1% Formic Acid. A linear gradient was used with a 10 minute run time, and samples were run overnight. Data was integrated using Skyline and analyzed in Microsoft Excel.

3.3 ANTIBODY SCREEN RESULTS

Nine plates containing 94 hybridomas, a total of 846 antibody containing supernatants, were screened for recovery in comparison to an unextracted sample to find antibodies that recover peptide efficiently. The antibodies were tested at constant volume and compared within each plate. A final 10th plate was used to test 94 antibodies that had either the potential to recover efficiently or could have had a problem in the original experiment. A final distribution list was created and shown to have antibodies tested across all plates for all targets as shown in table 2. Mouse 86a supernatants were retested due to all supernatants from this mouse having unacceptable recovery.

Table 2. Final distribution list of antibody supernatants for re-testing.

PLATE	P3CP		KLOTHO		KLOTHO		TOTAL
	FCHP	FTYT	GLFY	GPVG	LGYL	SSA	
1	0	6	5	0	1	2	14
2	0	4	3	0	2	4	13
3	1	4	2	0	0	3	10
4	0	4	2	0	1	3	10
5	0	4	3	0	0	2	9
6	2	3	2	0	1	4	12
7	0	0	0	6	1	0	7
8	0	0	0	7	1	0	8
9	0	2	2	3	0	0	7
	3	27	19	16	7	18	90
						86a	4
						Mouse:	
						TOTAL:	94

The final plate resulted in many positive supernatants; however, the goal was to find the best few antibodies for purification. The five highest recovery antibodies were selected for GPVG and FTYT, and the six highest recovery antibodies were selected for SSA. No antibodies were selected for LGYL or GLFY due to bad chromatography and extreme loss in the experiments as well as negative results in the final plate check. The final constant volume recoveries are seen in figure 3-3. GPVG has very high recovery whereas SSA antibodies recover the least. Despite more positive antibodies in the 846 supernatants, FTYT recovered well but less than GPVG.

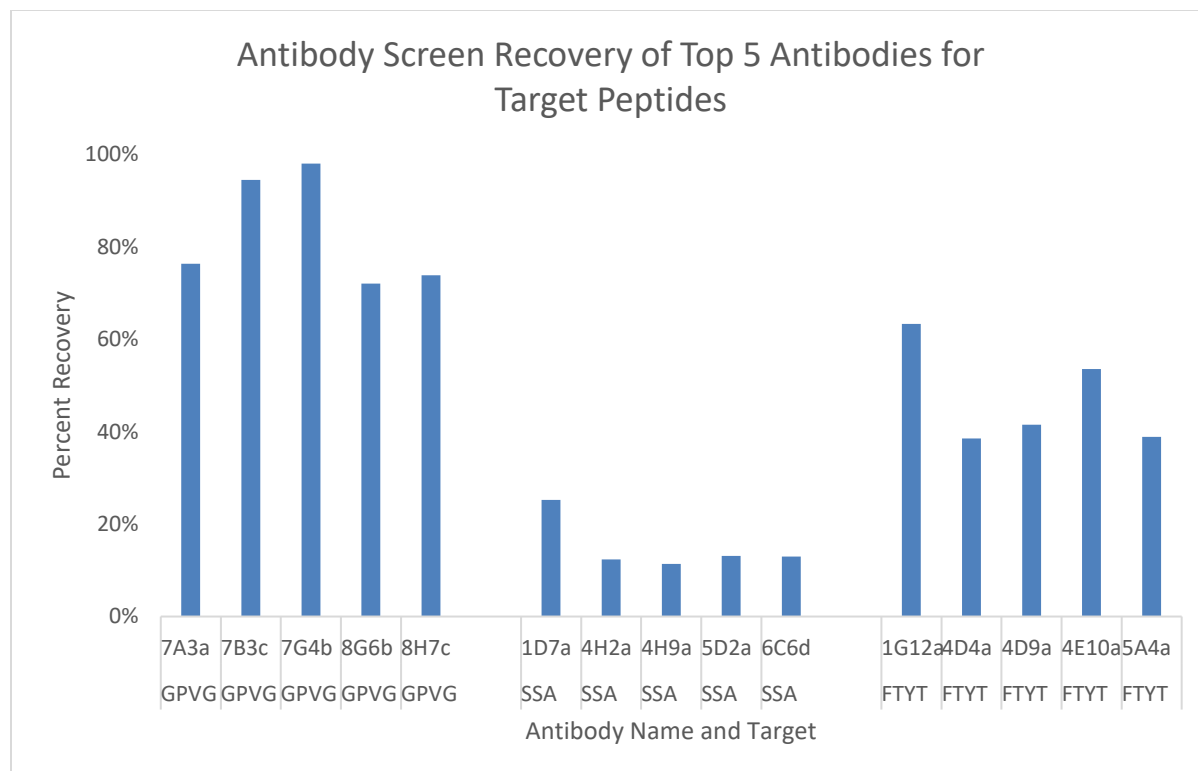


Figure 3-3. Top recovery antibodies for the best three targets: GPVG, SSA, and FTYT.

3.4 ANTIBODY SCREEN DISCUSSION

Over the course of a few weeks, 846 antibody supernatants were tested and whittled down to a top 16 antibodies for purification. The shared resource lab in Eastlake then purified 15 of the antibodies, 5 antibodies per target. One of the SSA antibodies listed for purification failed which would make it undesirable anyway for future workflows.

There are some areas in which this screen could have been improved. Despite previous work in screening chromatography columns for the best choice for all peptides, a screen for other columns and mobile phases might be helpful for testing highly hydrophobic peptides since this screen failed to provide satisfactory results for LGYL and GLFY. FTYT, with a hydrophobicity of about 24, was the most hydrophobic peptide that was successful in experiments. It would be

important to create a separate screen for peptides of higher hydrophobicity than FTYT to ensure more accurate results.

Previously, before the in-house antibody screen, data was collected by the Shared Resource Lab screening the antibodies. The method used was an iQue bead-based ELISA. All positive and negative antibodies were listed in an excel file; however, as with all ELISA-like assays, they are prone to false positives and false negatives. Although in general the results of the LC-MS/MS screen agreed with some of the iQue results, and every chosen antibody for purification was positive by both methods, there were large numbers of false positives using the iQue system.

Chapter 4. IMMUNOAFFINITY BINDING KINETICS

4.1 PREVIOUS ANTIBODY KINETIC WORKFLOWS

There are a few different ways to characterize antibody (Ab) – antigen (Ag) interaction and these include the apparent dissociation constant (K_D), the association rate (k_{on}), and the dissociation rate (k_{off}), which are all interrelated. As seen in the equation below, the apparent dissociation constant is equal to the dissociation rate divided by the association rate [23].

$$\text{Equation 1.} \quad K_D = \frac{k_{off}}{k_{on}} = \frac{[Ab][Ag]}{[Ab \cdot Ag]} = \frac{1}{K_A}$$

Two methods to determine the affinity constant (K_D) are a binding curve analysis and a Scatchard analysis; although, other ways exist such as individually determining the on and off rates. For ease, I chose binding curves due to the amount of variation seen when determining on and off rates by the plate method. Antibody binding curves are created with a constant antibody concentration and varying spiked peptide concentration [1]. The K_D is the concentration at half of the maximum binding, B_{max} [1]. Alternatively, the Scatchard plot uses similar data, but expresses the amount of antibody bound/free vs. the amount of antibody bound to produce a linear expression relating K_D and B_{max} where an unextracted sample is used to determine how much peptide is bound [1].

$$\text{Equation 8.} \quad \frac{Bound}{Free} = \frac{[Ag \cdot Ab]}{[Ab]} = \frac{(B_{max} - Bound)}{K_D}$$

The intercepts of a Scatchard plot contain the most detail with the x-intercept being the maximum binding and the y-intercept being B_{max}/K_D [1]. The apparent K_D found by using a hyperbolic binding curve to reach maximum binding is experimentally equal to the inverse of the y-intercept of the equivalent Scatchard plot [1].

There are a handful of different methods to measure the apparent K_D for antibodies, and some of these methods include but are not limited to ELISA, SPR, and LC-MS/MS. ELISA is relatively cheap and high throughput using absorption of light or percent transmittance as its signal of measurement; however, even under the best conditions ELISA is prone to errors such as inability to measure high affinity antibodies consistently as well as large dissociation rates, k_{off} [2,5]. SPR, unlike ELISA, uses refractive index changes to measure concentration of peptide on the interface of the sensor containing bound antibody, and Biacore produces a handful of useful systems to provide k_{on} , k_{off} , and K_D results [2,5]. Another producer of equipment capable of determining affinity constants is ForteBio who uses technology like SPR with peptide bound to the sensor measuring the binding of the antibody via a shift in wavelength due to thickness [7]. Unlike ELISA and SPR signal which is dependent on light for an indirect measure of binding, LC-MS/MS workflows directly measure peptide eluted from antibody following immunoaffinity enrichment, which provides signal when it exits the quadrupoles and hits the detector [9,10]. LC-MS/MS can be high throughput like ELISA, but LC-MS/MS use of an antibody in measuring peptides adds layers of specificity to the measurement [10]. Which method to use is an important decision when determining how to measure antibody kinetic constants due to what is being measured and how an antibody will be used in the future.

4.2 DETERMINATION OF ASSOCIATION RATE

Association was determined by an additive plate method using the BioRad hard shell PCR 96 well plate with the VP 771BT-LE-A magnet for plate washing, but it was paired with the DynalMPC-96S for transferring samples between plates. Digested serum was the matrix used, and 85 μL was added to the plate as well as 10 μL 0.5 pmol/ μL peptide and 5 μL 1:10 diluted

FSP:BGG tosyl beads. For the incubation period, every 10 minutes the plate was centrifuged for 3 seconds at 500 RPM to touch down before the next sample was added. The plate was sealed with aluminum silver seal and incubated end-over-end on the Labquake at room temperature. This additive method created the first sample added to be timepoint 120 minutes and the last sample to be added to be the 0 timepoint as the incubation lasted for 2 hours. At the end of the incubation, the plate was put on the plate washer on the VP magnet and washed twice with PBS/0.1%CHAPS. The samples then had 50 μ L 2.5% acetic acid/0.1%CHAPS added to elute, and the plate was incubated end-over-end on the Labquake for 8 minutes before touch down. Two cleanup plates were used for transferring, and each cleanup plate was centrifuged for 5 minutes at 3000 RPM. Final eluates were transferred into the Waters Aquity UPLC 700 μ L round well plate paired with VP 771LWAS magnet for injection.

Figure 4-1 shows the results of the association experiment. Peptide is still actively binding to the antibody after one hour, and the signal starts to plateau at about 2 hours. Due to the nature of the experiment, addition of samples to a single plate, there was some increased variation at time points in the experiment. It is recommended for incubation to be at least 90 minutes to allow for sufficient signal for binding experiments.

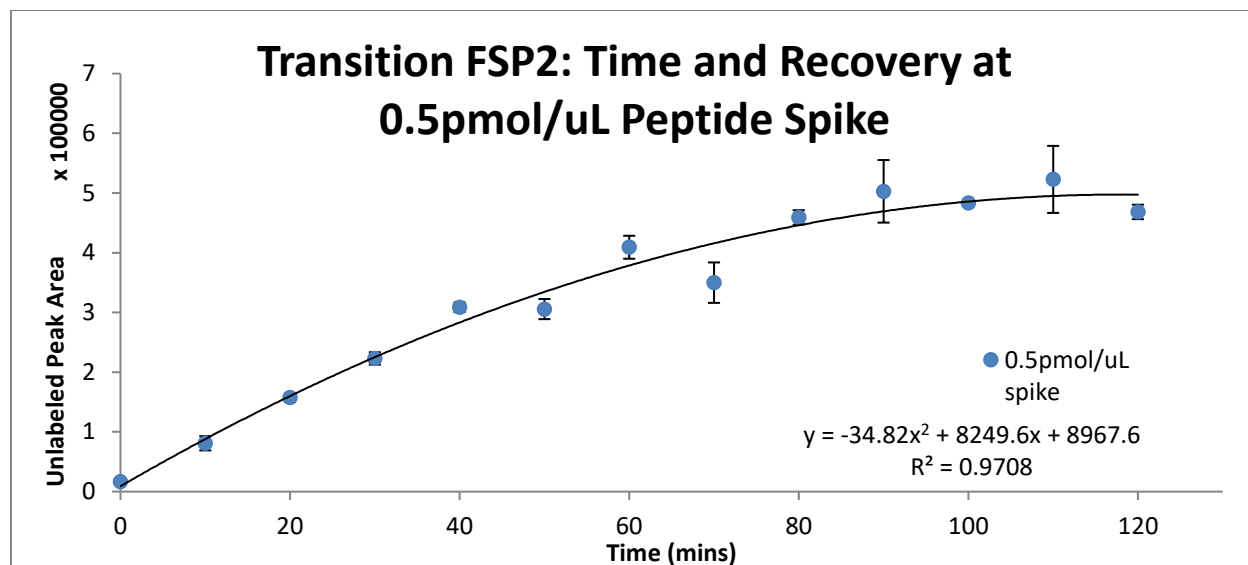


Figure 4-1. Association of FSP peptide for Anti-FSP antibody beads. Error bars show the standard deviation.

4.3 DETERMINATION OF DISSOCIATION RATE

Dissociation was determined using the tube method for immunoaffinity enrichment. A 1.5 mL Eppendorf lo-bind tube was used. Digested serum was the matrix used, and 85 μ L was added to each tube as well as 10 μ L 0.5 pmol/ μ L peptide and 5 μ L FSP beads diluted 1:10 with BGG tosyl beads and VIF beads. The tubes incubated for 60 minutes end-over-end in the Labquake, and every 10 minutes a sample was removed, placed on Invitrogen's DynaMag-2 and the matrix containing peptide was removed and replaced with PBS/0.1% CHAPS. The tubes were then placed back on the Labquake. At the end of the incubation the tube samples were removed, placed into a BioRad hard shell PCR 96-well plate, and the plate was put on the plate washer on the VP magnet. The plate was washed twice with PBS/0.1% CHAPS. The samples then had 50 μ L 2.5% acetic acid/0.1% CHAPS added to elute, and the plate was incubated end-over-end on the Labquake for 8 minutes before touch down. Two clean-up plates were used for transferring,

and each clean-up plate was centrifuged for 5 minutes at 3000 RPM. Final eluates were transferred into the Waters Aquity UPLC 700 μ L round well plate paired with VP 771LWAS magnet for injection.

Figure 4-2 shows the results of the dissociation experiment for Anti-FSP and Anti-VIF antibodies for their respective peptides. VIF dissociates much faster than FSP. The experimental time in minutes VIF takes to completely dissociate is 173 minutes whereas FSP takes 473 minutes.

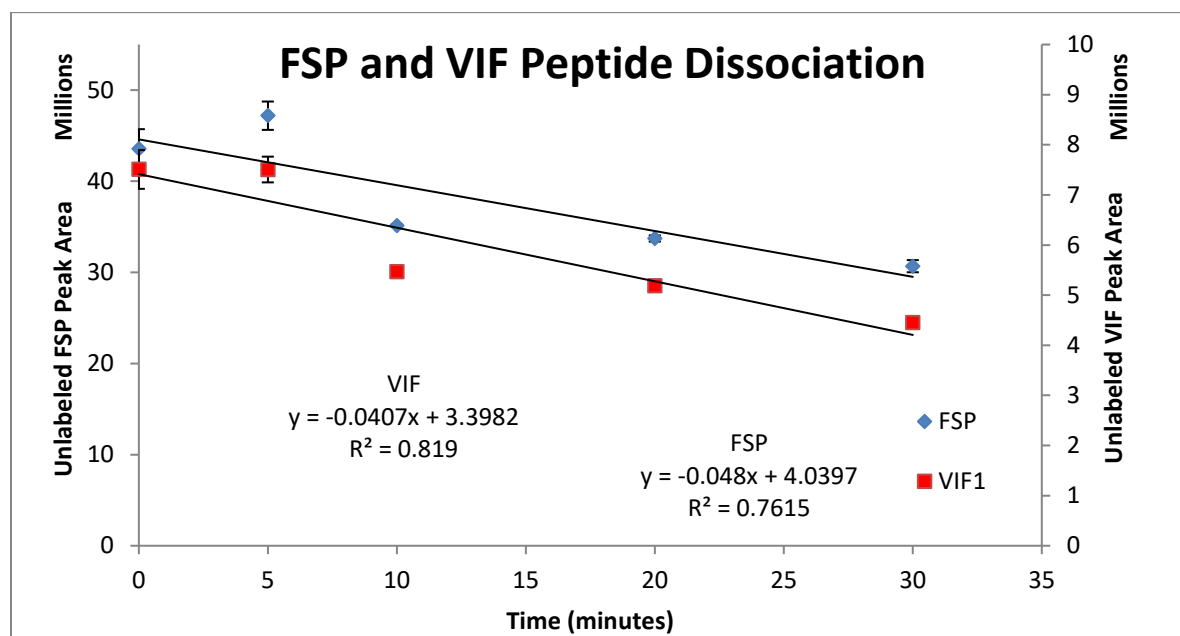


Figure 4-2. Dissociation of peptide FSP and VIF in PBS/CHAPS over time. Error bars show the standard deviation.

4.4 ANTIBODY CHARACTERISTICS VIA TANDEM MASS SPECTROMETRY

The plate method used for the antibody screen was employed for determination of K_D for antibodies of interest. Preliminary testing was done on existing thyroglobulin assay antibodies, Anti-VIF and Anti-FSP, and tosyl-activated beads were prepared following current thyroglobulin

assay protocols [17]. A volume of 85 μL PBS/0.1% CHAPS was added followed by an addition of 10 μL of endogenous peptide and 5 μL of beads into each well. The initial binding curve peptide spike concentrations were 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, 2.0, 3.0, 3.5 pmol/ μL unlabeled FSP and VIF peptide mix. The plate was sealed with foil and incubated for 2 hours at room temperature end-over-end on a Labquake. At the end of the incubation, the plate was touched down for 10 seconds at 3000 RPM on a centrifuge before the plate was washed via plate washer method. The plate washer method used the VP 771BT-LE-A magnet and PBS/0.1% CHAPS as wash buffer, which was primed once before the wash method was run. The wash method included a stationary period, two 150 μL washes with shaking, and aspiration of fluid. Once the beads were washed, 50 μL of the elution buffer containing Internal Standard was added to the plate, sealed with foil, and incubated end-over-end at room temperature on the Labquake for 8 minutes. The plate was touched down on a centrifuge for 10 seconds at 3000 RPM and placed on the DynalMPC-96S magnet. The supernatant was transferred to a fresh BioRad PCR plate labeled “clean-up plate 1,” centrifuged for 5 minutes at 3000 RPM, placed back on the DynalMPC-96S magnet. The supernatant was transferred to a fresh BioRad PCR plate labeled “clean-up plate 2,” centrifuged for 5 minutes at 3000 RPM, placed back on the DynalMPC-96S magnet. The final supernatant was transferred into the Waters Aquity UPLC 700 μL round 96-well injection plate paired with VP 771LWAS magnet for injection using the existing thyroglobulin assay method [17].

Figure 4-3 shows the results of the binding curves for FSP and VIF transitions. Data points are the average of triplicates. The curves were generated individually using mycurve.com before combining in one graph. As expected, Anti-FSP antibody beads diluted 1:10 with BGG beads bound FSP peptide to a higher maximum binding (B_{max}) than Anti-VIF antibody beads

despite Anti-VIF beads being 10 times more concentrated than Anti-FSP beads. The Anti-FSP beads reached a plateau whereas the Anti-VIF beads were close to not plateauing at a maximum binding concentration; although, the results were sufficient for analysis.

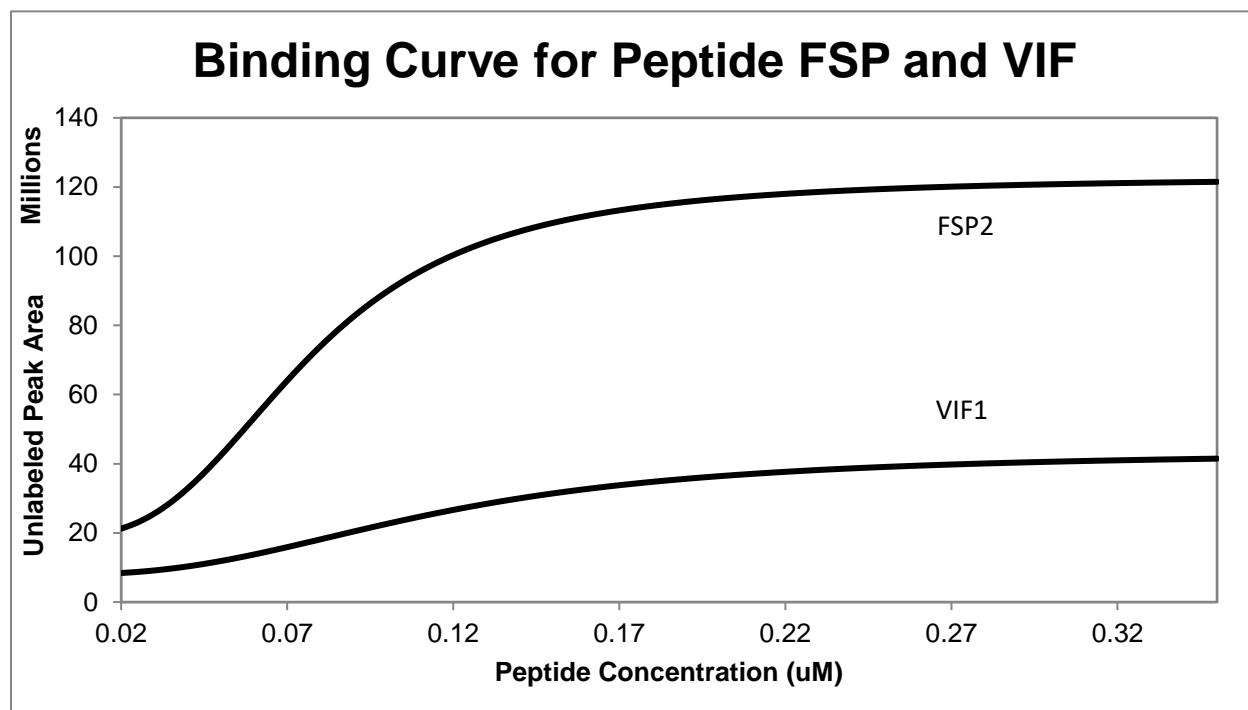


Figure 4-3. Binding curve of Anti-FSP antibody diluted 1:10 with BGG beads and Anti-VIF beads in PBS/CHAPS.

4.5 DETERMINATION OF AFFINITY

For determination of affinity, the data was collected using the antibody experiment in section 4.4, and data points were then plotted in a Scatchard plot for analysis. The y-intercept is the relationship of B_{\max}/K_D , but its inverse also is the K_D as determined by binding curve. As can be seen in figure 4-4, the better binding antibodies have a higher y-intercept and lower K_D

concentration. The Anti-FSP K_D was determined via Scatchard analysis to be $0.039 \mu\text{M}$ and Anti-VIF was $1.12 \mu\text{M}$.

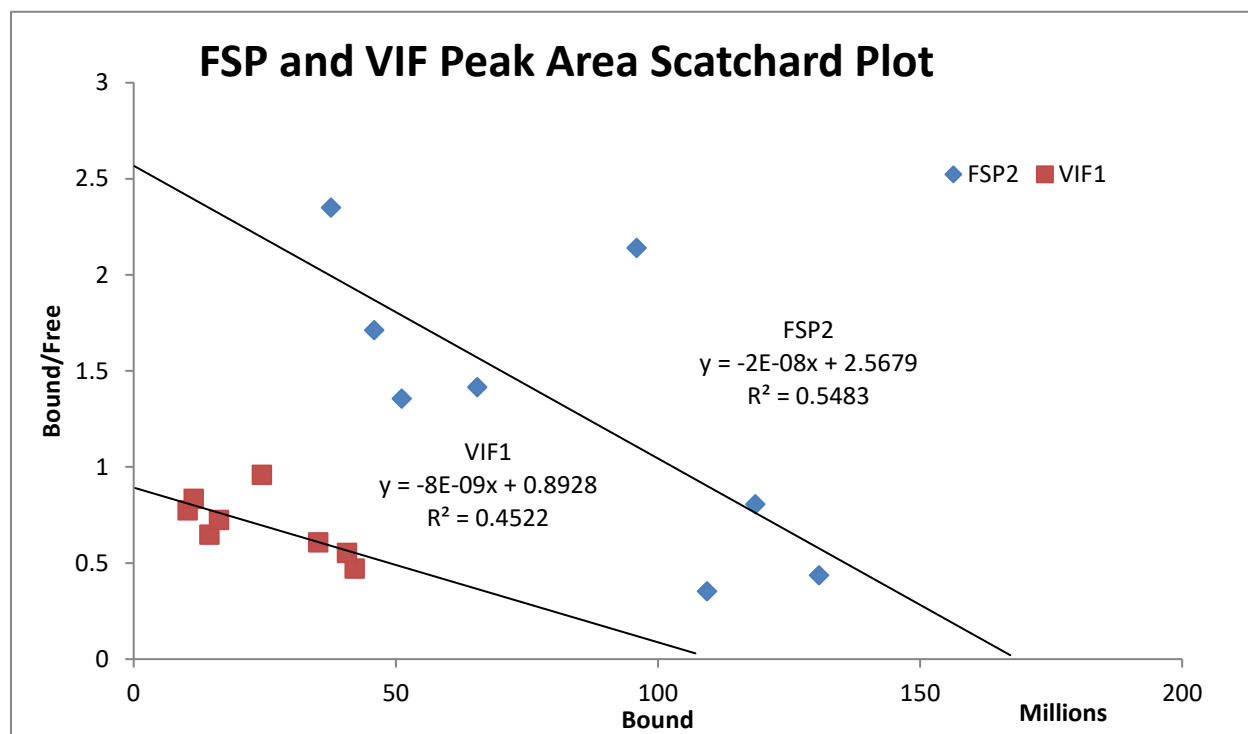


Figure 4-4. Scatchard Plot of Binding Data for Anti-FSP and Anti-VIF antibodies.

4.6 SCREENING AFFINITY

A full K_D binding curve requires many sample concentrations in triplicate, so a quicker way to determine or screen for affinity was advantageous. Figure 4-5 shows the same results of section 4.4 for the Anti-FSP and Anti-VIF binding curve; however, figure 4-5, unlike figure 4-3, has normalized data so the two graphs are superimposed. This is one way to show how the left shift results in better binding for FSP compared to VIF, given their respective antibodies. To screen affinity, it would be expected to want to use concentrations which have the greatest difference between antibodies, the first concentration with signal, the highest change between the two

antibodies binding curves, and a concentration at the maximum binding, circled in red on figure 4-5. With the nature of equilibrium there are some points with increased variability. At the largest delta between the two curves there is the highest amount of variability due to either one peptide binding to the antibody or two, but if a point is chosen slightly less the variability decreases as only one peptide is more likely to be bound. So, multiple concentrations were tested to decrease the overall variability with the FSP and VIF binding curves and still provide accurate K_D results (mathematics not shown). For initial experiments, the high concentration used was 0.3 μM ; however, 0.2 μM was used after initial experimentation due to potential of saturation of the detector for antibodies better than the Anti-VIF antibody. The chosen final concentrations are circled in blue on figure 4-5 for beads made using 2 $\mu\text{g/mL}$ antibody.

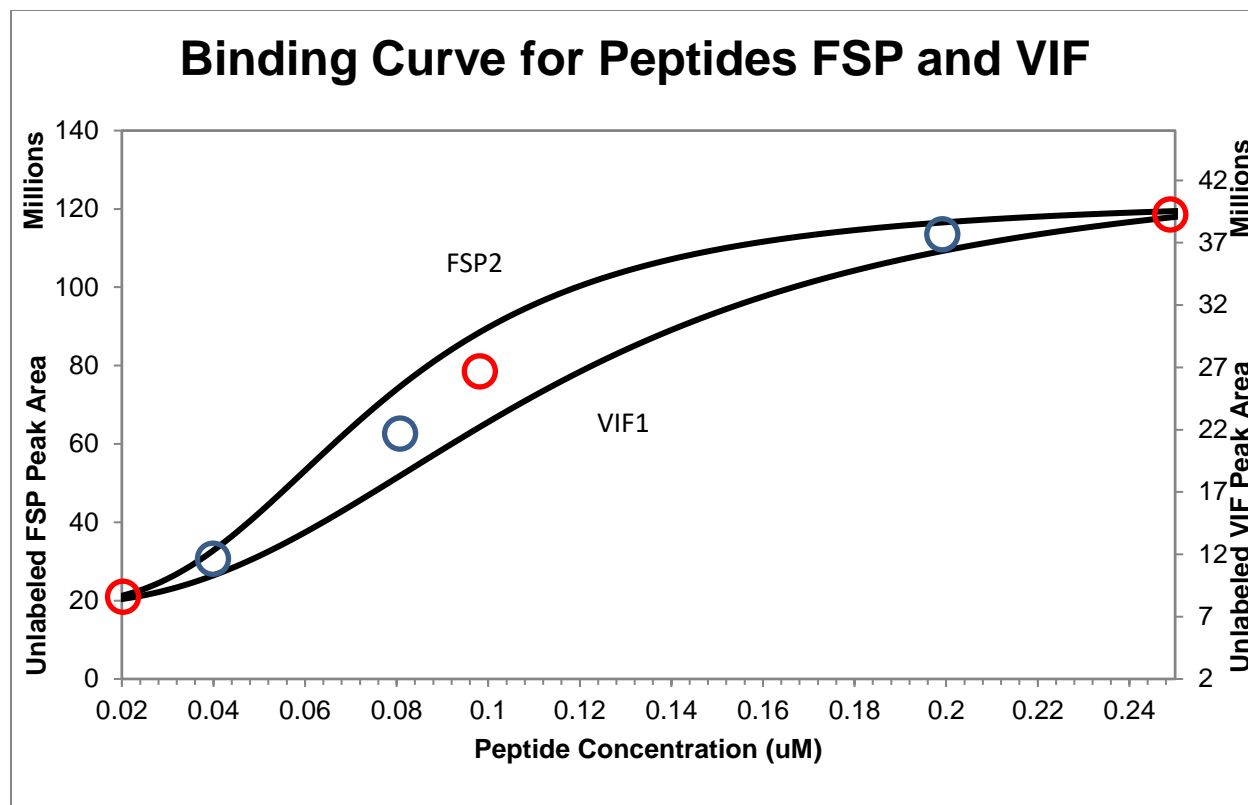


Figure 4-5. Normalized binding curves for Anti-FSP beads diluted 1:10 with BGG beads and Anti-VIF beads.

After determining what concentrations to use for an affinity screen, antibodies were acquired to test the screening procedure. The antibodies used were Anti-VIF antibodies produced in yeast compared to the Anti-VIF monoclonal antibody produced in rabbit. The plate method was used, and tosyl-activated beads were prepared following current thyroglobulin assay protocols separately for each antibody [17]. PBS/0.1% CHAPS was added at an 85 μL volume followed by an addition of 10 μL of endogenous peptide and 5 μL of beads into each well. The screen peptide spike concentrations were 0.4, 0.8, and 3.0 pmol/ μL unlabeled VIF peptide. The plate was sealed with foil and incubated for 2 hours at room temperature end-over-end on a Labquake following the plate method outlined in section 4.4.

Figure 4-6 and 4-7 shows the results of the affinity binding screen using the Anti-VIF antibodies. It was hypothesized that the Anti-VIF antibodies would not bind well when produced in yeast, which are denoted by numbers, compared to rabbit antibody. As shown in the figure very few antibodies had good signal even at the highest peptide spike concentration. The rabbit monoclonal antibody recovered 19.9% of the peptide spike with the next best recovery being yeast antibody 507 at 10.9% recovery. Figure 22 shows an easy comparison of percent recoveries between all the antibodies tested. Note, 1:10 diluted FSP beads bind better than all Anti-VIF antibodies tested.

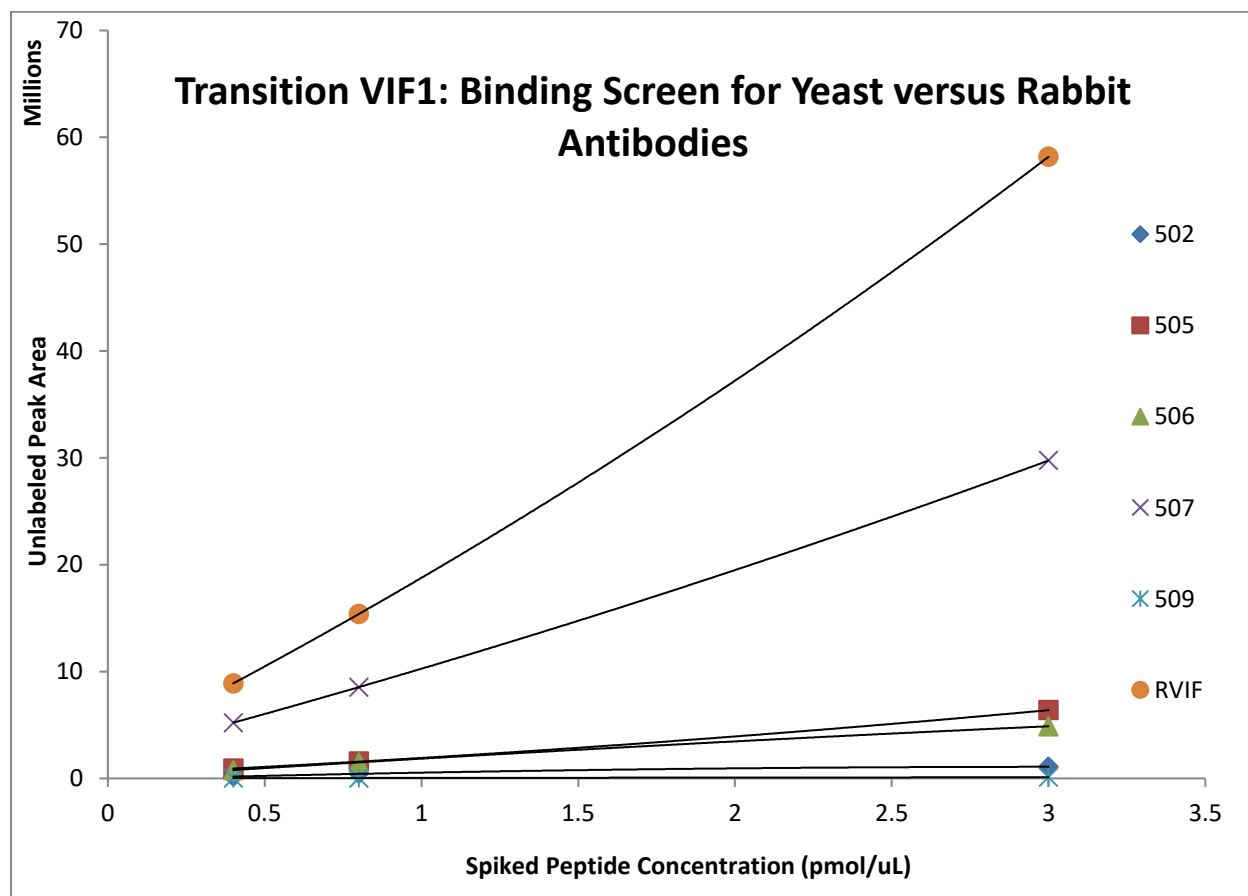


Figure 4-6. Binding curves for antibodies produced in yeast to recognize the epitope of the first three amino acids of the VIF peptide with comparison to the rabbit Anti-VIF antibody previously used in the thyroglobulin clinical assay.

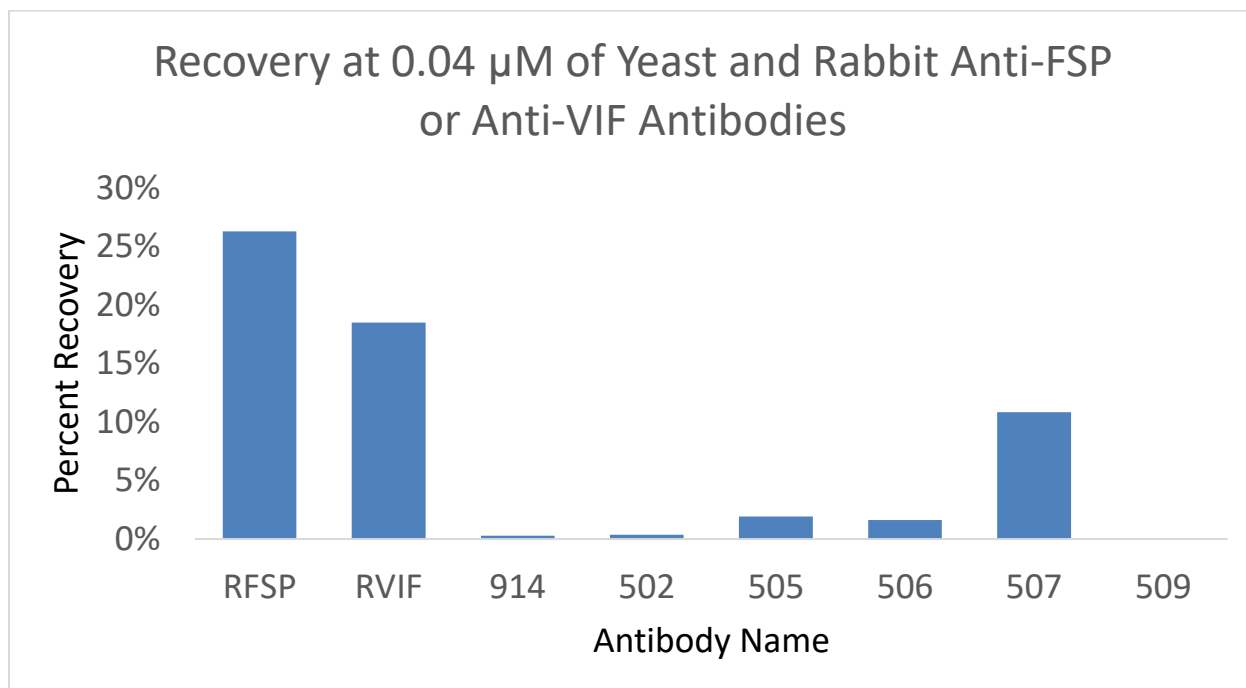


Figure 4-7. Percent recovery for yeast produced Anti-VIF antibodies in comparison to percent recovery of the two rabbit antibodies used in the thyroglobulin assay. Anti-FSP diluted 1:10 with BGG beads and Anti-VIF rabbit antibody beads were used.

Using the data from figure 4-6, the Anti-VIF antibody affinity binding screen, a Scatchard analysis was done in Excel. Figure 4-8 shows a bar graph of the best yeast produced antibody compared with the rabbit monoclonal antibody used in the clinical TG assay, and Table 3 shows the percent error between previous results obtained using a different testing method. The percent error for antibody 502 and 505 was extremely high causing investigation of how the previous result was determined. Previous results for K_D were determined using a ForteBio system. This system uses peptide bound to a sensor with IgG in solution. This is different to the

LC-MS/MS method described here which has IgG bound to a paramagnetic bead and peptide in solution. All other results were within 20% error.

Table 3. Results of the Anti-VIF affinity binding screen compared with previous k_{off}/k_{on} results done via ForteBio.

Antibody	% Recovery	Previous (uM)	Experimental (uM)	% Error
506	1.78%	14.18	14.3	0.86%
RVIF	19.89%	1.16	1.12	-3.11%
507	10.91%	2.46	2.08	-15.63%
509	0.04%	610.72	727.97	19.20%
502	0.43%	32.74	57.59	75.89%
505	2.10%	5.75	13.29	131.23%
Average Percent Error:				34.74%

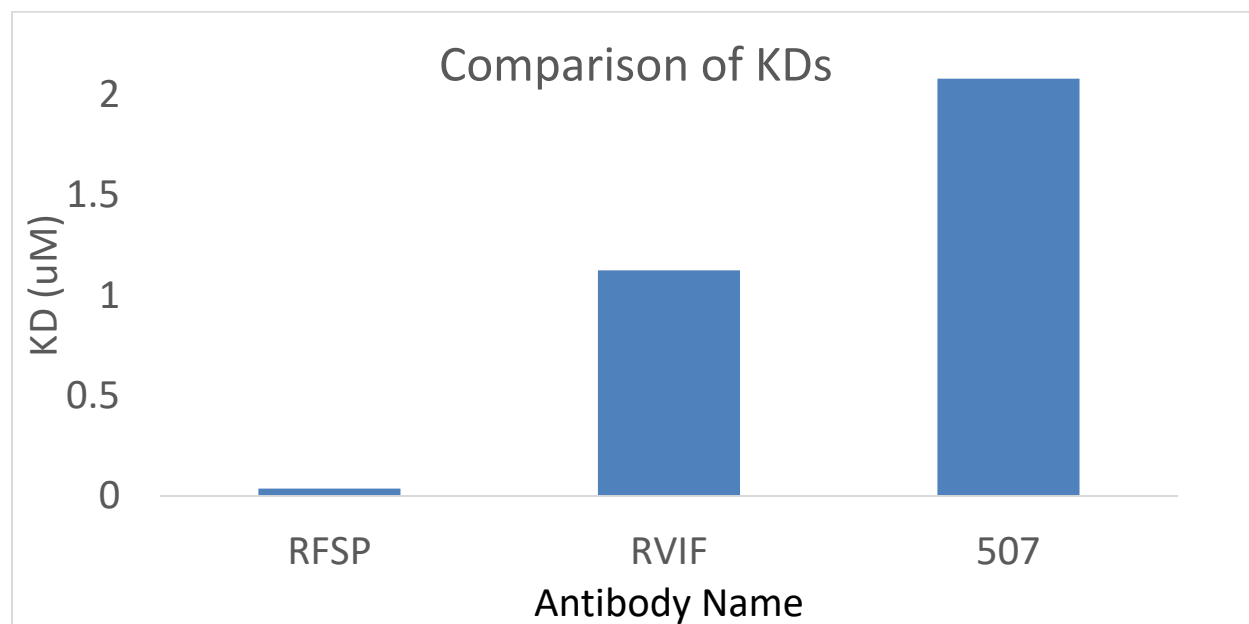


Figure 4-8. Comparison of KD of the two rabbit antibodies for FSP and VIF with the best yeast antibody for VIF.

4.7 BINDING RESULTS

The plate method used for the antibody screen was employed for determination of K_D for antibodies of interest. Tosyl-activated beads were prepared following current thyroglobulin assay protocols [17]. The plate method in section 4.4 was used for all determination of K_D experiments.

Peptide collision energy was optimized manually on the Waters TQS. Acquisition was scheduled to allow 4-5 transitions per peptide using a C18 T3 column with mobile phases 98% Optima water/Formic Acid and 98% Methanol/0.1% Formic Acid. A linear gradient was used with a 7.5 minute run time, and samples were run overnight. Data was integrated using Skyline and analyzed in Microsoft Excel.

Due to the concentrations of purified antibodies provided, some dilutions were necessary. The antibody screen of the 15 supernatants used beads which were made at 1 $\mu\text{g}/\text{mL}$ due to low amounts of purified antibody available. To adjust for this, the peptide concentrations for the screen were changed from 0.4, 0.8, and 3.0 $\text{pmol}/\mu\text{L}$ unlabeled peptide mix used when beads are conjugated with 2 $\mu\text{g}/\text{mL}$ to testing using 0.2, 0.4, and 1.5 $\text{pmol}/\mu\text{L}$ peptide concentrations.

As an example, figure 4-9 shows the binding curve screen for GPVG and figure 4-10 shows the binding screen Scatchard plot which was used for K_D determination; however, similar graphs were produced for peptide SSA and FTYT (data not shown). For GPVG, the best two antibodies for recovery and K_D were 8G6b and 7B3c, and this can be seen both in figure 4-9 as the highest two for binding as well as figure 4-10 as the highest two y-intercepts. The worst antibody for recovery and K_D was 7A3a for peptide GPVG.

Table 4 shows all the results provided from the binding curve screen and Scatchard analyses. These results are using 1 $\mu\text{g/mL}$ beads for the binding screen. The best two antibodies for each target are bolded, and they are as follows: for FTYT antibodies 1G12a and 4E10a, for GPVG antibodies 7B3c and 8G6b, and for SSA antibodies 4H2a and 6C6d. These 6 antibodies were chosen for further study.

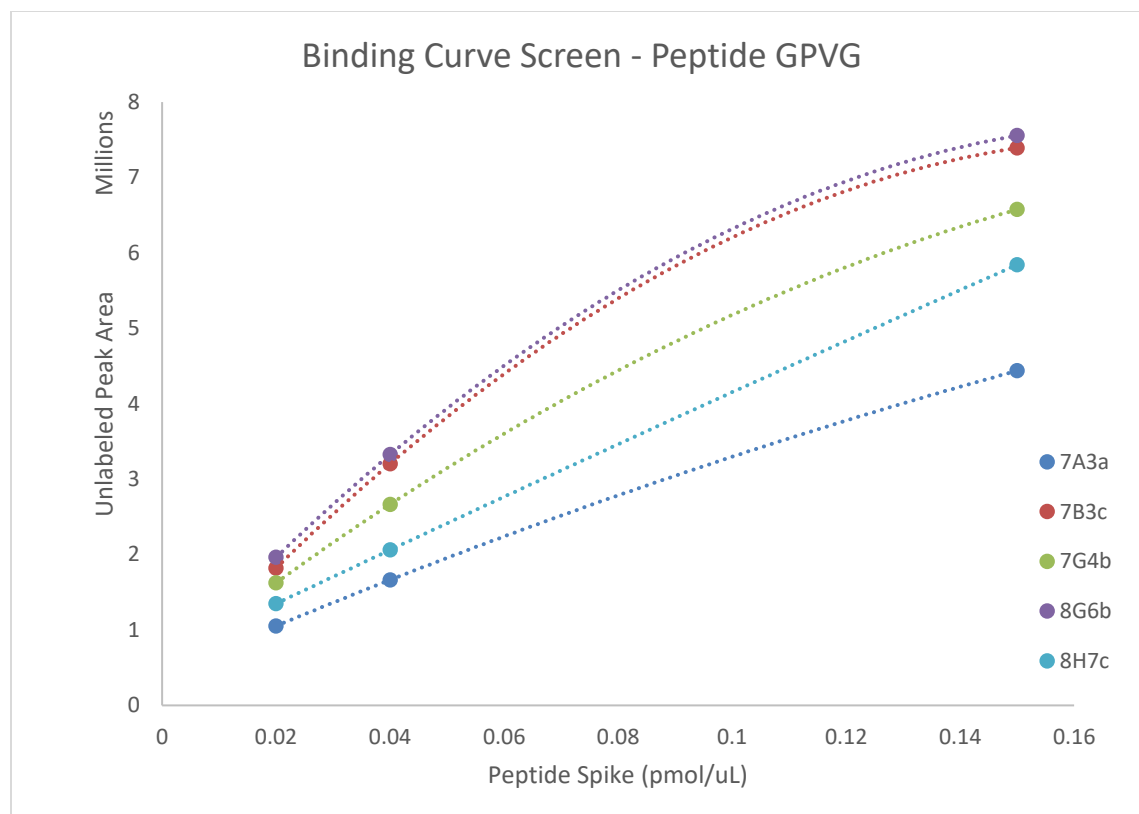


Figure 4-9. Antibody screened K_D binding curve for peptide GPVG for the 5 best purified antibodies from the antibody recovery screen.

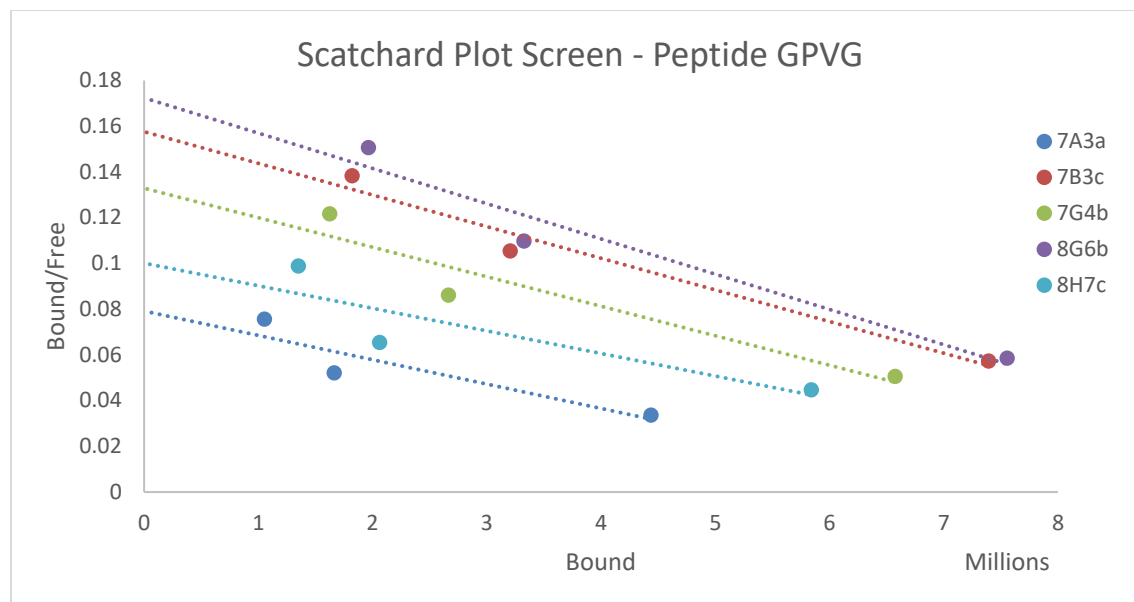


Figure 4-10. Scatchard plot screen of peptide GPVG for the 5 best purified antibodies from the antibody recovery screen.

Table 4. Antibody screened K_D for all 15 purified antibodies from the recovery screen. Bold antibodies correspond to the best two for each target, and beads were produced at 1 $\mu\text{g}/\text{mL}$.

Target	Mix	Antibody	K_D (μM)
FTYT	Mix1	1G12a	0.297503
	Mix2	4D4a	0.783044
	Mix3	4D9a	0.736667
	Mix4	4E10a	0.232749
	Mix5	5A4a	0.563257
GPVG	Mix1	7A3a	0.315828
	Mix2	7B3c	0.158628
	Mix3	7G4b	0.188123
	Mix4	8G6b	0.145026
	Mix5	8H7c	0.249774
SSA	Mix1	1D7a	6.377738
	Mix2	4H2a	0.269420
	Mix3	4H9a	5.065909
	Mix4	5D2a	3.877502
	Mix5	6C6d	0.725992

The same screening method was repeated using the best 2 antibodies for each target made at 2 $\mu\text{g/mL}$ antibody concentration on beads. The results are shown in figure 4-11 and 4-12 for peptide GPVG. Both antibody 7B3c and 8G6b bind well, so further study is necessary to choose between them. The results of the experiment can be seen in table 5. The best antibody for SSA was 4H2a with a recovery of 36.75% and K_D of 0.054 μM , and the best antibody for FTYT was 4E10a with a recovery of 5.69% and K_D of 0.251 μM .

Table 5. Antibody full K_D for best 2 antibodies per target. Beads were produced at 2 $\mu\text{g/mL}$.

Target	Antibody	K_D (μM)	Recovery
GPVG	7B3c	0.085041	23.08%
	8G6b	0.080623	22.90%
SSA	4H2a	0.054293	36.75%
	6C6d	0.150524	25.32%
FTYT	1G12a	0.342266	4.15%
	4E10a	0.251395	5.69%

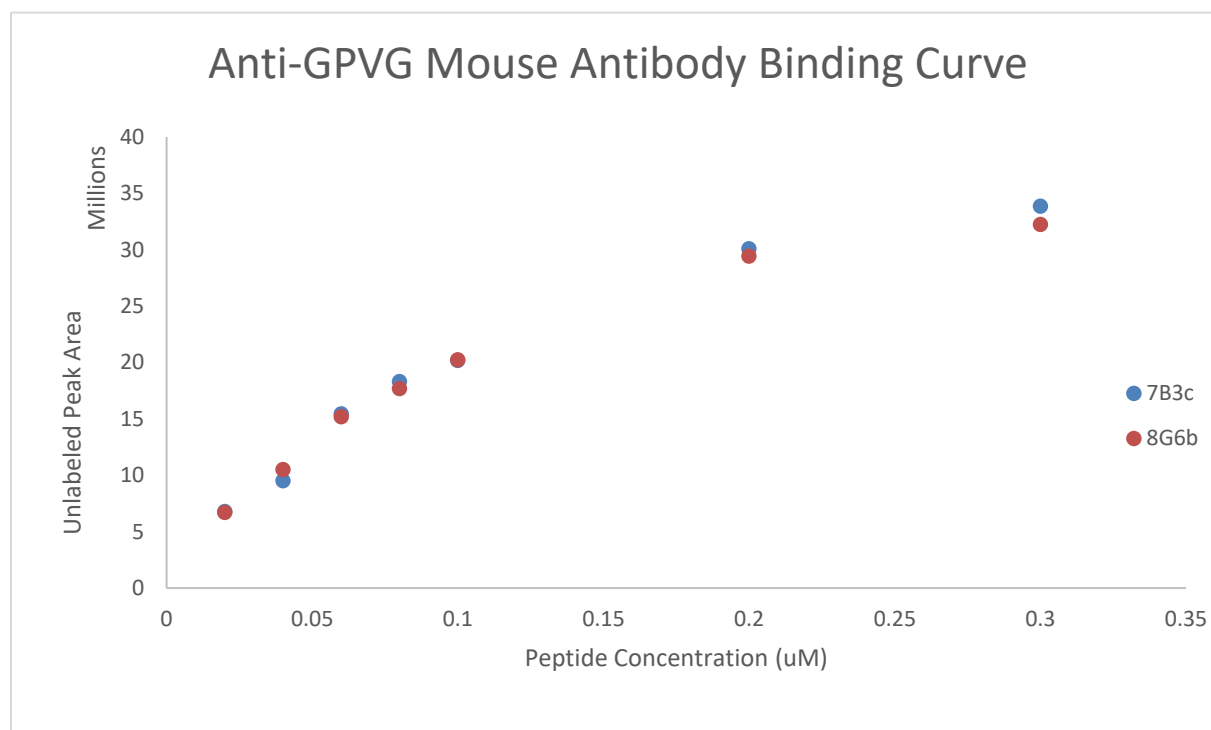


Figure 4-11. Full binding curve for the best two Anti-GPVG antibodies: 7B3c and 8G6b.

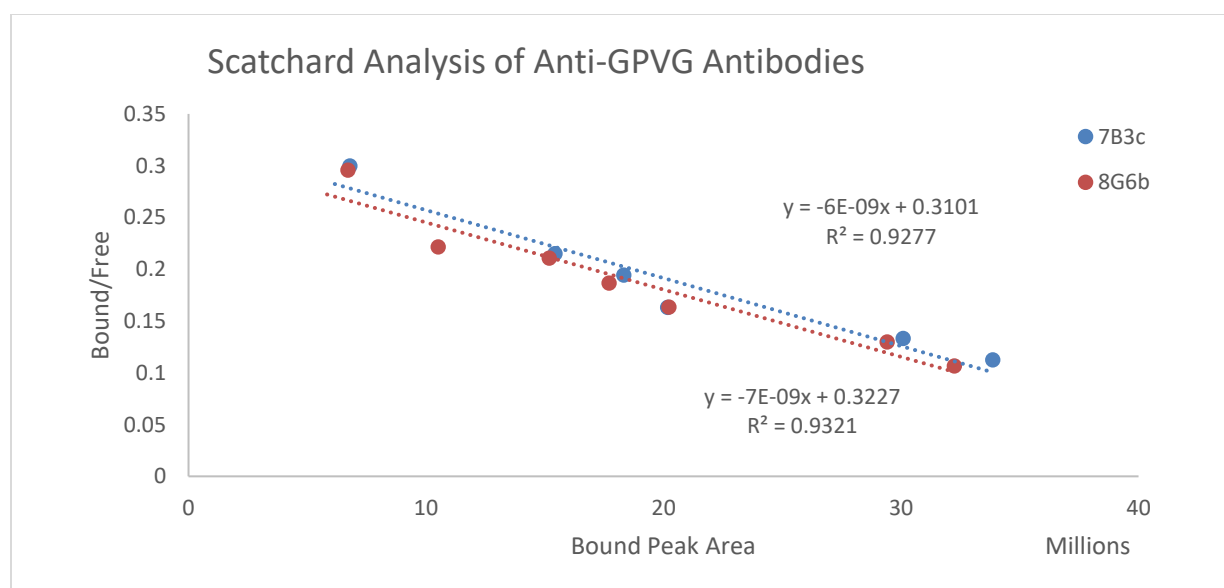


Figure 4-12. Full Scatchard analysis of the best two Anti-GPVG antibodies: 7B3c and 8G6b.

4.8 PRELIMINARY TESTING IN POPULATION POOL DIGESTED SERUM

The plate method used for the antibody binding screen was employed for preliminary testing in digested population pool serum containing $n = 400$ patients. Tosyl-activated beads were prepared following current thyroglobulin assay protocols [17]. The plate method from section 4.4 was used.

Figure 4-13, 4-14, and 4-15 show the results for each target: FTYT, SSA, and GPVG. For FTYT, there was higher signal seen using antibody 4E10a, and for SSA there was higher signal seen for antibody 4H2a. The two antibodies for GPVG similarly recovered in serum.

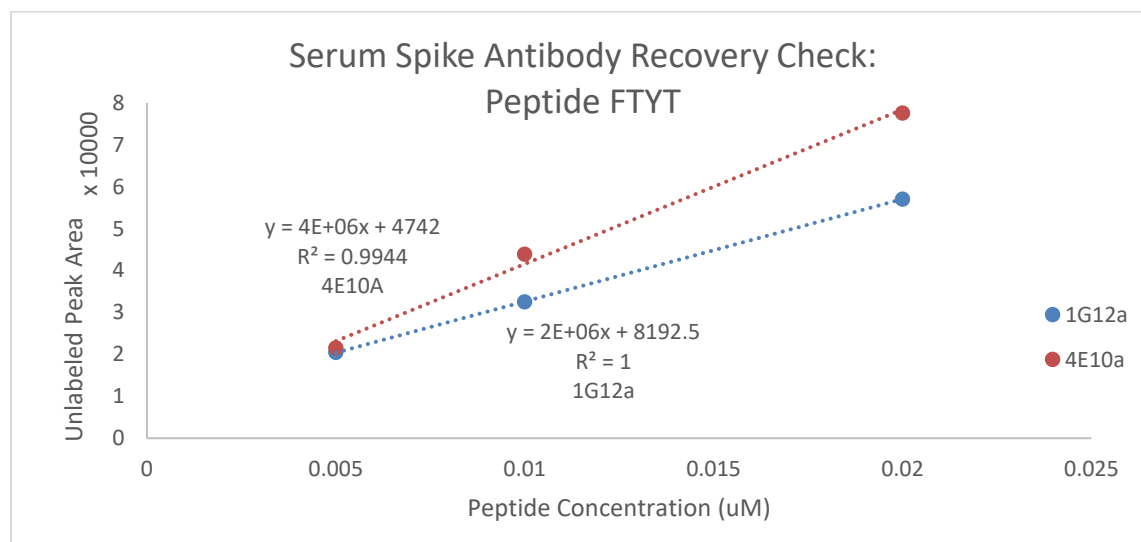


Figure 4-13. Preliminary testing using spiked population pool frozen, digested serum and Anti-FTYT antibodies 1G12a and 4E10a.

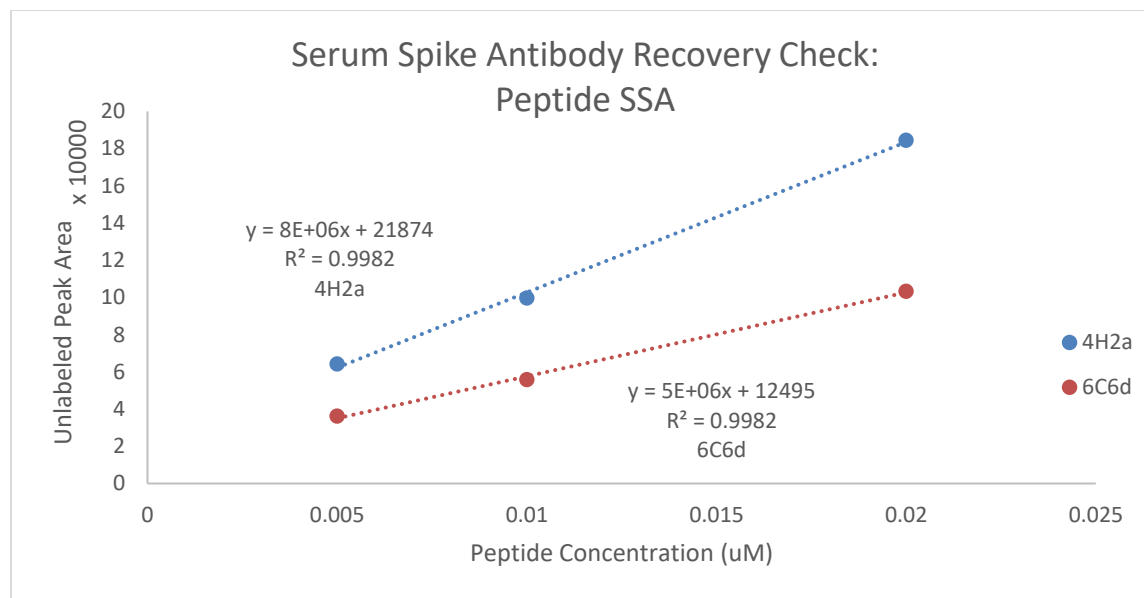


Figure 4-14. Preliminary testing using spiked population pool frozen, digested serum and Anti-SSA antibodies 4H2a and 6C6d.

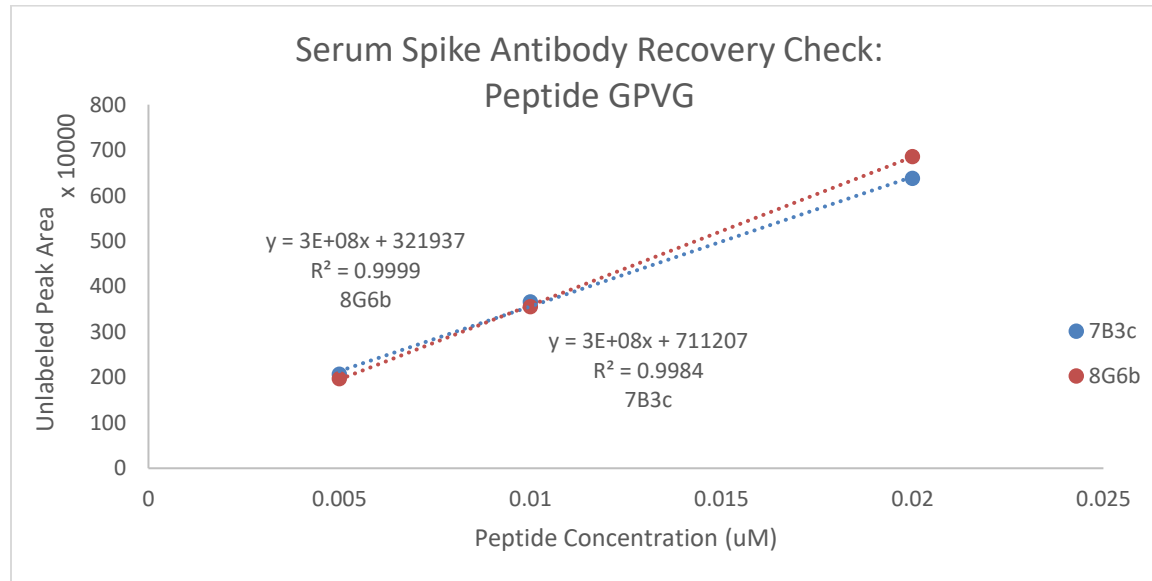


Figure 4-15. Preliminary testing using spiked population pool frozen, digested serum and Anti-GPVG antibodies 7B3c and 8G6b.

4.9 ANTIBODY BINDING DISCUSSION

The full K_D binding curve was originally tested on monoclonal rabbit antibodies for peptides FSP and VIF, both in use in the clinical thyroglobulin assay [17]. The experimental results for Anti-FSP K_D was determined via Scatchard analysis to be $0.039 \mu\text{M}$ and Anti-VIF was $1.12 \mu\text{M}$, and this compares well to previous results provided by the MiSCREEN listing Anti-FSP K_D as $0.04 \mu\text{M}$ and Anti-VIF was $1.16 \mu\text{M}$, which represents less than 5% error [13]. In this case, the MiSCREEN used SPR with antibody bound to the film and peptide in solution, and the results were concordant [13].

The K_D screen was then developed and tested against Anti-VIF rabbit monoclonal antibody as well as some yeast antibodies with known results. Previous results for K_D were determined using a ForteBio system using peptide bound to a sensor with IgG in solution [7]. This is different than LC-MS/MS method described here which has IgG bound to a paramagnetic bead and peptide in solution (an antibody recognizes one epitope on a peptide resulting in 1:1 binding if the peptide is bound to the sensor instead of 1:2 binding). The abnormal results seen for two antibodies could be due to low signal or experimental error not accounted for and the agreement could be due to lack of saturation of the antibody with peptide.

Over the course of a few weeks, 846 antibody supernatants were tested and whittled down using a constant volume recovery-based screen to the top 16 antibodies for further screening. Of the 16 antibodies chosen, 15 were purified, providing 5 antibodies each for the 3 peptide targets. Since the full binding curve and screen resulted in good preliminary testing results, the method was used to test the 15 antibodies, and the best 2 had full binding curves completed as well as spiked peptide recovery assessed in digested human serum. Since these are

newly produced antibodies, there is no data comparison, so, further study will be necessary to decide which antibodies to move forward for assay development.

Chapter 5. CONCLUSION

In conclusion, an antibody screen was developed, tested, and used to screen 846 antibody supernatants produced in mice to find the best 2 antibodies for 3 peptide targets. This manual method used fewer plates, less reagent, and less supernatant than previous LC-MS/MS antibody screens, and it employed a magnet during injection on the instrument to prevent plugging of the system. This in-house method could be easily translated to other antibody screens, assuming the antibodies bind well to the paramagnetic beads used in the screen (which prefer certain IgG isoforms) [16]. It is important when choosing a screening method to choose a method that will closely relate to how the antibody will be used to prevent false positives and negatives. In other words, it is important to test an antibody that will be used in an LC-MS/MS assay with LC-MS/MS to ensure it will work rather than screen using a different method which could waste money on discovering antibodies that would be unsuitable for assay development.

Additional research will be necessary to deploy these antibodies in a clinical assay format. C-62A is a guideline of how clinical mass spectrometry assays should be developed to ensure accurate measurements and patient safety [25]. Pre-validation testing, final decisions, and moving forward with following the C-62A guideline will be the necessary next steps before these potential antibody reagents can be used in a future clinical mass spectrometry assay.

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APPENDIX A

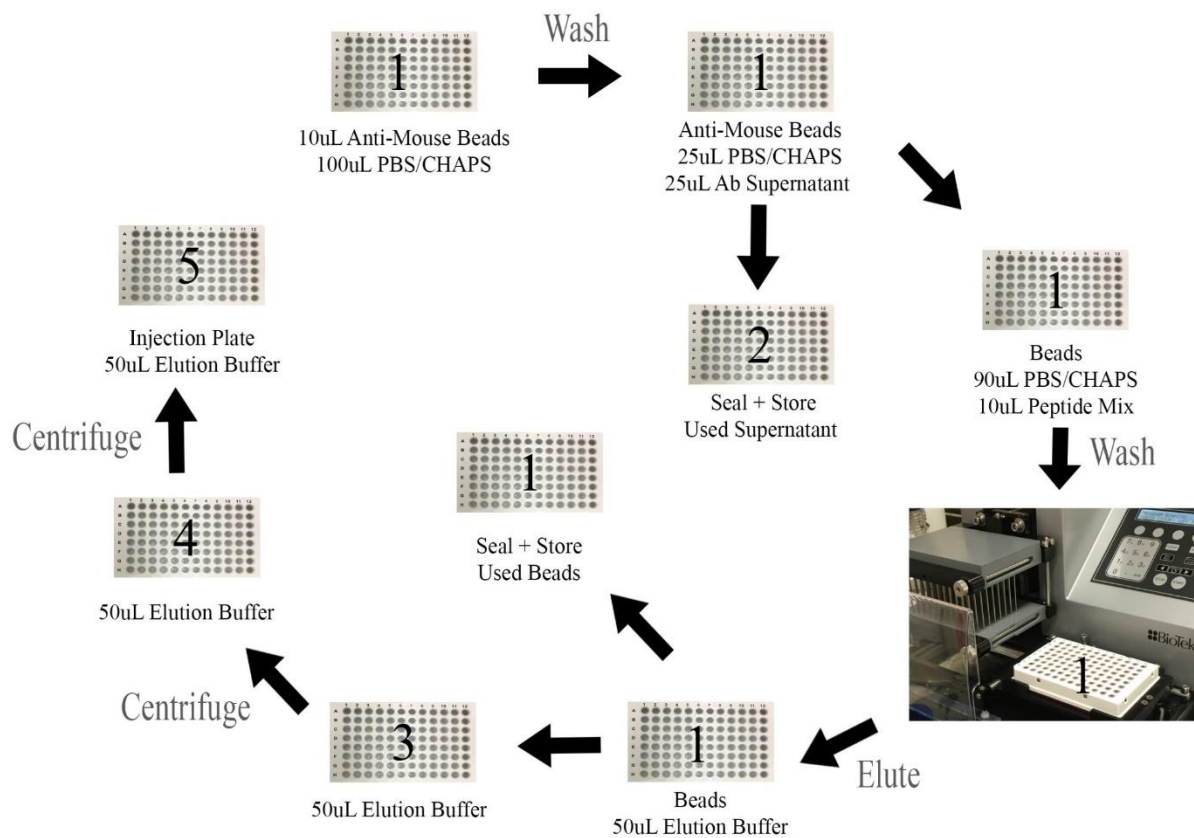


Figure A-5-1. Plate method workflow.

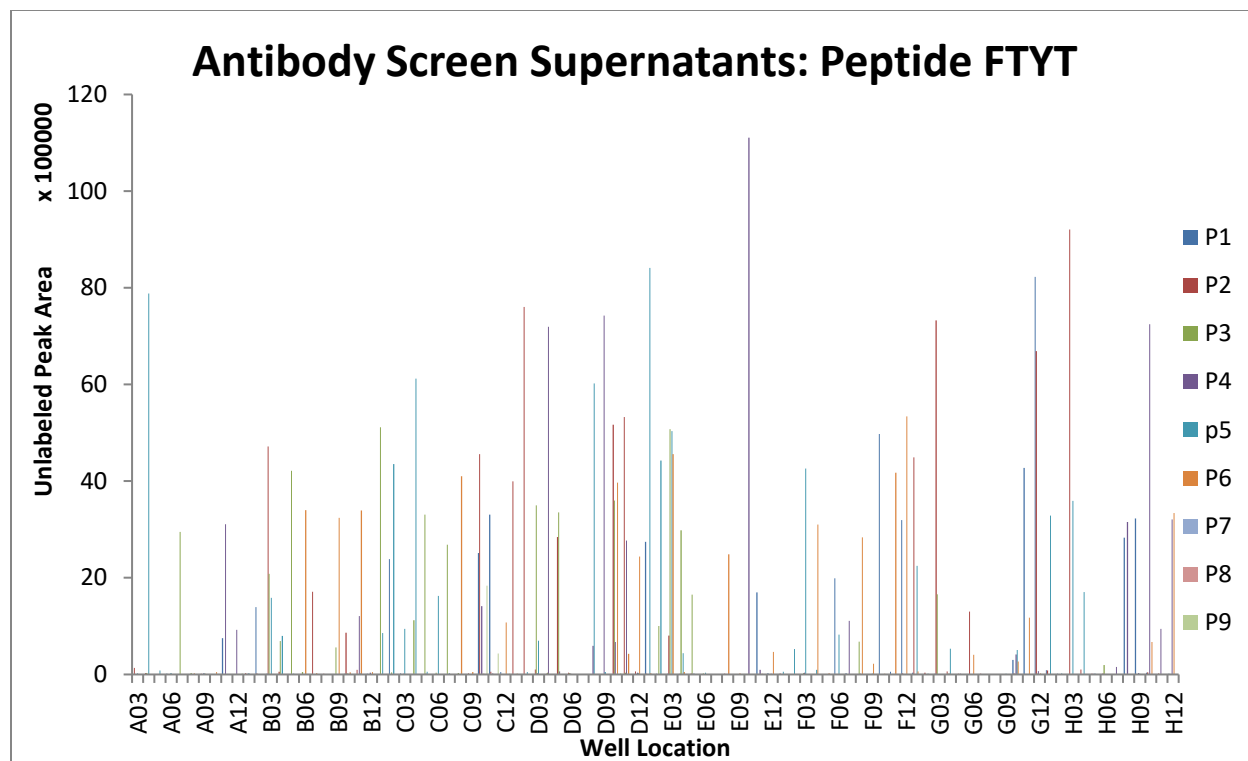


Figure A-5-2. Antibody Screen Supernatants for all plates for peptide FTYT.

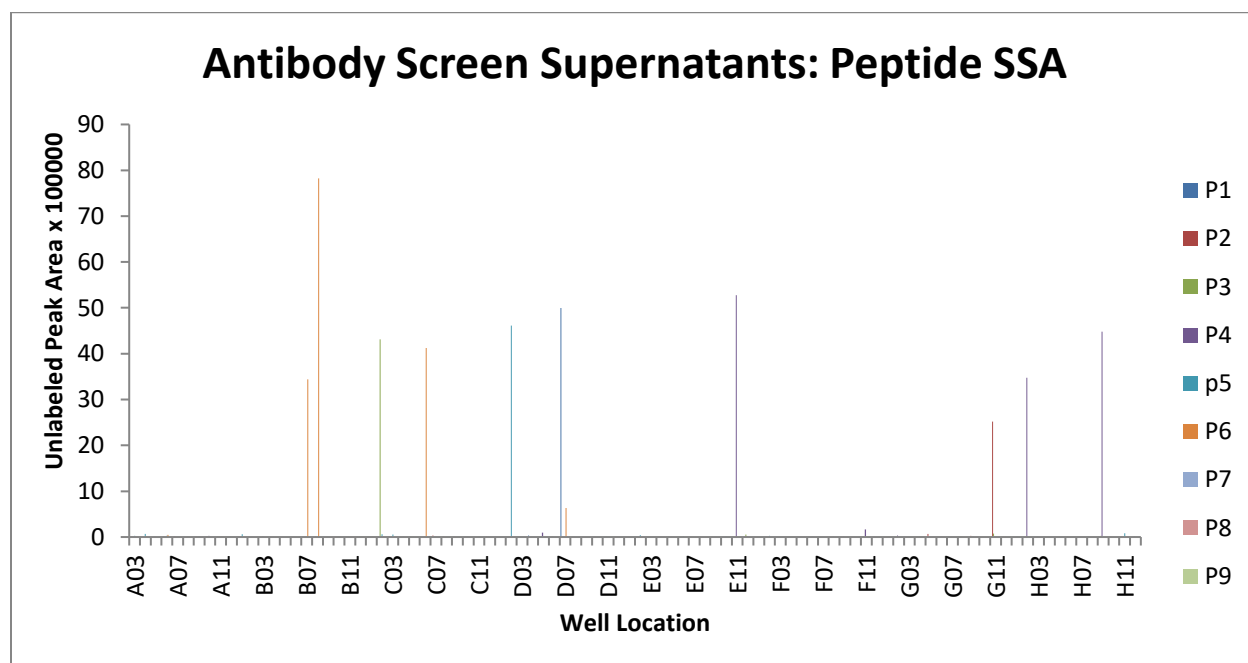


Figure A-5-3. Antibody Screen Supernatants for all plates for peptide SSA.

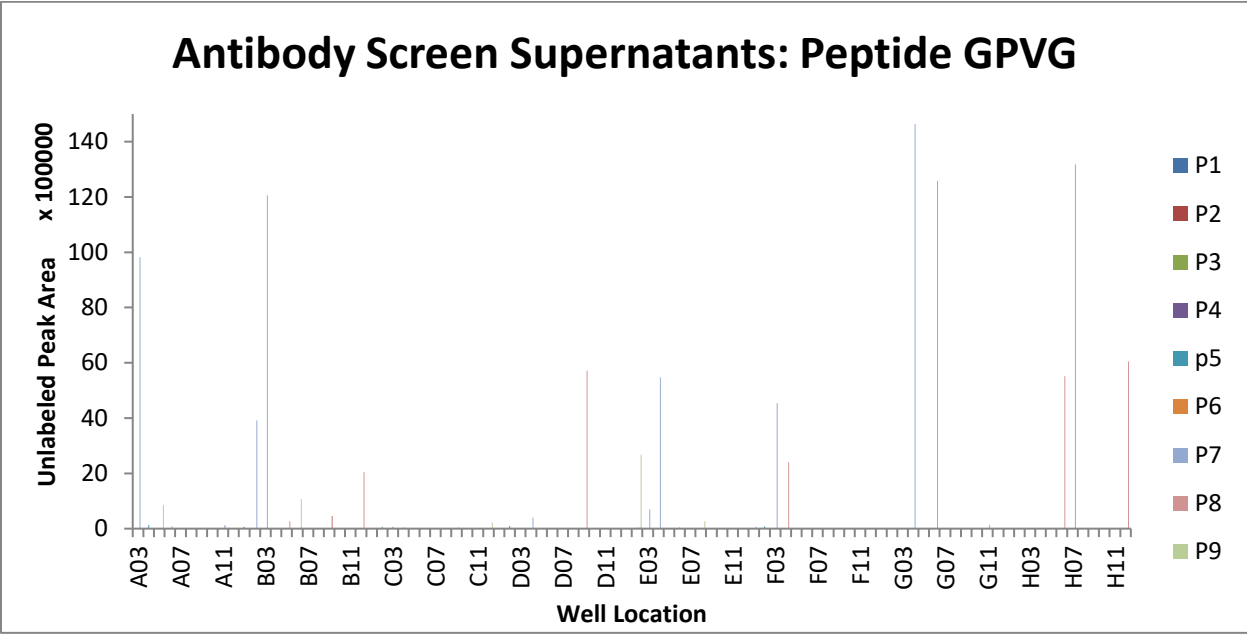


Figure A-5-4. Antibody Screen Supernatants for all plates for peptide GPVG.

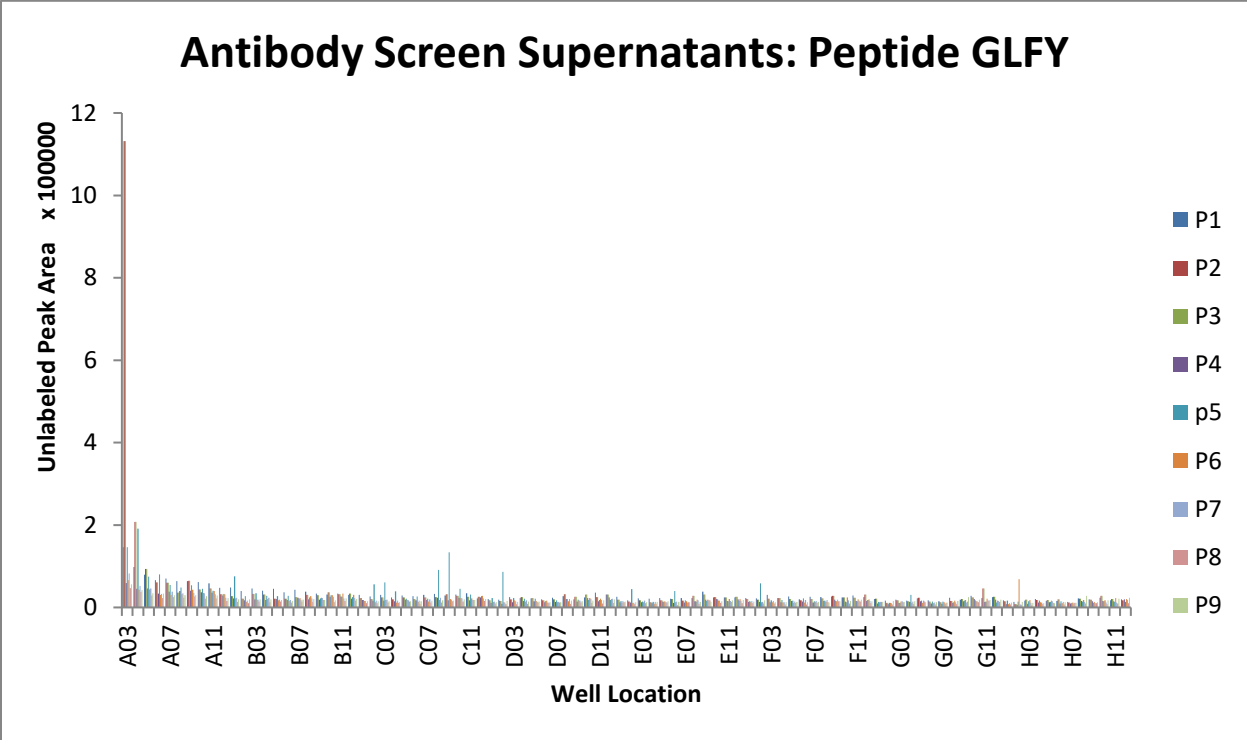


Figure A-5-5. Antibody Screen Supernatants for all plates for peptide GLFY.

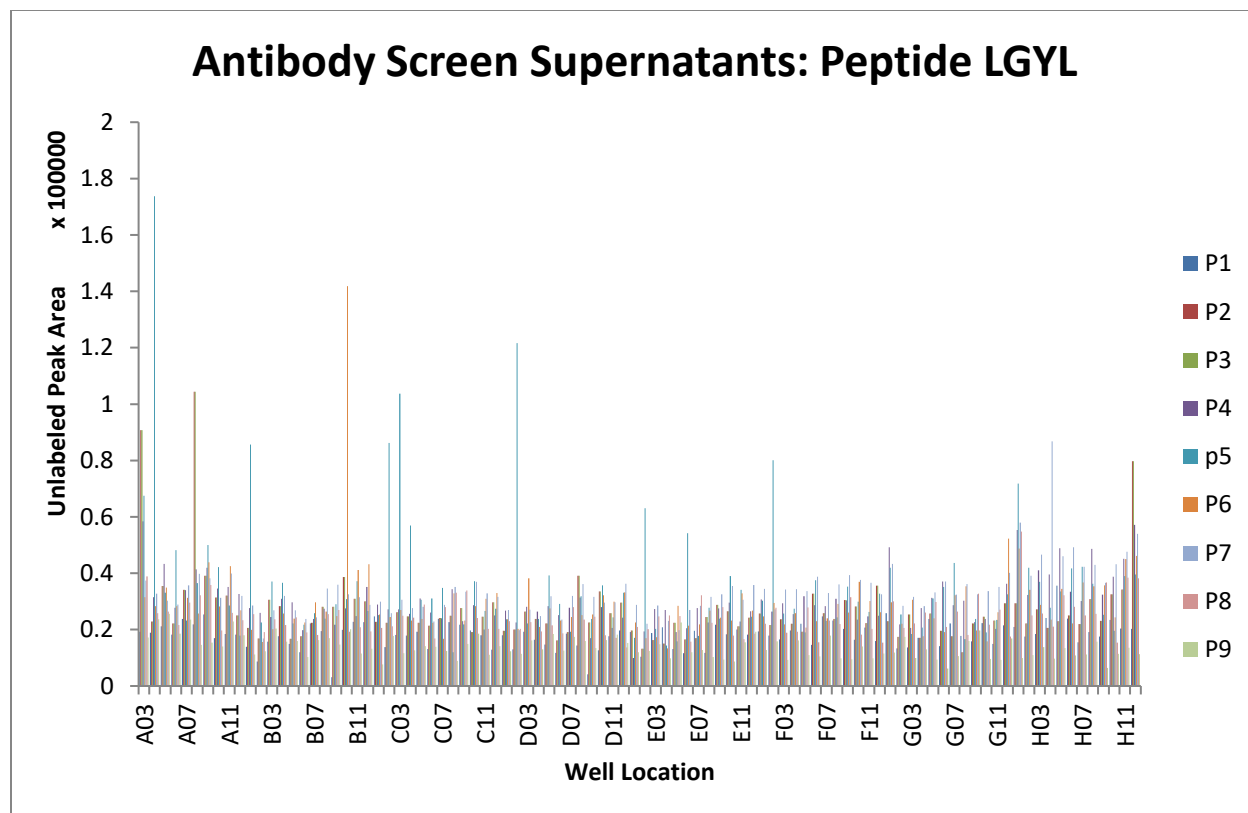


Figure A-5-6. Antibody Screen Supernatants for all plates for peptide LGYL.