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SANTA CLARA UNIVERSITY

Department of Bioengineering

I HEREBY RECOMMEND THAT THE THESIS PREPARED
UNDER MY SUPERVISION BY

Riley S Parsons, Mari A Ueno

ENTITLED

APTAMER-BASED HYBRID ASSAYS FOR EARLY STAGE DISEASE DIAGNOSIS

BE ACCEPTED IN PARTIAL FULFUILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

BACHELOR OF SCIENCE
IN
BIOENGINEERING

Steven W Suljak 6/3/16

Steven Suljak, Thesis Advisor — Date

06/06/2016

Prashanth Asuri, Acting Department Chair — Date

APTAMER-BASED HYBRID ASSAY FOR EARLY STAGE DISEASE DIAGNOSIS

By

Riley S Parsons, Mari A Ueno

SENIOR DESIGN PROJECT REPORT

Submitted to
The Department of Bioengineering

of

SANTA CLARA UNIVERSITY

in Partial Fulfillment of the Requirements
for the degree of
Bachelor of Science in Bioengineering

Santa Clara, California

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Aptamer-Based Hybrid Assays for Early Stage Disease Diagnosis

Riley A Parsons, Mari A Ueno

Department of Bioengineering
Santa Clara University
2016

ABSTRACT

The objective of the project is to use aptamers, oligonucleotides designed to selectively bind target molecules, to develop a methodology for building an enzyme-linked aptasorbent assay (ELASA). This assay is designed to detect the presence of a target protein nucleolin that is overexpressed on the surface of cancer cells, and it would act as a novel diagnostic method for the disease. The success of our project would also confirm the feasibility of using an ELASA as a diagnostic tool to detect the presence of thrombospondin-1 (TSP-1), a protein known to experience glycosylation changes in human endometrioid ovarian cancer tissue. Our methodology will be based on that of existing ELASA "sandwich" type assays for analogous proteins, which incorporate the use of aptamers and antibodies. Aptamers offer inherent benefits over antibodies, which are the primary agents used in traditional enzyme-linked immunosorbent assays (ELISA), while demonstrating comparable sensitivity. Our developed ELASA has the potential to be an innovative diagnostic tool that can improve disease prognosis due to its high sensitivity and low limits of detection as an analytical method.

Keywords: Aptamer, Nucleolin, Antibody, Endometrioid Ovarian Cancer, ELASA

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CHAPTER 1: INTRODUCTION

1.1 Background

The objective of the project is to use aptamers, oligonucleotides designed to selectively bind target molecules, to develop an enzyme linked apta-sorbent assay (ELASA). This assay would act as an alternative to enzyme-linked immunosorbent assays (ELISA). The ELASA will detect the presence of a target protein that has been determined to be an early indicator of disease growth, and for that reason, it could be utilized as a novel diagnostic method for the disease. The success of our project would also confirm the feasibility of the use of an ELASA to detect the presence of thrombospondin-1 (TSP-1), a protein known to experience glycosylation changes in human endometrioid ovarian cancer tissue, and an ELASA could therefore act as a diagnostic tool for this deadly disease.[1] Our method will be based on existing methodologies for ELASA "sandwich" type assays for analogous proteins, which incorporate the use of aptamers and antibodies. By using aptamers in the place of traditionally used antibodies as a diagnostic indicator of disease growth, this ELASA can become a useful alternative to more costly conventional diagnostic methods.

1.2 Statement of Project Objectives

We have three main objectives:

1. To develop a methodology that will test serum samples for the presence of a target protein using a novel ELASA diagnostic system.
2. Ensure that the ELASA system's detection limits and sensitivity will be comparable to existing diagnostic techniques
3. Confirm the resilience of the methodology in the testing of other protein targets such as TSP-1.

1.3 Review of Field Literature

Toh et al. discussed and promoted an emerging technique, ELASA, which is gaining popularity over ELISA, due to the inherent benefits of aptamers.[2] Antibodies, which play key roles in an

ELISA as both the immobilized capture agent and the signaling agent, are expensive to manufacture, become unstable at room temperature, and are limited to only immunogenic target molecules. In comparison, aptamers are cheaper, more stable, and can specifically target an assortment of molecules while also being able to distinguish between minute differences in molecular structure. As a result, ELASAs can be more affordable, have longer shelf lives, and be utilized for a wider variety of applications.

Toh et al. also explored various ELASA formats including the sandwich ELASA, which utilizes a capturing antibody or aptamer immobilized on a surface with a signaling aptamer that is modified for detection.[2] The information provided in the paper will guide the design of our ELASA. An ELASA assay that completely replaces antibodies with aptamers would be ideal, but consequently the target molecule would need two different well-studied aptamers that bind selectively to two different regions. However, this is not the case for many target molecules, so a combination of antibodies and aptamers must be used. Our target molecule, nucleolin, only has one aptamer, AS1411, which was utilized by Guo et al., so we must also use an anti-nucleolin antibody for our sandwich assay.[3] The associated antibody is commercially available, so it is obtain for research purposes. The article reviewed various ELASAs developed by other groups, and the materials and methods used in these studies will prove useful as we work to develop and optimize our own protocols. The sensitivity of these ELASAs were also provided, so they can act as a standard, to which we can compare our results, to see if our product can be competitive with existing ELASAs.

In a study performed by Guo et al., nucleolin was used as a target molecule to direct drug delivery via PEG-PLGA nanoparticles to brain tumors.[3] Nucleolin is a protein that is normally expressed in various parts of the cell. However, most notably, it is overexpressed on the surface of tumor cells, making it an effective biomarker for various types of cancer.[4] In order to specifically target nucleolin, Guo et al. added an aptamer AS1411 to the surface of the nanoparticles.[3] AS1411 is a well-studied aptamer sequence that has been isolated and identified for its specificity and selectivity for nucleolin. The 28 base-pair guanine-rich aptamer sequence is provided. This study initially sparked the idea to use nucleolin as our target molecule for our ELASA, as it is a highly researched molecule that is related to a popular disease-state. Although

we do not plan to utilize the AS1411 aptamer for drug delivery, it was useful to find a study that supports the efficacy of using this aptamer to target nucleolin.

1.4 Existing Technologies

Enzyme linked immunosorbent assays (ELISA) are one of the leading diagnostic techniques in clinical laboratories. It has been an industry mainstay due to the technique's efficacy in determining the presence of a wide range of target molecules within any given sample, even at very low concentrations. These factors, in tandem with its compatibility with inexpensive detection instruments like spectrophotometers, have led to ELISA being the preeminent diagnostic technique in oncology and microbiology.

ELISAs leverage the specific binding of antibodies to target antigens to indicate the presence of a certain target molecule. To produce a signal that can be in turn correlated to a specific target concentration via regression analysis, a secondary antibody coupled with a enzyme is then added to the solution. This antibody also binds to the target molecule and when the bound enzyme interacts with an added substrate, fluorescence is emitted and detected by a spectrophotometer.

1.5 Project Significance

Cancer continues to be one of the deadliest and most economically damaging diseases worldwide costing the cumulative world economy nearly $900 billion in 2008. In 2015, there were a total of 8.2 million global deaths resulting from cancer with 65% of those deaths occurring in developing countries[5]. In developing nations, the high cost of health screenings and diagnostic tests can gravely affect the health of the community. The loss of the primary source of income for a family due to sickness on top of the cost for treatment can be a crippling prospect for families. To combat these staggering figures, early diagnosis of cancer, which can dramatically improve prognoses, must become a priority.

The development of a successful methodology for an ELASA targeting nucleolin, would provide support for the use of ELASAs as a novel diagnostic method for numerous forms of cancer. Our methodology can then be applied to other target molecules that are relevant to various disease

states. One of these molecules, Thrombospondin-1 (TSP-1), is being studied by Dr. Steven Suljak at Santa Clara University's Department of Chemistry and Biochemistry. TSP-1 undergoes specific glycosylation changes during early-stage endometrioid ovarian cancer, and it can therefore act as a biomarker for the disease. These specific yet minute changes in the structure of TSP-1 can be recognized by an aptamer, and when an adequate aptamer pool is selected for this glycosylation state of the protein, they can be utilized in an ELASA, which can serve as a novel diagnostic method for this disease. Currently, five-year survival rates are lower than 50%, but early-detection results in a 90% survival rate for patients who are diagnosed while the cancer is confined to the ovaries.[1] Therefore, the development of a functional ELASA will support the efficacy of using aptamers in a diagnostic assay for various disease states, which can help improve survival rates.

Additionally, ELASAs can be competitive alternatives to the traditional ELISA kits, which currently dominate the market. Aptamers can provide inherent benefits over antibodies, which will be explained in a later section, and because of this, an aptamer-based assay can be more rugged and more affordable. Currently, easy-to-use ELISA kits are available on the market for upwards of $700, and they must be refrigerated, making them incompatible with use in developing nations.[6] As a result, an ELASA kit could become more accessible for developing nations that currently lack forms of diagnostic technologies due to insufficient funds and resources. In order to improve the quality of life for all and to continue the major growth of the emerging economies worldwide, it is essential that early diagnostic methods become more available.

CHAPTER 2: DETAILS OF KEY CONSTRAINTS

2.1 Critique of Existing Technologies

The enzyme linked immunosorbent assay, or ELISA, is among the most widely used diagnostic assay on the market. ELISAs work by combining the high affinities of antibodies to their target antigen in a controlled, wet lab environment. The antibody, which binds to the antigen found in a sample, can be conjugated, or linked, with an enzyme capable of releasing a colorimetric or fluorescent signal in the presence of substrate. B.K. van Weemen and A.H.W.M. Schuurs established this enzyme-based method as an alternative to radioactivity-based detections using the enzyme horseradish peroxidase (HRP) in 1971.[7] In their initial findings the assay was able to detect the presence of a particular antigen with sensitivities of 80 ng/mL. Over 50 years later, ELISA assays are widely available and in use in clinical laboratories with sensitivities more than 100-times smaller.

Currently available ELISA assays vary in methodology and configuration at a molecular level. At a high level, there exists three main ELISA formats: indirect, blocking, and antigen capture. The indirect format uses an intermediate, non-enzyme-linked antibody to bind to a target molecule, or antigen, affixed to a solid surface. Then, a secondary enzyme-linked antibody, is introduced, which sandwiches the intermediate antibody and indicates the presence of the antigen in question. The blocking ELISA assay leverages the binding coefficients of two competing antibodies, one of which is enzyme-linked, with an antigen, to produce a colorimetric signal that is inversely proportional to the level of antigen present.

Finally, an antigen capture, or “sandwich” ELISA, uses two pairs of antibodies, to capture an antigen in solution and signal its presence with an enzyme and its substrate. The capture antibody is either directly or indirectly immobilized onto a solid surface, and then binds to antigen in solution. Following a washing step, the enzyme-linked signal antibody is introduced, which binds to the antigen captured earlier. With the addition of the enzyme substrate, the level of antigen presence can be determined using absorbance or fluorescence spectrophotometry.

For the purposes of this project, the sandwich ELISA format and methodology will act as the general model. Sandwich ELISA formats are considered to be the most sensitive and robust on the market, and therefore are widely available for an array of antigens. Commercially available sandwich ELISA kits for the protein nucleolin, known to be over-expressed in cancerous tumor cells, tend to cost between $750 and $800 dollars and can reach a sensitivity under 0.057 ng/mL. Such ELISA kits are capable of testing tissue samples and other biological fluids for levels of nucleolin between 0.156 - 10 ng/mL.[8]

Despite a low limit of detection, low sensitivity, and selectivity for a target protein, commercially available ELISA assays have several drawbacks. Firstly, costs for ELISA kits tend to be very high, mostly due to the antibodies included. Antibody development tends to be a lengthy, delicate, and resource intensive process, resulting in a high cost passed onto the consumer. Secondly, antibodies are heat and pH sensitive, leading to strict handling guidelines and short shelf lives. Antibodies, when properly handled may last for up to two years. However, ELISA kits containing enzyme conjugated or lyophilized antibodies tend to have shorter lifetimes. Thirdly, antibodies like IgG, a prolific immunoglobulin found in human plasma, are large molecules weighing around 150 kDa. Their large sizes can lead to less specific and selective binding characteristics than smaller molecules capable of binding at higher resolutions.

2.2 Analysis of Key Constraints

In designing an oligonucleotide alternative to the traditional ELISA sandwich assay, it is important to be aware of key parameters that will dictate its commercial success and laboratory efficacy. The key metrics in the design of an alternative assay are its sensitivity, limit of detection, and specificity for the target molecule. As mentioned earlier, nucleolin ELISA assays are capable of reaching a limit of detection of 0.156 ng/mL. Equally important in assay development is quantifying low levels of analyte in a sample. Nucleolin ELISA kits offer a sensitivity of 0.057 ng/mL. Finally, it is imperative that non-specific binding be minimized in order to avoid false positive readings for the target molecule. Non-specific binding occurs when a secondary antibody binds to a molecule, which is not the analyte, leading to higher than expected signal for a given amount of target molecule. ELISA kits prevent this by blocking the well plate before shipment, and including washing steps in the protocol. Similarly, this

phenomenon can be reduced in an alternative assay with careful blocking and washing steps written into the assay protocol.

CHAPTER 3: DESIGN DESCRIPTION

3.1 Attributes of Oligonucleotides

Aptamers are oligonucleotides, such as single-stranded DNA and RNA molecules, that are designed to target specific molecules. These molecules take on a unique 3-dimensional conformation to tightly bind to its target molecule and discern between minute structural motifs. Because of their specificity and selectivity in binding, aptamers are commonly used in applications where antibodies have traditionally been used.[2] However, aptamers can bind to a wider range of molecules and because they are not immunologically active like antibodies they can be used within the body with less risk of adverse effects. Therefore, aptamers can be used in directed drug treatments in vivo.[3]

The enhanced stability of aptamers allows them to be economical and efficient. Antibodies must remain refrigerated and will permanently denature if left out in temperatures near room temperature or higher. While aptamers should also be stored at cooler temperatures, aptamers can denature in suboptimal conditions, but they can return to their functional conformation and continue to bind to target molecules. This allows products containing aptamers to have a longer shelf life and be compatible in a wider range of environments.

Additionally, the production of aptamers is simpler and cheaper than that of antibodies. Once a specific aptamer sequence is identified, they can easily be ordered from companies such as IDT and Thermo Fisher, who synthesize the desired sequences. Contrastingly, antibodies must be produced in batches through an arduous process that includes animal testing. This is not only more expensive and inefficient, but also this can result in batch-to-batch variabilities in antibodies, affecting the consistency of products.

The unique attributes that aptamers offer have allowed them to be used in a variety of applications. They are gaining in popularity in the diagnostic field for their abilities to specifically and selectively bind to molecules. While antibodies have been a central component in traditional diagnostic assays, aptamers are an advantageous alternative.

3.2 Assay Overview

Our hybrid assay utilizes a sandwich format depicted in Figure 1. In this type of assay, a capturing agent it immobilized onto a solid surface, such as a well-plate or chip. Here we utilize our antibody as the capturing agent. The target protein is then added, which binds to the immobilized capturing agent. A signaling agent is then introduced to bind to a separate region on the target, sandwiching the target protein between the capture and signaling agents. The signaling agent, in this case an aptamer, is conjugated with a signaling molecule, which allows detection via absorbance, fluorescence, or chemiluminescence. The observance of a signal would signify the presence of the target molecule, and without the target, no signal would occur.

Figure 1. Pictorial depiction of a sandwich assay

3.3 Antibody Immobilization

The first step to building our hybrid assay requires us to immobilize our capture antibody onto the solid surface of the well-plate. We decided to look at two different methodologies for the immobilization. The simplest method is to use passive adsorption, in which the antibody binds to the plastic surface of the well-plate via hydrophobic interactions. In this method, the surface does not have to be pre-treated and the antibody is simply incubated at 37°C for several hours to allow for proper binding. However, this method results in the random orientation of antibodies. Figure 2 shows the variability in orientation that can result from passive adsorption.

Figure 2. The random orientation that occurs during passive adsorption of antibodies onto a solid surface

Antibodies are asymmetrical molecules, where the Fab region depicted in Figure 3 is unique to each type of antibody and is the site of antigen-binding.[5] Contrastingly, the Fc region is common among all antibodies of a certain species and is not involved in the binding of the antigen. Therefore, if the Fab region is immobilized onto the plate, it is not available for antigen binding, which limits the assay's ability to capture the target molecule. This results in a lower effective concentration, or the concentration of functional antibodies immobilized onto the surface.

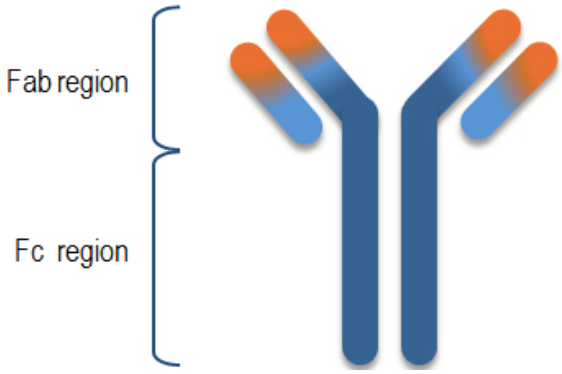


Figure 3. The labeled regions of an antibody

In order to address the drawbacks of passive adsorption, we also wished to explore the use of Protein A to control the orientation of our antibody. Protein A is a surface protein normally expressed in the cell wall of the *Staphylococcus aureus* bacterium, and has the unique ability to bind to the Fc region of most antibodies.[6] Because of this, Protein A can be coated onto a solid surface to immobilize the antibodies onto the surface in such a way that the Fab region is open to the environment for antigen binding (Figure 4). This results in an increased effective concentration and greater consistency. Analogous proteins such as Protein G and Protein A/G can also be used, but each have their strengths and weaknesses depending on the species from which the antibody was derived. Our anti-nucleolin antibody was rabbit-derived, so Protein A was the most appropriate for our assay. While Protein A offers a more effective immobilization

method, it is a more intensive process, as the surfaces need to be properly treated with Protein A. Luckily, this method has become popular in diagnostic assays and purification methods, so Protein A-coated surfaces are commercially available.

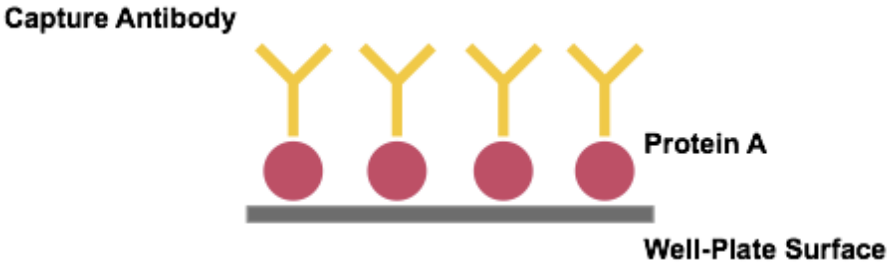


Figure 4. Protein A-coated surface for oriented antibody immobilization

We wished to build our assay using these different immobilization methods to observe any differences in results. While each method has benefits and drawbacks, in order to build the most effective hybrid assay, we wished to test both of these methods to see their effects on our overall product.

CHAPTER 4: EXPECTED RESULTS

4.1 Detection Limits

Detection limits of nucleolin ELISA assays range from 0.156 - 10 ng/mL. To compete and be efficacious, an ELASA assay must be able to detect analyte in a similar range. The development of our ELASA assay would be proven effective in terms of detection limits if able to produce a standard curve for nucleolin that lies within this range.

4.2 Sensitivity

We aim to compete with industry standard ELISA assays for nucleolin in terms of sensitivity. Therefore, to be successful on the market our ELASA assay must have sensitivities of 0.057 ng/mL or better. Given the attributes of oligonucleotides compared with antibodies, it is plausible that our assay could improve upon the sensitivities seen advertised in commercially available ELISA kits for nucleolin.

4.3 Efficacy as a Nucleolin Detector

On top of achieving similar sensitivities and detection limits to commercially available ELISA kits, our aptamer-based must accurately detect heightened levels of nucleolin and show no signal for negative controls. For this reason, we will rigorously test the assay with and without crucial components present, and check for any non-specific binding that might lead to a false positive in a clinical setting. The presence of nucleolin, capture antibody, and aptamer can be removed to test the characteristics of the assay. Whenever each component of the ELASA assay is negatively controlled, we would expect no significant signal to appear.

CHAPTER 5: MATERIALS AND METHODS

5.1 Materials

We utilized two different well plates as the solid surface for our assay. One was an untreated 96-well polystyrene plate, and the other was a Protein A-coated well plate from Thermo Fisher Scientific, Inc. in Waltham, MA. Buffers included a washing buffer of 0.1% TWEEN in phosphate buffered saline (PBS) and a blocking solution of 2% bovine serum albumin (BSA) in PBS. We obtained the anti-nucleolin antibody, AS22758, from Origene, Inc. of Rockville, MD. Recombinant human nucleolin protein was also purchased from Origene, Inc. The anti-nucleolin aptamer, AS1411, conjugated with Cy5 was produced by Integrated DNA Technologies (IDT) of Coralville, IA. All reagents are diluted to the desired concentrations in PBS. Finally, we utilized a 37°C incubator and a well-plate reader with fluorescence and absorbance capabilities during our testing.

5.2 Hybrid Assay Methodology

We begin our assay protocol by incubating 200 μL of the anti-nucleolin antibody in the well plate at 37°C for several hours. After the incubation is complete, each well is washed three times using 300 μL of washing buffer for each wash. This is followed by the addition of 200 μL of blocking buffer in each well and a 30 minute incubation at 37°C. When using the Protein-A coated well plates, this washing step is skipped because the plates are already pre-blocked. The wells are washed and then 200 μL of nucleolin is added to each well. After a one hour incubation at 37°C, the wells are washed and 200 μL of the aptamer is added to each well. A final washing step is conducted, and fluorescence readings are taken using an emission wavelength of 635 nm and an excitation wavelength of 665 nm, which were determined via a fluorescence wavelength scan performed on 10 μg/mL of aptamer.

CHAPTER 6: RESULTS

6.1 Full Assay Results

We ran our full assay methodology using both immobilization methods. In the passive adsorption assay, we ran n=4 of each assay condition, which included a full assay, partial assays containing only one or two assay components, and a blank. We also filled a well with 1 μg/mL of the signaling aptamer, which we used as a reference well. The signal we observed in this well would give us an idea of what signal would look like if we achieved it.

Figure 5 shows the fluorescence readings of this assay using the previously stated wavelengths. No apparent signal was observed for the wells containing all of the assay components. Additionally, no signal was observed in the partial and blank assay conditions.

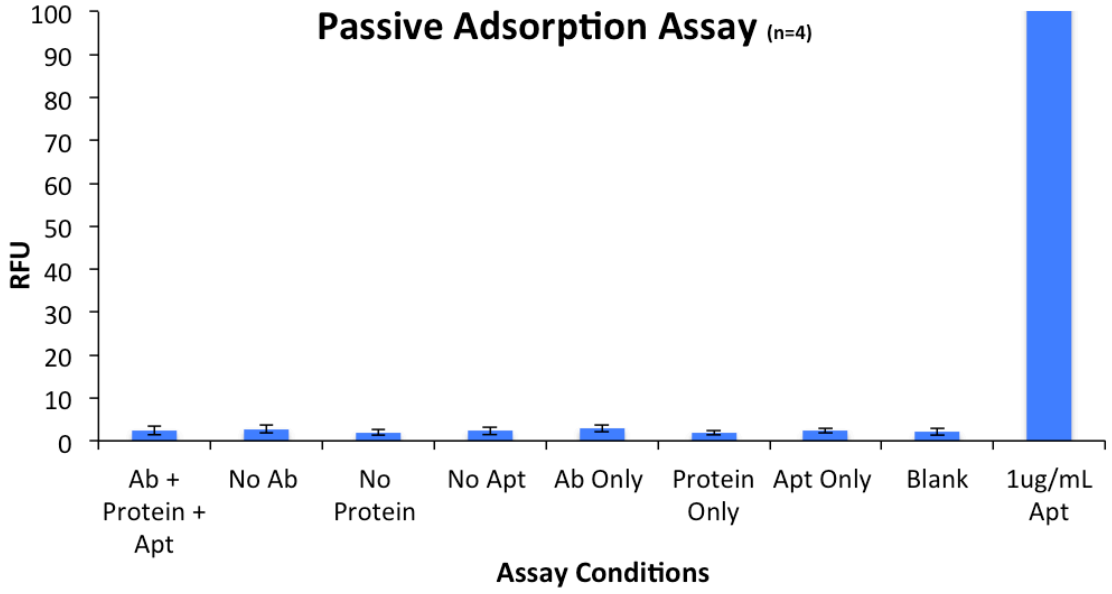


Figure 5. Fluorescence readings of various assay conditions using passive adsorption for antibody immobilization

Figure 6 shows the same assay conditions, but here Protein A-mediated binding was used as the antibody immobilization method. Again no apparent fluorescence signal was observed for the full assay condition, as well as the partial and blank assay conditions.

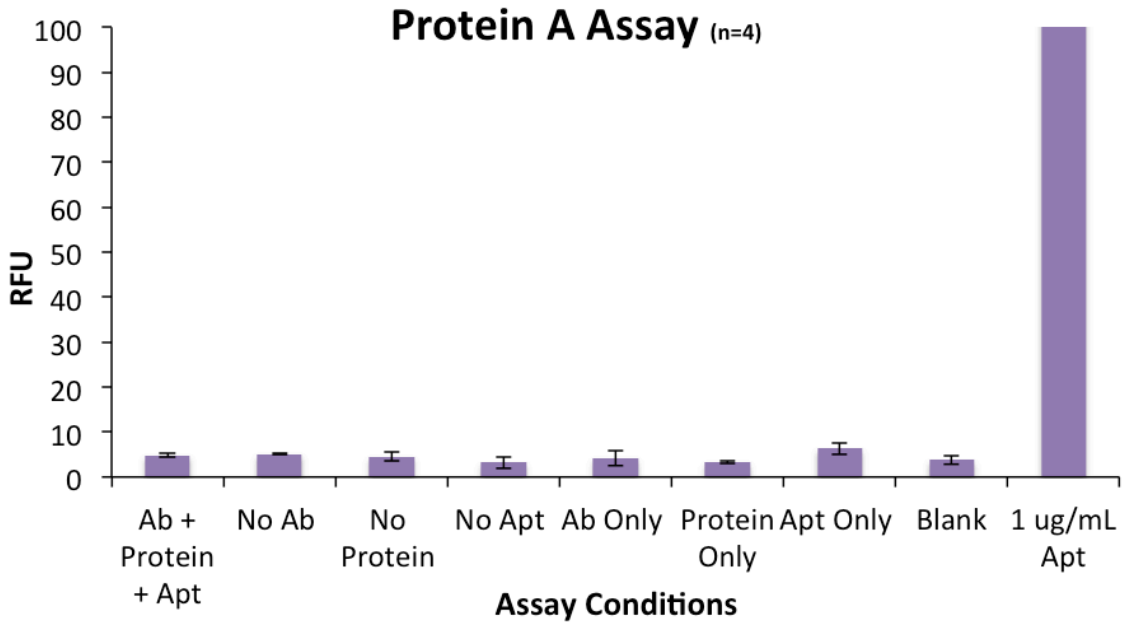


Figure 6. Fluorescence readings of various assay conditions using Protein A for antibody immobilization

6.2 Immobilization Confirmation

Since we could not achieve a signal using our full assay, we decided to evaluate the success of each step involved in building the assay. We began by evaluating the effectiveness of our immobilization methods. In order to confirm antibody immobilization using passive adsorption, we utilized a secondary antibody conjugated with horseradish peroxidase (HRP). First, we incubate our capture antibody in the well-plate to allow passive adsorption, and following a washing step the plate is blocked. Then, the plate is washed again and the secondary antibody is incubated at 37°C. During this process, the secondary antibody binds to the Fc region of the capture antibody that has successfully been immobilized onto the well plate surface (Figure 7). After the plate is washed, HRP substrate is added to the wells. When HRP is introduced to its substrate, a color change in the solution occurs causing the clear, colorless solution to turn blue. This color change is visible by the naked eye. Concentrated sulfuric acid is added to control the reaction and cause the color to change from blue to yellow. This yellow color can be detected and quantified via absorbance with greater sensitivity than the previous blue color, and it keeps the reaction from progressing too long.

Figure 7. The binding of HRP to the capture antibody for immobilization verification

Figure 8 shows the absorbance readings obtained from this experiment with various concentrations of the capture antibody. While keeping the secondary antibody concentration constant, an increase in capture antibody concentration resulted in an increase in absorbance. These results confirm the immobilization of our antibody onto the surface of the well plate.

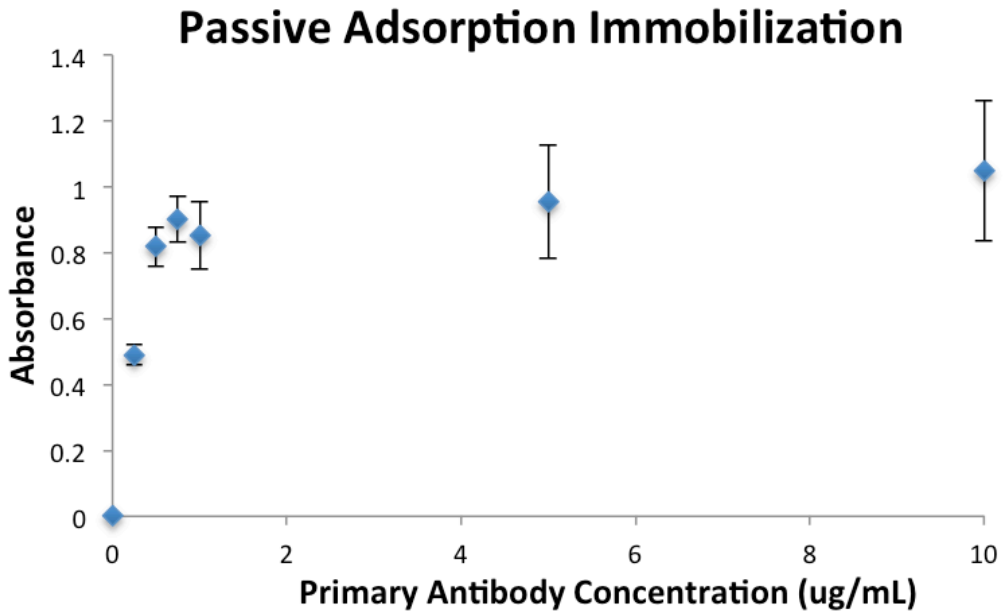


Figure 8. Absorbance readings taken at 450 nm for various concentrations of antibody immobilized via passive adsorption

6.3 Varied Protein Concentration Results

Since we did not achieve the expected signal in the full assay condition, we performed the assay protocols again, but we used increased and varied nucleolin concentrations. By varying the concentration, we could see which concentrations of nucleolin could achieve sufficient binding to both the antibody and aptamer to produce a signal. We also added an excess of aptamer to ensure that this component would not limit the success of our assay. In Figure 9, no signal was

recorded for any of the protein concentrations. Again, a well containing the fluorescently labeled aptamer was used as a reference.

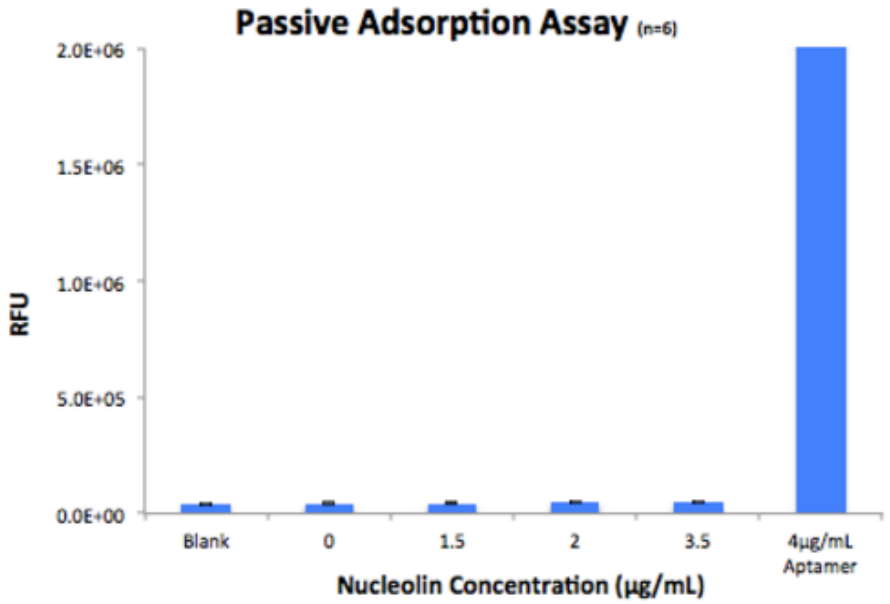


Figure 9. Fluorescence readings for passive adsorption assays carried out with various protein concentrations

CHAPTER 7: DISCUSSION

7.1 Lack of Signal

Although we expected a positive correlation between nucleolin concentration and fluorescence, this was not realized in our experimental results. Signal remained low for wells containing nucleolin, and mirrored the results seen in the "blank" or negative control well. For this reason, we hypothesize that there is a lack of expected binding behaviors between either the capture antibody and nucleolin, aptamer and nucleolin, or both. If either pair failed to bind with a low disassociation constant, this would negatively impact the end results of the assay. As the sandwich conformation of the assay is constructed, it is imperative that each addition component binds selectively with the target protein.

It is also possible that the lack of signal in our assay is due to flaws in our protocol that led to interference between the binding of aptamer and protein or protein and antibody. Between each incubation step, the well plate is washed thoroughly with a dilute surfactant to decrease the chance of non-specific binding. Although such washing steps are critical, it is possible that either the physical or chemical forces introduced lead to a disassociation between assay components, thus diminishing the overall signal detected.

7.2 Non-specific Binding

A positive takeaway from experimentation with our assay protocol is a lack of non-specific binding. Non-specific binding occurs when assay components, like antibody or aptamer, bind non-specifically and unexpectedly to other areas than their intended target. This increases the signal of the assay disproportionately to the level of protein in a sample. Therefore, in a commercial application, an assay with high non-specific binding would lead to a false positive reading, potentially leading to a misdiagnosed decision.

In order to mitigate non-specific binding and false positives in our assay, the protocol was constructed with a blocking step. Addition of bovine serum albumin into the well plate covers

areas where non-specific binding might occur, ensuring that antibody and aptamer will bind only at discrete, expected sites upon the tested protein.

With low levels of fluorescence seen in our blank wells, we were able to prove that such blocking steps were successful in preventing non-specific binding. The entire assay protocol was also tested with certain components, like antibody and aptamer, removed. Similarly, this returned a low level of fluorescence, suggesting that non-specific binding was not occurring at a significant level.

7.3 Future Work

Several auxiliary experiments can be run to further identify the possible sources of error within our assay protocol that are resulting in an unexpected lack of signal. In order to determine the level of binding occurring between aptamer and protein, and also antibody and protein, analysis of each pair can be completed with capillary electrophoresis and surface plasmon resonance.

Capillary electrophoresis is a separation technique that can effectively discriminate between molecules and bound compounds with respect to charge and size. By incubating nucleolin in solution with antibody, and separately with aptamer, the level of binding can be determined using fluorometric detection. A tested material is drawn through a glass capillary, microns in diameter, with an applied potential. As the solution is pulled across a clear glass window, laser light is applied at a wavelength specific to the excitation wavelength of the signaling molecule in solution. Fluorescence readings then indicate when a molecule elutes from the capillary. This process can help to quantify the level of binding between an aptamer and its target, as heavier bound states will elute later than unbound free molecules.

It should be noted that in the case of testing binding between antibody and protein, another secondary antibody, conjugated with a fluorescing molecule, must be used. Unlike in the case of the anti-nucleolin aptamer, the capture antibody lacks a conjugated signaling molecule. Therefore, in an analytical method that relies on fluorescence, like capillary electrophoresis, a conjugated secondary antibody, an auxiliary source of fluorescent signal, must be included in the incubation process between antibody and protein.

Another analytical method capable of determining levels of binding between each assay component is surface plasmon resonance (SPR). This technique relies on monitoring the angle of reflection of an incident light source that is affected by the binding of a target to a capture molecule. In the context of our assay design, the capture antibody or aptamer could be affixed to a solid state, often times gold plated surface, and be monitored for their respective binding to nucleolin. As more molecules bind to the target protein, the reflective angle changes, allowing for a precise calculation of the disassociation constant (K_d) between two species.

Prior to exploring the binding between each assay component using more sophisticated technology, it might be worthwhile to determine levels of binding between the capture antibody and nucleolin with a more basic method. Using a signaling antibody, included in a commercially available ELISA kit, the binding of a protein to the immobilized capture antibody on the well plate surface can be determined. By following our protocol steps preceding the addition of aptamer to the well, and then adding a conjugated antibody instead, the source of error can be more reliably isolated before completing more robust testing.

APPENDIX

A-1. References

[1]Abbott, Karen, et al. "Identification of candidate biomarkers with cancer-specific glycosylation in the tissue and serum of endometrioid ovarian cancer patients by glycoproteomic analysis." *Proteomics* 10 (2010): 470-481.

[2]Toh Saw Yi, et al. "Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay." *Biosensor and Bioelectronics* 64 (2015): 392-403.

[3]Guo, Jianwei, et al. "Aptamer-functionalized PEG-PLGA nanoparticles for enhanced anti-glioma drug delivery." *Biomaterials* 32.31 (2011): 8010-8020.

[4]Hovanessian, Ara, et al. "Surface expressed nucleolin is constantly induced in tumor cells to mediate calcium-dependent ligand internalization." *PLoS ONE* 5.12 (2010): e15787.

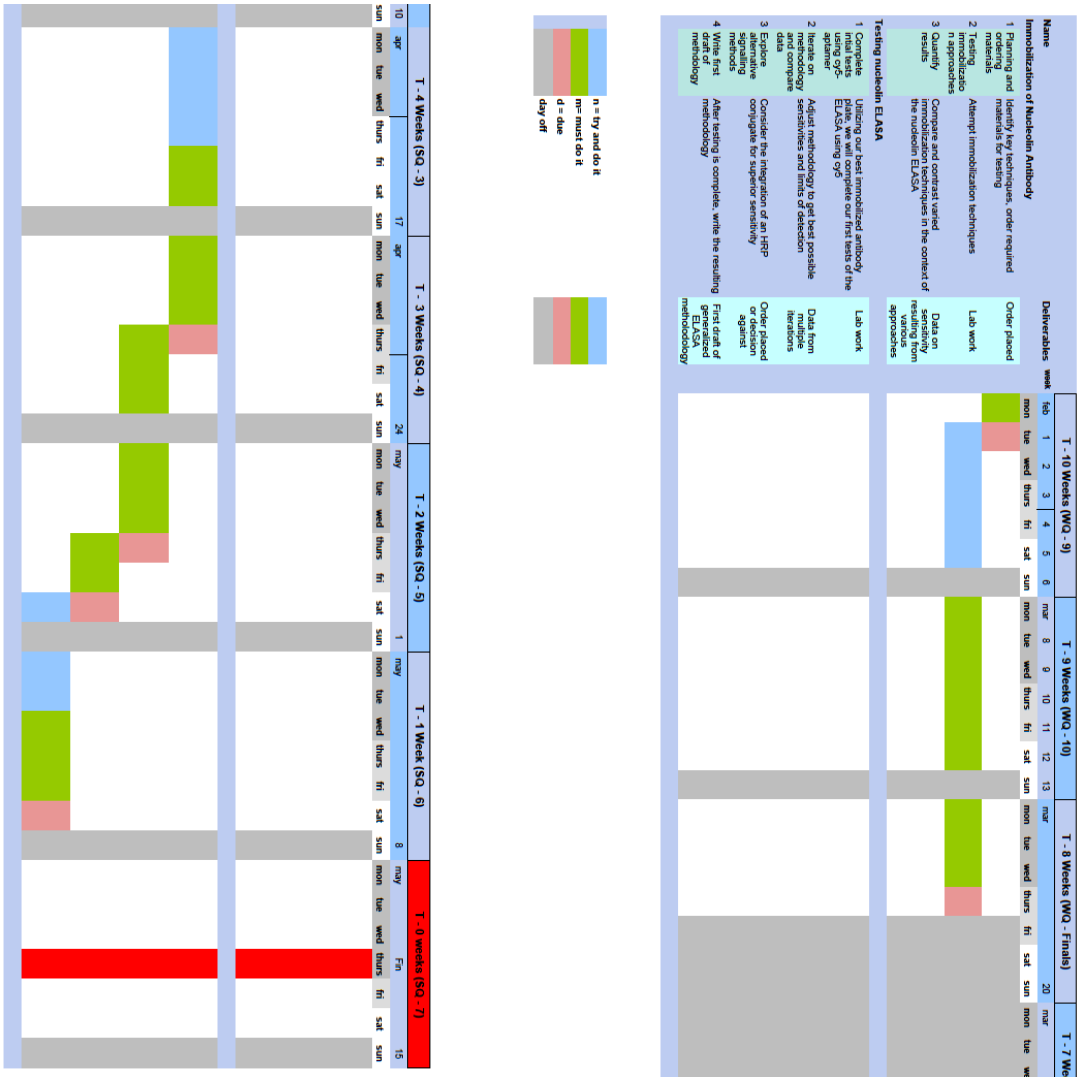
[5]Stewart, Bernard W., and Chris Wild, eds. World Cancer Report 2014. N.p.: n.p., n.d. World Health Organization.

[6]"ELISA Kit for Nucleolin (NCL)." Uscnk.com. Wuhan USCN Business Co., n.d. Web.

[7]Van Weemen, BK, and AHWM Schuurs. "Immunoassay Using Antigen-Enzyme Conjugates." FEBS Letters 15.3 (1971): 232-36.

[8]"Human NCL / Nucleolin ELISA Kit." LifeSpan BioSciences. LifeSpan BioSciences, n.d.

[5]Butler, J.E., et al. "The physical and functional behavior of capture antibodies adsorbed on polystyrene." *Journal of Immunological Methods* 150 (1992): 77-90.

[6]Lu, Bin, Malcolm R. Smyth, Richard O'Kennedy. "Oriented immobilization of antibodies and its applications in immunoassays and immunosensors." *The Analyst* 121 (1996): 29R-32R.

A-2. Spring Quarter Gantt Chart