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# Development of a Lab-on-a-Chip Microfluidic System for the Detection of C-Reactive Protein in Whole Human Blood and an Antibody Biomarker for the Detection of Alzheimer's Disease

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DEVELOPMENT OF A LAB-ON-A-CHIP MICROFLUIDIC SYSTEM FOR THE

DETECTION OF C-REACTIVE PROTEIN IN WHOLE HUMAN BLOOD AND AN

ANTIBODY BIOMARKER FOR THE DETECTION OF ALZHEIMER'S DISEASE

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**ABSTRACT**

This thesis is divided into two sections. The first section focuses on

developing a better method for the detection of C - reactive protein (CRP) from whole

blood. CRP plays a key role in determining the risks associated with various

cardiovascular diseases. However, detecting this protein is a challenge since it

accounts for < 1% of all known blood proteins. This protein can be detected by using

a sandwich ELISA inside of a microfluidic chip made from Polydimethyl Siloxane

(PDMS). PDMS chips are created that will first remove blood cells and platelets from

whole blood. For the removal of blood cells, different filter papers that specialize in

blood separation are tested based on the volume of filtrate recovered, amount of blood

cells filtered out and whether the cells rupture or not. From these tests, it was

determined that the Vivid Plasma Membranes GX and GR from the Pall Company was

the most suitable filter based on blood cell removal and quality of filtrate.

To further compliment this, these chips need to extract high abundance plasma

proteins (HAP) including human IgG antibody and human serum albumin. These

experiments focused on using protein A as a method for the removal of IgG due to its

high affinity with the antibody. This will be then coupled with IgG that has been pre-

attached to Protein A specifically modified to attach to Human Albumin. Experiments

were conducted in order to obtain the appropriate incubation time needed for removal

of antibody/protein as well as the effects on surface concentration and amount of

incubation reservoirs. The final step was to combine these two aspects in order to

create a microfluidic chip that accurately detects CRP by filtering out blood cells and

removing high abundance proteins. This resulted in a microfluidic chip that was able

to detect CRP from whole blood while filtering out blood cells and removing the bulk of the high abundant plasma proteins in order to obtain a high signal. This was performed by the addition of multiple incubation areas for high abundance protein removal embedded on the chip. Detection was carried out via fiber optic cables and a standard curve was created that associated a specific CRP blood concentration to an intensity found via fiber optics. By use of this method, a correlation between the concentration of CRP in a sample of blood, as well as a limit of detection for the system could be obtained.

The second section focuses on identifying an antibody fragment as a bio-marker against tau protein. Tau protein is a known to play a role in the development of Alzheimer's disease. This is due to the rapid addition of multiple phosphate groups attaching to the protein. This section focuses on the identification of antibody fragments that detect a normal form of tau and one affected by the addition of phosphate groups. These fragments are recombinant antibody fragments created via phage display. Antibody phage is inserted into a well, in which the phage that is able to bind to tau will bind to it. These phages are then injected into an E. coli culture which will produce antibodies known as single-chain variable fragments (scFvs). These antibodies are extracted and tested on whether or not they attach to the specific tau antigens. After performing these experiments, two scFvs have been found that attach to both forms of tau: one with phosphate groups and one without. Titration curves and DNA sequences for both antibodies were also created. With these antibodies, they can be used as an effective biomarker into the early treatment of Alzheimer's disease.

# ACKNOWLEDGEMENTS

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**SECTION 1**


DETECTION OF C REACTIVE PROTEIN IN WHOLE BLOOD


**CHAPTER 1**


INTRODUCTION


In previous years, in order to effectively detect a disease or a serious condition, a large sample would have to be extracted and sent into a laboratory. From there, numerous experiments that would take days, even weeks to complete would have to be performed. Not only was this work time consuming, but it also could mean the difference between life and death to a certain individual. Today, testing can be performed with small chips and paper-based cartridges with little fluids required. This is a breakthrough in detecting and treating certain diseases and ailments. This is what is known as Lab-on-a-Chip (LOC). In LOC experiments, large scale experiments are performed on the small scale with small volumes of sample and reagent, is not only found in current research, but used in practical applications[1,2]. This can be beneficial in rapid detection and care of various ailments. One such ailment is heart disease[3]. There are various factors that can cause heart disease which include high cholesterol[4]. However, one important compound is C - reactive protein (CRP). CRP is found in the blood and is a factor in determining whether or not an individual can be affected by heart problems[5]. If a certain sample has a level of CRP that is over a certain amount,

then this individual would be susceptible to heart disease.  Currently, those same laboratory methods are used to detect CRP, which include extraction of a good amount of blood and waiting at least a few days for results[6].  The following project will focus on these aspects of LOC's in order to better detect CRP.

### 1.1. Lab on a Chip

In the previous decade, there has been an effort to make the detection and treatment of diseases and ailments easier and faster. Currently, the best methods to detect diseases are in laboratories with expensive equipment[6]. There are various issues that do come up when performing these experiments.  First of all, bulky equipment is used and a lot of it can be pretty expensive to buy and/or repair. Second, with this large equipment, it can be certain that large amounts of sample and or reagent have to be used. This amount can be in the area of a few milliliters. The third issue is the time needed to perform each experiment. These tests can last for a few weeks if needed. Because of these issues, a new method needs to be addressed. Basically, a system for detection has to be designed, that not only is light and portable, but must also be fast in detection and must take as little fluid as possible.

Currently, there is a new method of detecting ailments with all of these points in mind called Lab-on-a-Chip, or LOC for short. LOC essentially takes experiments that can only be done in labs and miniaturizes them onto a small chip[1,2]. Because of this, all the reagents used would have to be enough to be present in the chip, preferably in the microliter range. In addition to all of this, because of the smaller volumes of liquid, the time needed to perform these experiments is greatly reduced. This allows for better detection that most people can do in a relatively short amount of

time. This is beneficial not only for home testing, but also for allowing testing to be performed in under-developed countries, where access to top of the line health care is all but non-existent[7].

Because LOC devices are relatively new, there are a limited amount of technological advances that are widely known. The most known system to use all three of these properties (miniaturization laboratory equipment, small sample and reagent size and quicker reaction times) are glucose meters. These meters measure the amount of glucose that is found in the blood[8,9]. Other examples of these include pregnancy tests[10] and disease testing kits that range from strep throat[10] all the way to HIV[11]. These devices prove to be beneficial in the determination of something that does not need a large scale laboratory or a large amount of time to complete.

How can each of those three aspects create a better system for detecting diseases and ailments? Here, each aspect can be looked at one at a time. The first aspect is the amount of equipment. As stated previously, the detection of most antigens are performed in huge laboratories with numerous large and expensive equipment[6]. With LOC, chips are created using inexpensive materials (polymers and paper, as a few examples)[3]. These chips include all of the reagents and detection areas that are needed for the experiment to be performed[8]. Also, platforms can be made that are either portable or hand held, eliminating the need for a laboratory[8].

The second aspect involves using as little reagent and sample as possible. In laboratories, it is common to use milliliters of sample and reagent to perform experiments[6]. But in LOC applications, the amount of fluid used is in the microliter range[8]. Even in ELISA (enzyme linked immunosorbent assay) experiments, which

will be explained later, the total amount of liquid used in each experiment could be upwards to around 1 liter[12]. But in LOC experiments, with the exception of a sample, the majority of the fluid is in the chip. And with the chip being as small as possible, the amount of fluid can amount to less than 10 milliliters[1,8]. The third aspect involves time. In normal laboratory settings, with the previous two aspects, these experiments can take upwards to a few days to complete. With LOC processes, these same experiments can be done in the matter of an hour or less with comparable results. For these reasons, it can be seen that LOC technology is a novel idea as a better way to detect diseases and ailments.

One source of interest with LOC devices is the method of detection of various pathogens in blood. Since there are about $4.7 - 5.5$ liters of blood in the body and it reaches virtually every place in the body carrying oxygen, carbon dioxide and nutrients, it is one of the body's most essential fluids[13]. There are thousands upon thousands of different proteins and pathogens that can be found in blood, some of which can be dangerous for people. There have been numerous methods to use LOC to detect these antigens, but one of the problems with using LOC devices with blood is that the makeup of blood is very complex[14]. Plus, with the small amount of liquid used in LOC, it becomes difficult to separate the antigen that is desired from all the other components in blood[14]. In order to see how complex blood is and the problem associated with it, the upcoming sections will explain the composition of blood with comparison to C-reactive protein.

## 1.2.    Detection of C-Reactive Protein in Blood

Since CRP is normally found in blood, one of the issues concerning the detection of CRP on LOC is the amount of interference caused by other components in the blood. These components not only include large particles, including blood cells (both red and white) and platelets, but also smaller particles, including various blood proteins and antibodies[14]. Because LOC applications require the use of minute amounts of reagents (up to 1 mL), traditional blood plasma separation, which include centrifugation is not applicable. Also, it should also be noted that because of the need to these applications to be portable and compact, an on-site separator also has to be incorporated onto the chip. Because of these reasons, a direct blood plasma separator has to be integrated onto the chip[15-17].  In the upcoming sections, different methods of blood separation will be explained and checked in order to see if it is feasible to integrate these systems onto an LOC device.

One other issue is in the plasma itself. CRP is only one of thousands of proteins found in blood and these proteins can cause interference with detecting CRP[18]. Because of this, a method to extract a good amount of proteins before detection has to be implemented inside of the chip.  This can be performed by incorporating the various channels that transfer the plasma from the cell separator to the detection site[14]. Using the channels, these proteins have to be removed before they enter the detection area. There are different methods which can be performed to accomplish this. These will be explained in the upcoming sections.

One other point to get across is that there are many methods to detect CRP in blood, including different methods based on LOC concepts[19,20]. This project will take

a look at these different concepts and compare them to how well they can be performed. With all of these concepts, a newer and better method to detect CRP in blood on a chip will be tested.

### 1.3.    Removal of Cells and High Abundance Proteins

As previously stated in section 1.2, one of the issues that surround the capturing of CRP is because of the fact that it is normally found in blood[6]. This causes a lot of issues because of the amount of other abundant objects in blood[18]. These objects of important not only are of concern on the micro scale (red and white blood cells, platelets), but also on the nanoscale (plasma proteins). These objects are able to cause all types of obstruction when they are placed in a detection area that would be used to capture CRP. Because of this problem, it is essential to remove these aspects in order to obtain a reliable result.

Starting from whole blood, it is ideal to remove the blood cells and the platelets first[17]. The reason for this being that it is easier to remove larger objects, (cells, platelets), compared to the proteins. Since the cells are noticeably larger, they do tend to interfere with the signal the most. This can be done either by intentionally blocking the receptors, or by accidental removing of whatever is supposed to bind to CRP, or whatever antigen is being detected. There are numerous methods to separate blood cells from plasma, but most involve using large devices and sample amounts[21]. In terms with LOC applications, especially if it is being used in a non-laboratory setting, it would be ideal to use only a minute amount of blood and basically separate it on a chip. There have been projects in the past that have focused on using filter paper as that medium because of its flexibility to be in any shape and size, its low cost

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and with most papers, its effectiveness in separating blood cells from plasma[16,17].
This project will integrate this method of using filter paper for blood plasma
separation.

The other obstacle in this project is the amount of proteins found in plasma[18].
There are hundreds of plasma proteins at varying concentrations.  One of the issues
surrounding this is that like blood cells, these proteins are still able to cause
interference within the detection area.  Because of this, like the blood cells, they have
to be removed in order for CRP to be fully detected.  However, because of their small
size and that this has to be performed in the chip before detection and after blood cell
separation, current methods of protein removal, which can include centrifugation[21],
cannot be applicable.  Instead, there have been studies, including former
investigations[22-24], which use antibodies on surfaces, like an ELISA[12] (Enzyme-
Linked Immunosorbent Assay) that can capture these various proteins.  This project
will focus on these principles in order to effectively remove these high abundance
proteins (HAP).

### 1.4.    Objective

The objective of this investigation is to create and improve on a LOC process
that would better detect C - reactive protein in blood.  This project will be in
cooperation with previous projects that have been used to begin to detect CRP[22-24].
These projects include a better method to filter blood cells, capture HAPs in
blood/serum that can cause interference and use these two methods to implement a
system to detect CRP onto a chip design that was previously used in a few other
projects.

The first part of the project is to find a better way to filter blood cells.  This is important since they consist of the largest objects in blood and take up the largest amount of space.  There have been previous methods in which to extract plasma from blood into a microchip[23,24], but a lot of projects do not go into a lot of detail that explains the need to better filter blood.  These reasons include the amount of force it takes to extract blood into a channel, how much plasma can be extracted from filter papers and if the paper is sturdy enough to withstand not only the amount of blood cells on it, but also the force required to effectively extract plasma without ruining the filter.  For this, there will be research performed that will determine the quality of the filter paper that will be used for the microchip.

The second part of the project is to find a better method of HAP removal.  Like the above section, there have been attempts to effectively remove these proteins[23,24].  However, as will be explained in Chapter 2, there are flaws with these methods.  This is based on the amount of proteins in plasma and how they can affect the detection area.  This is not only because of the amount of the different types of proteins, but rather the amount the most abundant proteins in blood plasma: human serum albumin, and human immunoglobulin antibody G.  These two proteins account for more than 75% of all the total proteins in blood[18,28].  Because of this, which will be explained in the upcoming sections, a better method of antibody removal must be performed.

The final part will be to put both parts together in order to better a currently proposed method for CRP detection in whole blood.  With these two parts formed into one, actual CRP tests in blood can be performed.  In the previous projects, most experiments were not performed with whole blood because of special procedures and

the uncertainty of the amount of proteins in a certain sample. Most projects either relied on a buffer solution with CRP in it, or serum with CRP. Because of this, one could not say whether or not it could be performed with blood. One reason is that serum does not contain fibrinogen, a protein known for clotting, whereas plasma does. Also, the previous filter effects have to come into play. It has to be certain that only plasma can go into the channel, and not either blood cells or hemoglobin, caused by hemolysis, or blood cell rupturing. These factors have to come into play, in order for this project to be successful.

Lab-on-a-Chip is a novel idea, where a person can perform diagnostic checks without the use of a laboratory and a large amount of sample and/or reagent. But the above topics have to be addressed in order to make certain that it can be justified that blood can be used for LOC applications. Due to the amount of blood cells and the amount of plasma proteins, these have to be removed first hand in order to be able to detect CRP, a known protein that is linked to cardiovascular disease. With this in place, a better method of detecting CRP on a microfluidic chip can be performed.

# CHAPTER 2

## REVIEW OF LITERATURE

### 2.1.    Types of Lab on a Chip Applications

The first known instance of Lab-on-a-Chip (LOC) was in the December 1979 issue of Electron Devices, where a miniature gas analysis system was implemented on silicon using photolithography and chemical etching[1].  This was a system used for gas chromatography and used to detect and separate various hydrocarbon mixtures[1].  However, there has been an increased interest in microfluidics starting in the mid to late 1990's[2].  One of the earliest known examples of LOC technology was a project between the years 1999-2003 at Delft University in the Netherlands where a microchip was created that allowed for the analysis of different bioprocesses (i.e. fermentation)[2].  These microchips was created by using glass and used silicon and glass etching in order to create the channels to allow fluid inside the chip.

One other application for LOC systems is for testing the quality of certain fluids (urine, semen, blood, water, etc…).  By allowing these measurements, one could find out how healthy a certain environment is.  For example, a biochip has been developed by Siemens that would be able to test the quality of water[25].  This test would be able to detect certain hormones, antibiotics, bacteria and pesticides.  The unit is roughly the size of a laptop computer and can be able to identify up to 25 known substances in a 30 minute timespan.  This biochip is covered with surface antibodies that react to corresponding substances contained in the sample liquid.  This is followed

by an electrochemical reaction which emits a signal. With this technology, it would be beneficial to any industry that handles wastewater on a regular basis (water treatment plants, hospitals, and even living conditions with horrible drinking water)[25].

However, the most common application of LOCs is in the use of medical diagnostics. In recent years, there has been a rise in the amount of the need of portable diagnostic equipment that is easy to use with accurate results. One option that helps with LOC devices is the integration with point of care testing (POCT). POCT is testing that is performed at or near the site of where a patient is located. These tests are important as that they can give direct results to the patient at his or her precise location[26]. This is both beneficial to the doctor and the patient where that the results for any diagnostic test can be fast and immediate. Also, since the tests are performed with the patient at the doctor's side, there is no need of a laboratory in order to obtain results. There are many types of POCTs that are currently in use. Some of the most common tests that require POCTs include testing for pregnancy, the amount of blood glucose, testing for strep throat and influenza via the throat[10,26]. These are only a few of the tests that POCTs can deliver.

However, with the advances in medical diagnostics, there is a greater need to deliver POCT as a method of detecting different conditions and diseases. This is extremely beneficial in home care, doctors working on site and in places where modern medical facilities are not readily available. One such use for POCT that has been on the rise is the use of analyzing blood. Blood contains numerous amounts of proteins, viruses and compounds that that serve a specific function within the human body[27,28]. One of the first POCT devices that incorporate LOC technology are

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glucose meters. Here, a small amount of blood is drawn from the body and sent up a wick coated in glucose oxidase, which is an enzyme that reacts to the glucose found in the blood sample[8,29]. An electrical current then interacts with the glucose reaction, which then generates a signal. This signal then is read as a value that corresponds to a glucose concentration in blood. The higher the signal, the higher the number[29]. This allows clear, quick and accurate results for people who suffer from diabetes, in order to make sure that their glucose levels are either healthy or not[8,29]. This is only one example of an LOC device which is a good POCT tool for blood testing.

Other examples of POCT for LOC devices that uses blood include various chips that are able to detect a whole plethora of proteins and ailments[30-33]. These include a device that uses an LED to detect proteins and viruses fixed to a gold plate[30,31] and a device that could detect conditions that include HIV and diabetes[32]. In the example of the LED and nanoparticles, proteins and antibodies are fixed on an array and a blood sample is passed over it. After incubation, a gold plate with holes on it goes over the sample and light is emitted from an LED through the holes and onto the sample. An imager detects the emitted and reconstruct the image of the detected chip into intensity values[31]. One final example is a chip which separates blood cells and analyses the properties of the separated cells[33]. These examples of POCT chips which use LOC technology are useful in that different viruses and proteins and can be extracted and analyzed. With this technology, it will not be long until these detection values will have a correlation to how much of an extent a person has a certain ailment. However, one thing in common with these LOC devices is not only that blood is used, but also that blood cells have to be separated from plasma in

order to obtain accurate results.  The next section will explain the makeup of whole

blood, why separation is an issue and what types of separation are there.

### 2.2.    Whole Blood Makeup
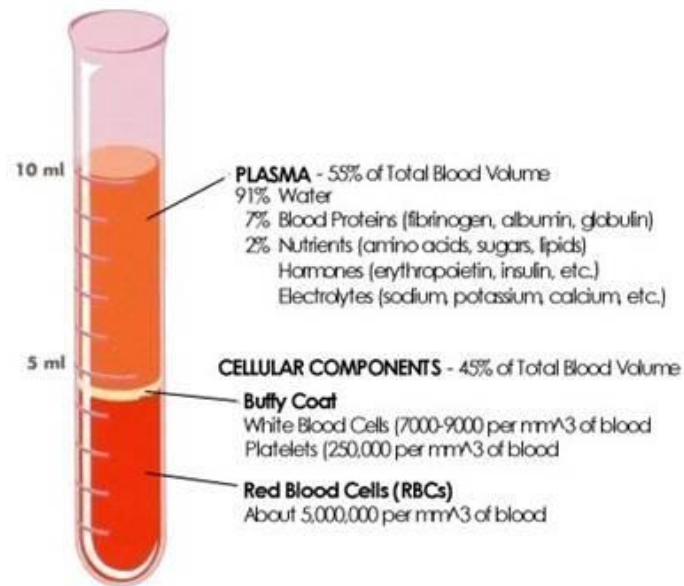
**Figure 2-1:** Makeup of whole blood[27]

In the human body, there are many factions that contribute to the makeup of

whole blood. The first includes red and white blood cells and platelets. These three

components are the biggest objects that can be found in blood. Red blood cells

transport nutrients and oxygen to the muscles of the body, while also transporting

carbon dioxide to the lungs and waste to the kidneys. They account for 45% of the

total volume of blood. White blood cells are part of the body's immune system, which

fight off foreign pathogens and platelets are used for blood clotting when the blood

canal is ruptured in order to prevent blood from escaping the canal. Both of them

account for < 1% consists of the volume of blood. The remaining 55% is known as

plasma. Plasma consists of water and all the proteins that are common in blood. With

the absence of the protein fibrinogen, which aides platelets with clotting, this fluid is

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called serum[18,27,44]. Figure 2-1 shows a comparison as to how the makeup of whole blood is constructed.

The issue with blood in an LOC process is that these three components can interfere with any detection signal when a certain antigen is to be obtained. This is due to the large size and abundance of the cells. Most proteins have an average diameter size of 200-400 μm, while blood cells as a comparison have a diameter of 6-8 μm[18,44]. In any experiment in which blood is used, it will cause interference in any type of detection because of its size. These cells could both act as a barrier and block certain antigens from being detected or if there is a method of detection already in place in the chip, it could effectively remove that area of detection. Either way, because of this disruption, it will be necessary to remove said blood cells and platelets before the blood goes into the chip. The following section will explain the various methods of blood filtration and plasma separation.

### 2.3. Methods of Blood Cell Separation

#### 2.3.1. Blood Centrifugation

The most common method for blood cell removal and plasma separation is centrifugation. This involves taking a vile of blood and placing it inside of a centrifuge. Using centripetal force, the centrifuge spins around at a high RPM and the cells head towards the bottom of the vial. After about 20 to 30 minutes, a vial that appears to be red now has three visible layers: a bottom red layer showing the cells, a small middle layer containing platelets and a clear, almost tan layer containing plasma or with EDTA, serum[21]. Figure 2-2 on the next page shows two vials with blood inside of them. The right vial is normal blood without any separation of blood cells

occurring.  The left vial is blood that has underwent centrifugation.  Notice the two

distinct layers; while the third layer, the platelets, are not shown due to the amount and

size of them in blood.  The bottom of the vial contains all of the blood cells, both red

and white, while the top tannish translucent liquid is blood plasma after the removal of

blood cells.



**Figure 2-2:**  Blood separation via centrifugation (left vial) compared to whole blood
without separation (right vial)

While centrifugation is an ideal method to separate blood cells effectively, it is not an

ideal method of blood separation to use in POCT with an emphasis on LOC

technology.  The main reason for this being that external equipment has to be used.  In

this case, a centrifuge has to be used.   With LOCs and POCTs, it is imperative that all

experiments are performed on the chip.  This should include any blood separation

processes where the testing can be performed outside of the laboratory.  Since a

centrifuge is normally not included on or in an application involving LOC and that

most centrifuges can be big and bulky, this method is not applicable with this type of

process. Also, the amount blood required to be separated is greater than what is

required in LOC applications. In order to effectively see any separation, at least 1 mL

of blood has to be sufficient in order to see this separation of plasma and cells. One

could use less blood, however for most experiments that use blood centrifugation; at

least 1 mL is required in order to effectively separate blood. The most effective

method to separate plasma from blood would be to perform this inside of the chip

itself.

### 2.3.2. Lab on a Chip Methods

Currently, there are numerous methods for the separation of plasma from

whole blood with volumes less than 1 mL on LOC devices. One of these methods

involves a process known as dielectrophoresis. "Dielectrophoresis (DEP) is a

technique to handle neutral particles in liquid medium using polarization of particles

and medium in an inhomogeneous electric field," according to Nahashima[34]. This

means that a force is placed upon a particle, in this case blood cells, and moves them

when exposed to an electric field. In this article, blood is inserted inside of the chip

and an electric current is run through the chip, which separates the cells from the

plasma. This is performed by placing electrodes on one side of the chip and

connecting it to an electric current. As soon as a voltage is applied, the cells move

towards the electrodes and plasma is extracted through small channels into a small

reservoir. The result of this being fully separated plasma without the rupturing of

blood cells[34]. Even though this is a good system for LOC applications, it cannot be

useful for POCT diagnostics. For one reason, this system relies on an outside electric current in order to separate the cells. This defeats the purpose of having the system portable and helpful in on site diagnosis. Also, because of the electrodes, it is rather difficult to integrate it with a system that also accounts for CRP detection. There are too many electrical parts this can cause interference with the detection system due to the electric field. One other constraint is that during the fabrication of the chip, the electrodes have to be fabricated inside of the chip. These are gold electrodes that must be between glass and epoxy before blood is inserted[34]. This creates a problem in that the chips are more expensive to make because of the electrodes. For this reason, other methods for blood plasma separation have to be considered.

One other method that can be considered when trying to filter blood in a microchip is by building tiny micro structures embedded inside of the chip that can help separate the blood cells[35]. Based on the research performed by Shim, a microfluidic chip was created in order to separate blood cells from plasma using tiny microstructures and packed beads in the chip[35]. Like the previous example, small channels were etched inside of the chip, but this example does not require the use of electrodes to separate the chip. Instead, small channels were created inside of the chip complete with fluid beds to hold the blood in the chip for filtering. These beds were filled with packed beads which created spacing just large enough for plasma to travel through, but not the blood cells. Movement throughout the chip was performed by capillary action in the chip, where the plasma would seek and fill the open spaces caused by the beads. Plasma was then extracted by way of an opening where air could be pumped into the chip and forces the plasma out of the chip[35].

Even though this is a possible method of blood cell removal, in terms of this project, it is not a viable method to remove blood cells. The reasoning for this is the fabrication, or building, of the microchip. According to Shim, a nickel plate had to be coated with a photoresist in order to create the molds. These molds were formed by exposing the photoresist under UV light and then developing the molds[35]. One of the experiments on mold creation involves this method, but the technology provided by the laboratory does not allow the formation of these particular channels. This is due to the fact that in order to create narrow channels, the UV light source in this experiment has to be focused and direct in creating these channels. A broad UV light source can cause errors in creating the channel shape by making the channel widths wider than what it should be[22]. This will be explained in further detail in chapter 3. Also, because of the extra step in that beads have to be used; there still can be gaps in which blood cells can escape. Because of these reasons, this method of blood filtration is not feasible for this thesis project.

Other methods of blood cell separation include hydrophoretic filtration, in which high structures inside of the chip inhibit blood cells from passing through[36], planar microfilters[37], microfilters with agarose gel[38], which does not use external forces to remove blood, devices that require cross-flow filtration[39] and ones that utilize the Zweifach-Fung effect, (where particles tend to migrate to an area of a higher flow rate when approaching a fork in the channel)[40,41], which uses more external forces upon the blood. Again some of the problems with these devices are the amount of time, energy and cost to create and the issues of implementation onto a LOC system. However, with the examples that do not require external forces to

separate blood cells, this becomes more difficult as a way to extract plasma is needed. Because of this, there has to be a simpler way to not only remove blood cells, but also extract plasma into the microchip.

In order to incorporate blood filtration in a microfluidic chip for use in a POCT device, it not only has to be cost effective in monetary value and labor, but it also must be easy to set up and integrate without external factors.  For this reason, one of the most effective methods is to use blood filters or membranes that can effectively separate blood cells from plasma. Filters/membranes are a more logical solution because of its availability, ease of use and cost. In addition, since the filtration is to be included in the microchip, the filters can be altered so that it is small enough to either fit inside or on top of the microchip. However, one does have to look out for the possibility of potential blockage in the filter paper based on the size of the blood cells compared to the pores embedded on the paper[42].  Despite this disadvantage, the restructuring of the chip to encompass the filter will be minimal. Because of the idea of filter paper, the user will be able to handle smaller quantities of blood (up to 100 μL) and eliminates the need for a centrifuge. Unlike previous Lab-on-Chip methods that involve small microchannels which filter blood, to include that and have the experiments on the same chip will require a larger chip altogether. This would require a redesign of the device that performs the immunoassay in order to adapt to the changes in the chip design. Because of these reasons, it would be beneficial in incorporate filter paper as part of the blood – separation process.

Since there are numerous blood filters, it would be essential to test as to how well each one will fair. While some filters are just a simple filter, others are more

complex based on its mechanics and how effectively it can filter out blood cells. For this reason, two separate tests will be conducted for the effectiveness of the filters: a filtrate volume test, a blood cell characterization test.

### 2.4. Plasma Makeup and Protein Removal

One other concern deals with what happens after the plasma fully separates from the blood cells. This concern deals with the makeup of the plasma. Currently, there are thousands of known proteins and antibodies in plasma[45], most of which again interfere with whatever the method of detection system is used. The amount of these proteins in blood is usually around 8% of the volume of plasma, while the remaining 92% is water, with dissolved materials which include glucose, vitamins, minerals, carbon dioxide and urea[43]. While there are hundreds upon hundreds of proteins that are known to cause interference, three main proteins usually cause the most interference. The first important protein is fibrinogen, which explained in the previous section, is the cause of blood clotting along with platelets[18,44]. It is a long protein that has an approximate length of 45 nm and it is composed of multiple peptide chains which link together and form this long protein[44]. Compared to the other proteins that will be mentioned later on, fibrinogen can cause interference with detecting antigens in blood. Even though the amount of fibrinogen in blood is low compared to other proteins (4-7% of total plasma proteins)[44,45], they could still be a factor in obstruction due to their size and ability to form clots. However, most of the LOC experiments rely more on blood serum. Serum is essentially blood plasma but without any fibrinogen. Because it does not contain fibrinogen, serum will not easily coagulate when outside of the blood canal. Despite this, there is still an issue with

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serum due to two other proteins, known as high abundance proteins (HAP): human albumin and human immunoglobulin antibody.

Albumin makes up roughly 50 - 60% of the total amount of proteins in plasma, making it the most abundant protein in blood[28,45]. Because of the abundance of albumin, it does not allow the majority specific proteins to be easily detectable. In short, albumin acts as a barrier that does not allow antigens to be detected easily. Natural occurring antibodies, known as immunoglobulin, which comprise of a group of proteins known as the globulins, which account for $20 - 30\%$ of the total proteins in the body[28,45]. These antibodies come in various forms including immunoglobulin A (IgA). Immunoglobulin B (IgB), immunoglobulin E (IgE), immunoglobulin G (IgG) and immunoglobulin M (IgM). The most abundant of these antibodies would be human IgG[45]. There are two factors that rise with IgG is a factor when dealing with assays that require blood. The first of which is their size. Antibodies are normally larger in size than most proteins, which, like albumin and fibrinogen, represent a barrier against and sort of detection. The second factor arises in that in many assay, other known antibodies are used to detect specific antigens.  This will be explained later, but in short, human IgG can force these antibodies out of position, causing a false signal in the detection area. Because of these two aspects, it would be ideal to remove them before any detection happens. But since the aforementioned blood cells also have to be removed and are bigger, it would be easier to remove these proteins after the removal of the cells.  Table 2-2 explains the three different types of HAPs and the average amounts in plasma.

| Type of Protein | Concentration in Blood (mg/mL) | Percentage of Plasma Proteins |
| --- | --- | --- |
| Albumin | 40 - 45 | 50 – 60% |
| Globulins | 20 - 25 | 20 - 35% |
| Fibrinogen | 2 - 4 | 4 - 7% |

**Table 2-1:** Ranges of the amount of high abundance proteins in blood[28,44,45]

In laboratory applications, there are various methods to fully separate and remove these proteins. The most common methods are via precipitation[46] and ultracentrifugation[47]. Precipitation involves the use of a chemical (trichloroacetic acid, acetone, ethanol, etc) in order to aggregate certain proteins can clump them together[46]. This process requires the use of a centrifuge in order to force this sediment down to the bottom. Another method, known as ultracentrifugation, requires no chemicals, but still relies on a high-powered centrifuge in order to effectively separate and remove proteins[47]. These proteins can be extracted via drying of the precipitate, or extraction of the supernatant (liquid). While this method is shown to work, there are two drawbacks to these methods. First, with a centrifuge, the use of performing all the experiments on a chip would be useless, since protein extraction must be performed before the serum is in the chip. Also, with precipitation, some chemicals are not as selective in the precipitation of proteins. This means that all available proteins could be in the precipitate, which can cause problems which detection. Because of this, a method of HAP removal has to be utilized inside of the chip and is selective only to those HAPs.

To help further explain the reasoning behind the removal of blood cells and these abundant proteins, it would be helpful to talk about a protein that is normally found in the bloodstream. This protein is far less abundant in blood than that of

albumin and human IgG. Also, this protein is regarded to have a link to various cardiovascular diseases, diabetes and liver failure. This essential protein is known commonly as C - reactive protein[6,48].

### 2.5. About C-Reactive Protein

C - reactive protein (CRP) is a ring-shaped protein that is found in the blood stream. This is a protein that is used in immune system of the human body. Its role is to bind to the surface of infarcted, or dead, cells and bacteria and alert components in the immune system, including macrophages, which in turn, clear these cells. Because of this property and their role in the body, CRP is normally used as an inflammation marker[49]. CRP is synthesized in the liver and travels down the blood stream in search of inflamed areas[49,50]. The first mention of CRP came from William Tillett and Thomas Francis in 1930 as a pathogen because of its appearance in various cancers[49,50]. The CRP protein structure consists is of pentagonal shape consisting of 5 monomers with an average weight of 25.106 kDa per monomer (total weight: 125.53 kDa) and has 224 amino acids in its monomer structure[49].

In a laboratory setting, CRP is used as inflammation marker for the determination of patients that are susceptible of different ailments. For a long time, it was determined that there was a link between CRP and various cancers, including colon cancer. However, many studies have shown that CRP is only known to determine whether a patient has a risk of developing cancer, rather than whether a patient is at risk of cancer[50]. In recent years however, a link has been found between CRP and cardiovascular disease. This was shown with patients with high blood cholesterol content in blood, which can lead to coronary heart disease[49,50]. However,

since CRP levels can increase with any type of inflammation, it is not very specific in terms on the immediate risks of cardiovascular disease.  However, it can be shown that there is a link between the two based on sample size and amount of tests performed.

In blood serum, the amount of CRP varies with the individual; however there are CRP levels that exhibit whether or not due to age, inflammation or heart risk. Normal CRP concentration in serum is usually between 5 and 10 µg/mL. However, higher levels can be found in older patients and mild inflammations. This is usually between 10 and 40 µg/mL concentration of CRP. Over a concentration of 40 µg/mL CRP is where more active and severe cases of inflammation can occur[6,49].  In the case of determining a risk of heart disease, the American Heart Association has developed a quick reference guide.  According to them, a CRP level under 1.0 µg/mL correlates into a low risk category, 1.0 – 3.0 µg/mL correlates into an average risk category and over 3.0 µg/mL correlates into a high risk category[6]. However, this is based on the concentration in blood. Since there is a 11:9 ratio of serum to blood cells (54% serum, 45% cells)[27], this scale can be modified to mean that low risk is less than 1.85 µg/mL CRP concentration of serum and high risk is greater than 5.56 µg/mL CRP concentration of serum. However, multiple tests have to be confirmed, including a second CRP test and a cholesterol test to better determine one's own risk[6].

### 2.6.    CRP Detection

#### 2.6.1.  Current Detection Methods

Currently, there are numerous methods in which CRP can be detected. However most of these do not involve LOC technology. Perhaps the most common method used is using a blood analyzer[6]. A blood analyzer takes a sample of blood

and analyzes the sample. The most common method of analyzing is by use of a nephelometer. A nephelometer measures the particles in a solution by shining a light into the solution and reflecting the light onto a light detector. Each protein can be found by either emitting a different wavelength or by seeing the how much or the angle of light is reflected back[51]. For measuring CRP, there are two types of tests. The first being a normal CRP test which measures the amount of total CRP in a sample and the second being a hs-CRP (high sensitivity CRP) test which is more useful in determining the risk or cardiopulmonary disease[6].

While this is an effect method to measure CRP, it does not work well for LOC applications. The first of which is its size. This is a machine than can weigh anywhere up to a couple of hundred kilograms, so most of them are not portable. Second, all of the experiments are performed on the machine and not on a small chip. The blood goes into a vial and then into the machine for screening. This brings up the third drawback. Most of these blood analyzers up to, if not more than, 1 mL of blood. Most LOC devices use far less fluid than what a blood analyzer uses. Fourth, most of these analyzers only use plasma or serum. This means that the blood cells cannot be in the detection area. While all experiments require this, blood must be pre-separated before it can be placed in the analyzer. This usually involves centrifugation, hence the large volume required. Finally, time is always a factor. From blood extraction to results, the time elapsed could be as long as a few days, which does not take into consideration logging data and bookkeeping. Based on the type of machine, it can take anytime from a few minute to a couple of hours[4,51]. Even though it is a well-known system, it is not ideal for LOC applications based on the preceding.

Other large machines include liquid chromatography, for example, high

performance liquid chromatography (HPLC). Liquid chromatography allows a user to

determine what types of proteins are in a sample by mixing a sample with a solvent

and sending through a column with packing material that separates the proteins in

solution[52]. The detection of specific proteins is good, but there are also drawbacks to

this. Mostly, it can detect proteins, but not the concentration of certain proteins. Also,

all the samples are diluted before they are measured so an accurate measurement

cannot be found. Furthermore, one test run can last up to a day[52]. Because of these

reasons, any liquid chromatography experiments cannot be performed to detect and

measure CRP.

However, in terms of LOC experiments, there have been different platforms

that have been created in order to detect CRP. One platform uses immunoglobulin Y

(IgY) as a method to capture CRP in not only serum, but also in blood. The time taken

to perform this test has been reduced significantly to only a couple of minutes and has

a high detection range[53,54]. Other LOC applications use nanoparticles coated with

anti-CRP antibodies that would attach and allow for accurate detection[19,20]. These are

only a few examples on how far LOC has developed into a well-known method for

detecting CRP in whole blood.

However, while reviewing a few of these products, it is worth noting a few

limitations to them. First, some of the reagents can either be expensive in cost, as in

the case with gold nanoparticles[19], or expensive in time in which to engineer the

reagents[54]. In the case with IgY, there is a stark contrast with it compared to IgG.

IgG is a mammalian antibody that is used cooperatively with the immune system. IgY

is similar, but it is found in reptilian and avian (bird) blood. This means that is cannot

bind either well or at all to mammalian proteins or antigens[55]. Because of this, these

antibodies need time to be effectively engineered in order to make them more useful

for mammalian experiments. There is one other limitation to these experiments: the

type of fluid used in the experiments.

A number of LOC systems can either use serum or whole blood for analysis.

But this whole blood needs a certain anti-coagulant to be present before it is dropped

onto the chip, for example, with the ABX Micros CRP 200 by Horiba[56] and the CRP

analyzer by Labcompare[57]. The most common anti-coagulant is EDTA

(Ethylenediaminetetraacetic Acid). EDTA is commonly found in laboratories and is

mixed with blood as soon as the blood is extracted[56]. The need for an anti-coagulant

in experiment is that the blood does not curdle during experiments. Over time, due to

platelets and fibrinogen, blood can become more gel-like and solidify[58]. Anti-

coagulants slow down and occasionally stop this process in order to preserve blood.

This method is still great for laboratory work, but in field work, or a home setting,

where EDTA is not readily available, this can cause some problems. Because of this, a

better method for detecting CRP from a drop of blood directly from the body has to be

considered.

### 2.6.2. CRP Detection with ELISA

One of the most common methods in detecting CRP is what is known as an

enzyme-linked immunoassay (ELISA). In a basic ELISA, the antigens are attached to

a certain surface, in most cases, the bottom of a micro-titer plate. Afterward, a specific

antibody is attached to the antigen followed by a substrate to detect the antibodies

attached to the antigen. This is a simple method to be able to detect certain antigens[12].
A full detailed example will be discussed in the upcoming sections. For this part
however, it is essential to know the basics of an ELISA.

There are multiple types of ELISA that can be used for experiments. The first
type, explained above, is known as a direct ELISA. This ELISA is beneficial because
a certain antigen can be detected by the use of antibodies specifically designed to
attach to that antigen[59]. The second type is known as an indirect ELISA. This ELISA
is used to detect an antibody, rather than an antigen. To perform this experiment, a
known antigen is attached to the bottom of a well.  An unknown solution that may
possibly contain an antibody that can attach to that antigen is placed into the well.
From there, antibodies designed to attach to that antigen will attach.  This antibody is
what is known as a primary antibody.  Later, a secondary antibody is used to detect
that first antibody, in which the substrate can detect these secondary antibodies[59].
The third type is what is known as a sandwich ELISA.  This type can either be direct,
or indirect, but the main difference being that instead of an antigen binding to a well,
an antibody specific to that antigen binds to the well. Then a solution with many
antigens or proteins (blood serum) is sent though, allowing the antigens that are
specific to the antibody already attached to attach. A second, and sometimes a third is
used to detect that antigen again and also be used to activate the substrate once it
comes into contact[59]. A final type of ELISA is known as a competitive ELISA.
Unlike the first three examples, this type of ELISA uses competing proteins or
antibodies to either detect changes in detection, or to remove/narrow down possible

choices for unknown proteins or antibodies[59]. Below is a diagram highlighting the

main differences between the first three ELISA types.
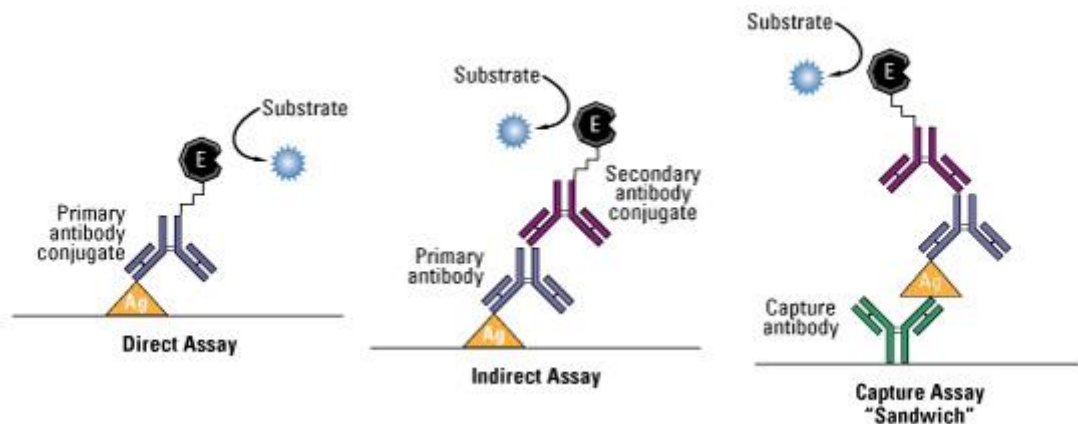


**Figure 2-3:** Diagrams highlighting the differences between a direct ELISA (left), an indirect ELISA (center) and a sandwich ELISA (right)[59]

Because of the fact that a certain antigen is being detected, it would be better to use a

sandwich ELISA in order to capture and detect CRP. Also, since this project is also

concerned about the concentration of CRP, it would also be helpful to use a

competition ELISA, where more than one concentration of CRP. With these two types

of ELISAs, it would be the most optimal method to detect and calculate the

concentration of CRP in blood. Previous lab projects have combined to obtain a

working microchip that envelops this concept[22-24]. Based on this, work from these

previous models will be brought together in order to create a more modern version of a

CRP detection chip that incorporates blood cell removal and ELISA detection.

### 2.7. Previous Lab Work

Over the past few years, there have been systems developed by the URI

microfluidics laboratory that used LOC technology in order to capture and detect CRP.

The first system was developed by Peng Li in order to detect CRP on a microchip.

This system implemented an ELISA detection system and was one of the first systems

to integrate sequential loading, where all the reagents were inside the chip and the reagents were fed through the detection area by way of a micropump[22]. This system accurately detected CRP in buffer. The second system was developed by Michael Godfrin in order to find a method of detecting CRP in blood serum with the removal of albumin and human IgG[23]. The third system was developed by Michael Franzblau as an updated version of the system developed by Peng Li with incorporated both sequential loading and CRP detection in blood. This system was also novel in that the detection was automated, rather than relying on human interaction. An example of this system is found in figure 2-4[24]. Pictures displaying this system is shown below in figure 2-4. Other projects developed include a hand-held CRP detector developed by John Jones[60] and an ongoing project in detection of CRP and other reagents on paper[61,62]. This project encompasses the first three projects mentioned and uses this to develop a better method to detect CRP. The reason for this is explained below.



**Figure 2-4:** Prototype of CRP detection POC device created by Michael Franzblau

### 2.8. Reasoning for a Modified CRP Lab-on-a-Chip System

In order to better develop this goal of detecting CRP in whole blood, it would be beneficial to incorporate the projects developed by Li, Godfrin and Franzblau into an updated and more reliable system. In order to see if there needs to be any improvements on these systems, a simple experiment combining the chip developed

by Franzblau[24], the protein removal developed by Godfrin[23] and the detection system developed by Li[22] to see if this experiment was feasible. Further explanations of each of the three experiments will be clarified in the next chapter.

In a brief synopsis of the experiments, the chips were first functionalized with BSA (bovine serum albumin) protein, glutaraldehyde and protein A[22]. This allowed the removal of the high abundance proteins and the detection of CRP. The chip was divided into three sections: a top section for removal of IgG, a middle section for removal of albumin and a bottom section for CRP detection[24]. Anti-albumin IgG in the middle and Anti-CRP IgG in the bottom were added and incubated for 5 minutes[22-24]. The chip was washed with PBS (phosphate buffer saline) and serum with CRP was added to the chip. It first incubated in the IgG removal area for 5 minutes, then in the albumin removal area for 5 minutes and finally in the detection area for 5 minutes. After a PBS wash, anti-CRP antibodies labeled with FIT-C were added and incubated for 5 minutes. After a PBST wash, PBS was added and detected using fiber optics[22-24]. A negative control with serum but no protein removal and a positive control with CRP in PBS buffer were also used in conjunction. This experiment was performed with 5 and 10 µg/mL concentration of CRP in serum (2.6 and 5.2 µg/mL concentration of CRP in blood)[48-50].
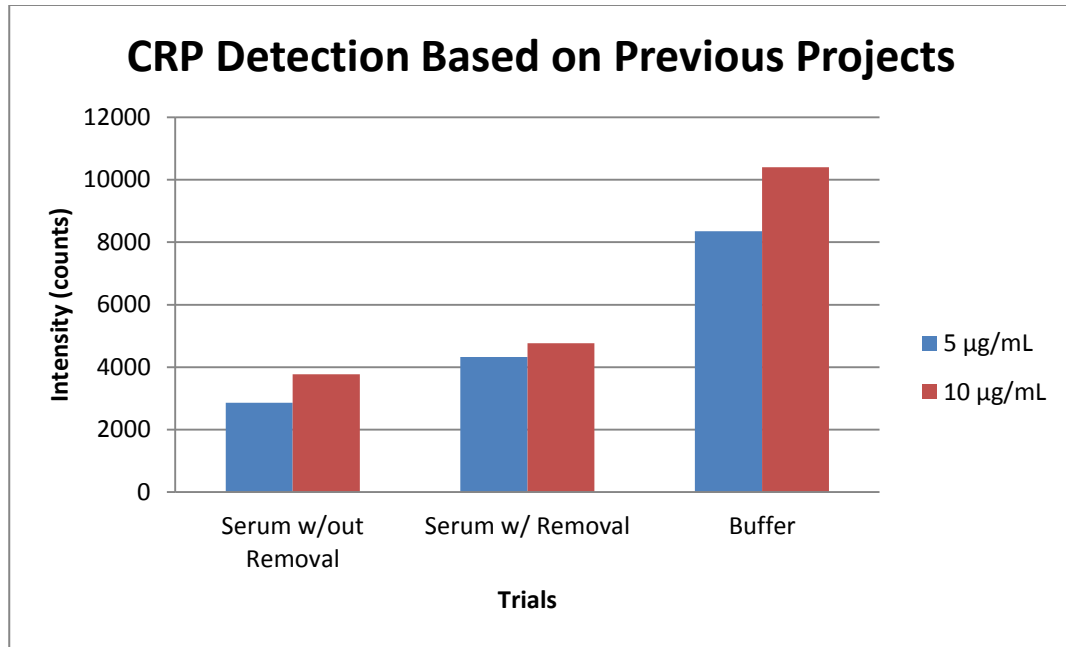
**Figure 2-5:** Comparison of CRP detection in previous projects

Figure 2-4 depicts the detection of CRP in serum using the previous methods designed. The center bars show the result given when the experiment was performed. The values of the signal are based on the intensity, or how much light was adsorbed by the fiber optics used in detection. To the left are the signal values for the same experiment with no removal of albumin and IgG. The signal of the tests are noticeably higher than that of the buffer, but in comparison to the signal of just CRP in buffer (right bars), it tells a completely different story. The values of the tests were half that of the buffer experiments. This relates that there was a noticeable presence of IgG and albumin in the detection area. The probable cause of this would be the incubation of the removal of IgG and albumin. Five minutes for the removal of the two most abundant proteins in blood is not an ideal amount of time if there is still a large amount of antibodies. Also, there was not a significant change in signal as concentration increased in the experiment as compared to CRP in buffer. One other thing is that even though it was implied that blood could be used in the experiment,

there were not enough tests in order to see if this was functional.  Because of these reasons, this method of high abundant protein removal has to be dealt with.

In order to go about fixing this system, each of the aspects from getting from separating blood cells to detection of CRP has to be looked at.  This includes seeing how well blood cells get filtered out of the chip to seeing as to what is the most efficient method of IgG and albumin removal.  This project will take a look as to how well IgG attaches to protein A and how it can be bettered.  This will help in the removal of albumin from serum as well.  With these aspects, a newer more efficient method of removing CRP from whole blood can be obtained.

**CHAPTER 3**

METHODOLOGY

In order to explain the process behind this project, this section will be broken up into three different sections. The first section will be about the process of making the chips. The second section focuses on blood separation. The third section focuses on the removal of high abundance proteins in order to detect C – reactive protein.

### 3.1. Microchip Fabrication

One of the main issues is deciding what type of chip to work with. Different chips can be constructed by using different materials. Based on the material being used, these chips can be divided into two categories. This is based on how fluid travels through the chip. The first category is known as passive flow chips. These chips use capillary forces to move a liquid down a channel without the need of an external force to move the chip. The second type is known as active flow chips. These chips use an external source, such as a pump, in order to move the liquid towards its potential area[63-65]. The upcoming paragraphs will examine both processes and determine which process would be better suited.

The first process mentioned is passive flow. This method does not require the use of a pump, but rather forces in the material, either through diffusion or capillary forces, drive the fluid to a destination[65]. In most cases, this is performed by wetting an area for the fluid to go through with a hydrophilic solution, while wetting the boundary in a hydrophobic solution[64]. The most common materials that are used to

make the chips are any material that can easily draw water, including paper, twine and fabric[64]. The advantages to using a passive system are that the materials needed to make the chip are inexpensive and the design and printing of the chips can easily be performed on the computer. The disadvantages to using passive systems are that there is no optimal fluid control since there is no external source other than the chip to guide and control the flow of the fluid.

The other process mentioned is active flow. This method requires the use of an external source for the fluid to flow. These sources include pumps, electrical impulses and magnetic impulses[65]. Basically, if an external force is needed to drive the fluid, then it is an active flow system. Unlike a passive flow system, where the material acts as channel for the fluid to flow, in active flow, the channels are formed inside of the chip, by way of a mold[22-24]. This allows the fluid to easily flow inside the chip and keep intact and not leave much of a streak or residue behind. These chips are usually made out of glass, plastic or nay polymer. The advantages for using active flow is that the user has full control over the liquid in the channel, they are easy to make and fluid can be easily placed in and taken out of the chip. The disadvantages are that it is more costly to make, based on the making of one mold, and numerous amounts of polymer or glass, the process to create the chips usually takes longer than that of a passive system and external equipment is needed to perform the experiments.

Based on the reasons listed above, it was more beneficial to go with an active system than a passive system. The reason for this being that the fluid is easier to control in an active system. The fluid is allowed to stay in one place, which would be ideal for allowing more CRP to be detected. Also, with a pump or a syringe, the user is

allowed to move the fluid to all the different parts of the channel. With a passive

system, the best way to move liquid would be to add more liquid to the chip. In the

case of adding blood to the paper, some aspects of the experiments include the

removal of blood cells and high abundance proteins. The issue is that with using a

passive system, if the plasma is in constant flow, then it would be a lot more difficult

to contain it in order to remove all of the high abundance proteins. This will be

explained in full detail later on in the removal of high abundance protein section.

Because of the ability to better control the fluid in the chip, the method of active flow

to detect CRP will be investigated.

### 3.1.1. Mold Creation

The first part on creating the chip is creating the mold. The mold acts as a

template in order to create the microchannels. Each mold will have an etched side,

where indents will be formed in order to create the channels that the substrate will be

set upon. For this project, the molds were made based on two methods: an older

method using silicon wafers and SU-8 photoresists via soft lithography developed by

Peng Li[22] and a newer method by using aluminum molds via hard lithography

developed by Michael Franzblau[24].

### 3.1.2. Silicon Molds

To prepare the molds using the first method, the silicon wafers (SI-Tech, Inc.,

Topsfield, MA) have to be pre-treated. This is performed by placing the wafers in a

piranha solution. This removes any debris and organic material on the chip. This

solution consists of a 50/50 mixture of hydrogen peroxide ($H_2O_2$) (Ricca Chemical

Company, Arlington, TX) and sulfuric acid ($H_2SO_4$) (Acros Organics, Geel, Belgium)

and is an oxidizer against organic material. These wafers are placed in the solution on an Orbit Shaker (Lab-Line Instruments, Melrose Park, IL) for about 15 minutes or until no bubbles are present on the wafer. The wafer is then soaked in distilled water three times for five minutes per soak. The wafer is then dried using compressed nitrogen and stored until adhesion of the photo resist.

Once the wafers have been cleaned and dried, the photo resist is then applied to the wafer. The wafer is first cleaned with compressed nitrogen and placed on the spin coater (Laurell Technologies, Corp., North Wales, PA). $2 - 3$ mL of AP300 adhesion promoter (Silicon Resources, Inc., Chandler, AZ) is added to the wafer. The wafer is spun for 25 seconds at 300 rpm and then for 30 seconds at 3000 RPM once to spread the resist and twice to dry it. Nitrogen gas is blown gently onto the wafer to dry the wafer. Then, SU-8 2050 photo resist (MicroChem Corp., Newton, MA) is added to the center of the wafer. The wafer is spun a second time first at 300 RPM for 45 seconds and then at 600 RPM for 50 seconds. The wafer is removed and placed on a leveling tray, resist side up and allowed to level off for 1 hour. Finally, the wafer is placed on a hot plate (HS40A, Torrey Pines Scientific Inc., Carlsbad, CA) in order for the photo resist to harden. The baking consists of a three part sequence. First, the hot plate must be at 50°C before the wafer can be on the hot plate. Once the wafer is on, the temperature will rise from 50°C to 95°C for 23 minutes at a rate of 2°C/min. At 95°C, the wafer will continue baking for 3 hours. Finally, the temperature is lowered at a rate of 1°C/min until the plate is at room temperature. These wafers are then stored until a pattern for them will be etched.

The next step is to create a pattern to be placed on the chip. This is done by using Adobe Acrobat. A sheet is made with four black boxes, with white etchings that relate to the dimensions of each wafer the channels are drawn using white lines, along with openings for the fluid to enter and a detection area for the immunoassays. Each document can hold up to four stencils, or photo masks.  Examples of the photomasks used can be found in the Appendix (A.1 & A.2).  Once the making of the photo mask is complete, the document is saved and sent via e-mail to CAD/Art Services, Inc. in Bandon, OR, which would print the masks on transparent plastic to be used for etching, with the clear sides being the areas where the channels will be etched.  These examples are shown on the below in figure 3-1.
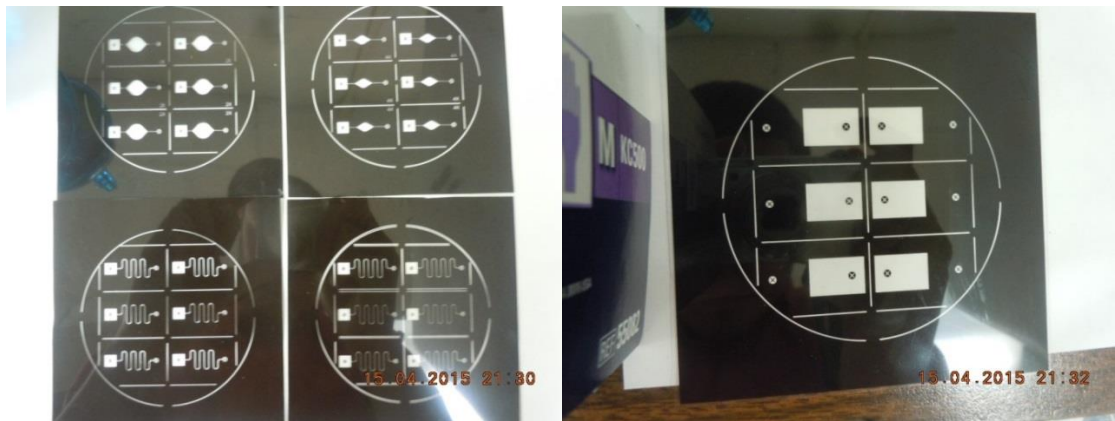


**Figure 3-1:** Transparent masks for silicon wafer molds for the channel side (left) and filter holder (right)

The last step will be to etch the pattern on the wafer. To do this, the photo mask is placed over the resist, making sure that there is no gap in between them. They are then placed in a UV-light source (i-line, Karl Suss MJB-3, Suss Microtech, Garching, Germany), where UV light at 365 nm wavelength will harden the exposed photo resist, while the covered sections will remain untouched. This exposure will last approximately 6 minutes for resist thickness of 400 μm. This does not include two 1

minute rest times in between. In total, for a 400 µm resist thickness, there is a 2.5

minute exposure, followed by a 1 minute break, then a 2 minute exposure, a 1 minute

break and finally a 2.5 minute exposure. The wafer is then placed on a hotplate at

45°C and then ramped up to 85°C at a rate of 2°C/min. It is then baked at 85°C for 40

minutes, before coming back down to room temperature at 1°C/min. Finally, the

molds have to be developed. For this, three solutions have to be used. First, the wafer

is placed in a series of two SU-8 developers (MicroChem Corp., Newton, MA), on an

Orbit Shaker and shaken until the non-exposed photo resist comes off (approx. 10-20

minutes).  Finally the wafer is placed in a container of isopropyl alcohol (Fisher

Scientific, Fair Lawn, NJ) for 1 minute and then blown dry. The last step would be to

bake the chip at 150°C for 30 minutes, with a 1°C/min heat up and cool down before

and after[22].  A full protocol can be found in appendix A.3.  Figure 3-2 shows the

process and figure 3-3 shows the final molds.
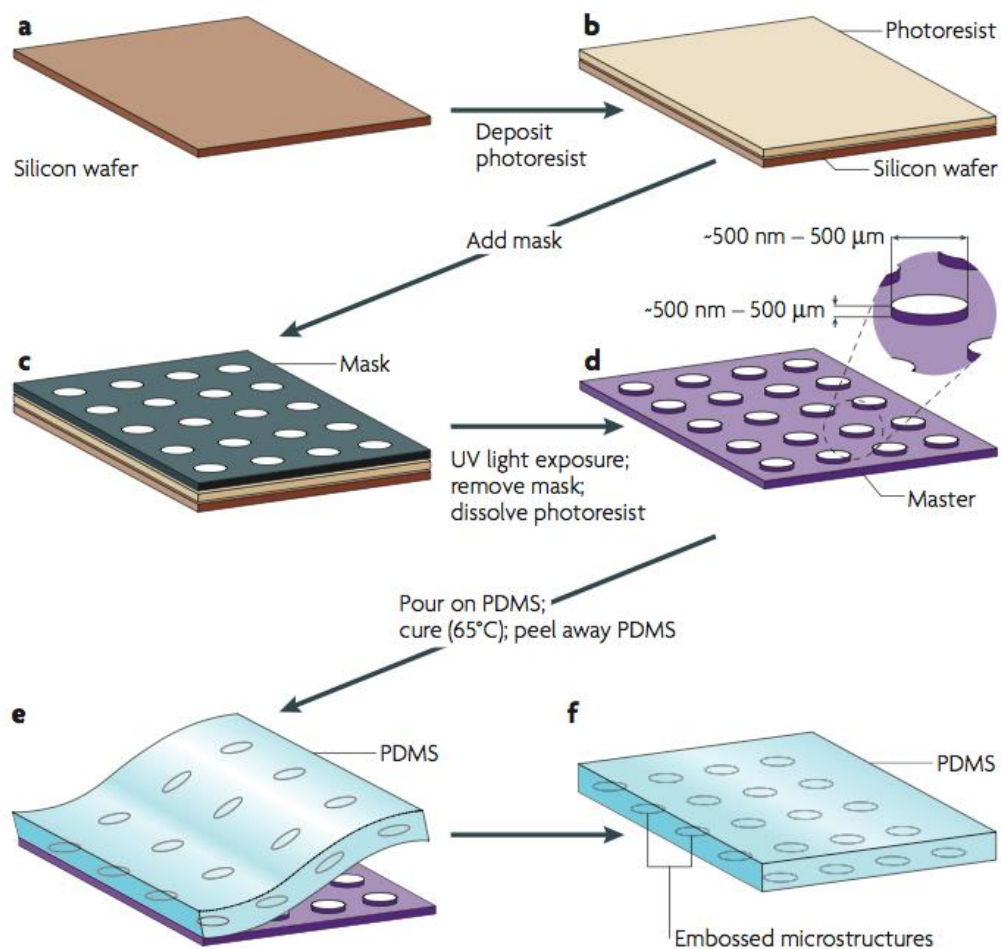
**Figure 3-2:** Example of microchip fabrication using soft litography[66]



**Figure 3-3:** Completed silicon wafer molds used for LOC chip production for multi-fluid assays (left) and single fluid assays (right)

Even though this is a known and good way to create the molds, there are

drawbacks to this method. The first drawback is the UV light. The light source used in

the experiment is very broad and dim when etching. This is problematic because the dimensions of the channels will be larger than what they should be and due to the lack of intensity, not too high. In the case of more elaborate channels, where this light source would not create better channels, the wafers and masks had to be sent to the laboratories at the Massachusetts Institute of Technology in Cambridge, where a better, more direct light source was used.  The second drawback is the repeated use of the wafers. On average, the wafers are used about a dozen times until they either break due to the heat and negligence, the photoresists peels off or the wafers become warped. The third drawback is the various chemicals being used, which are known to be not only toxic, but can cause skin corrosion. The fourth drawback is that one of the necessary components, the AP300, was discontinued.  The final drawback was that from designing the mask to finishing the molds, the process took approximately between 2 to 3 weeks to complete.  Because of these issues, a more ethical procedure in order to create better molds had to be established.

### 3.1.3.   Aluminum and Polymer Molds

In this method of mold fabrication, the channels were all etched out on a small block of either aluminum or a polymer material. The first step in creating the molds was to create a design by use of a CAD program (in this case, SolidWorks was used). The finished program was then translated into a programming language via MasterCam, which was then relayed to a computer numerical control (CNC) milling machine where the mold was carved out of either aluminum or a polyetherimide known as Ultem, which was developed by SABIC. The end result revealed a durable,

etched block that consisted of the etched channels that would be implemented in the chips[24].

There were a few things to look out for when creating these molds. First was that the molds had to have raised structures which indicated where the channels were supposed to be. Second, the molds had to be designed within the parameters of how the milling machine could create them. The bits that were used to sculpt the channels were McMaster-Carr end mills (part# 8832A008) that had a diameter of 203 μm and a depth cut of 350 μm. Because of this, all the channel heights hat to be at the most 300 μm and the thickness between any two raised structures to 205 μm, in order to stay within the parameters of the device[24].

However, there are many advantages to using aluminum molds as compared to silicon molds. One advantage is that the time which the entire process is performed is cut by, on average, a week. One of the reasons why the silicon wafers take longer is that the photo masks take up to a week or two to be fully completed. Without that step and the fact that the chip designs are only converted into code, it takes less time to make these molds. Second, these molds are more durable than the previous silicon ones. With silicon molds, there is always the possibility that the molds can shatter and warp, the photo resists can break off and the molds could easily get lost. This means that a new mold is usually created after only a couple of months. With aluminum and polymer molds, on the other hand, there is no need to worry about any of this because the molds are usually between 0.25" and 1" thick. And since the raised channel structures are part of the chip, there is no fear in that these structures would break off from the mold. Finally, unlike the previous molds, these molds can withstand higher

temperatures. With silicon molds, there is always the chance that warping can happen on the molds due to heat. Because of this, the chips have to be cooked at 95°C in order to prevent this. Aluminum molds are more thermal resistant, especially since they are thicker, so they can withstand temperatures greater than 150°C[24]. Because of these reasons, it is safe to say that these aluminum molds are an improvement over the previous silicon molds.



**Figure 3-4:** Aluminum molds used for LOC chip production for single fluid assays (left) and multi-fluid assays (right)
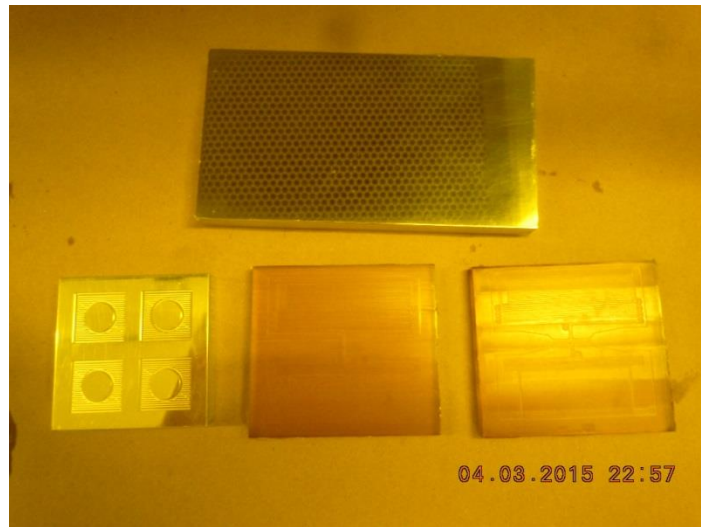


**Figure 3-5:** Aluminum and polymer molds used for LOC chip production for multi-fluid assays. Clockwise from top: bottom of chip, channel sides (2), blood filter holder

### 3.1.4. Fabrication of the Chips

Regardless as to how the molds were created, the method of making the chips remained constant. There are many substrates in which chips can be made, based on the different properties. Etching chips from glass is a common method that is used because glass can be durable, is transparent and does not react when in the proximity of biological substances[34]. However, glass is brittle and can shatter, is expensive to obtain and etching usually requires hydrofluoric acid, which is extremely corrosive. The best method would be to use polymers as a chip substrate because they are usually nontoxic, inexpensive, can withstand high temperatures and can be easily liquefied and solidified. One of the best known polymers is polydimethylsiloxane (PDMS). PDMS is a transparent silicon polymer that is usually inert under chemical and thermal changes. It is relatively inexpensive to use PDMS and the fact that it starts as a liquid with a high viscosity and low surface tension means that the polymer can flow over the mold in order to make sure all the nooks on the mold are covered.

To make the actual chips a mixture of liquid PDMS (Slygard 184, Dow Corning, Midland, MI) and curing agent are added in a 8:1 − 10:1 PDMS : Curing Agent ratio. Depending on the size of the mold and the thickness of the final chip (between 0.4 and 0.6 mm thickness), the mass of the PDMS mixture varies between 30 and 50 grams. This was found by taking the approximate volume (cross-sectional area X slab height for one side) and multiplying it by the PDMS density (0.965 g/mL) and doubling it. The reason for this mass is that this is the total mass for the top side (channel side) and bottom side (flat side). The liquid mixture is poured onto each mold in equal proportions. During the mixing and the pouring of the PDMS, air bubbles

form in the mixture. It is essential that these bubbles are removed as that they cause irregularities in the channel formation and cause interference in the detection signal. For this, the molds are placed in a vacuum chamber (Sybron – Nalge, Braunschweig, Germany) for 1 hour to remove all bubbles from the molds. This process can be performed quicker by leaving it in the chamber for 15 minutes until all the bubbles reach the surface and then leaving it outside the chamber for another 15 minutes. Once all the bubbles are gone from the mold, the mold is placed on a hot plate at 95°C to bake for 2 hours.

After the PDMS has hardened, they are removed from their respective molds and trimmed of excess PDMS using a 4" razor blade scrapper (McMaster-Carr catalog #26765A31). Holes are punched at the channel openings using a 1.5 mm biopsy punch (Miltex catalog #33-31A) to allow fluid into the chip and tubing to latch into the holes created. The next step will be to bind both sides of the chip together. This will be performed by using the plasma asher (Femto, Diener Electronic, Reading, PA). To use the plasma asher, both sides of the chip are placed on a tray that is then inserted the asher. The chips are inserted mold side up because they tend to be the clean side free from air bubbles and in the case of the channel mold, it is where the channels are located. The process consists of four steps. First, the chips are inserted, the window between the chips and the outside sealed and air is pumped out the chamber. This process takes about 3 minutes, in order to make sure no air is in the chamber. Second, oxygen gas is pumped into the chamber. This step takes approximately one minute. Third, the chamber is activated, which plasmas a thin layer of oxygen onto the surface of the chip. This process takes 3 minutes to perform. Finally, once the 3 minutes are

up, the gas and pump are turned off and air is added to re-pressurize the chamber. The

chips are then taken out and are bonded together with the two sides facing up being in

contact. The chips are pressed together and are placed on a hot plate at 95°C to bake

for 1 hour in order to facilitate binding. If the chip requires a paper pad to be used as a

waste area, the paper is fitted onto the channel chip with the flat chip brought in to seal

them. If a filter is needed, the top of the chip is placed in the asher with the filter

holder and the process continues. The filter is placed on top of the holder, with the

smooth side facing up, then the chip is pressed onto the holder and placed on the hot

plate[24]. The full protocol can be found in appendix A.3. Since there are multiple

chip designs that will be implemented, each upcoming section will explain the

geometries of each chip. Figure 3-6 below highlights the process of making the chips

and 3-7 on the next page shows the equipment used in the creation of the chips.
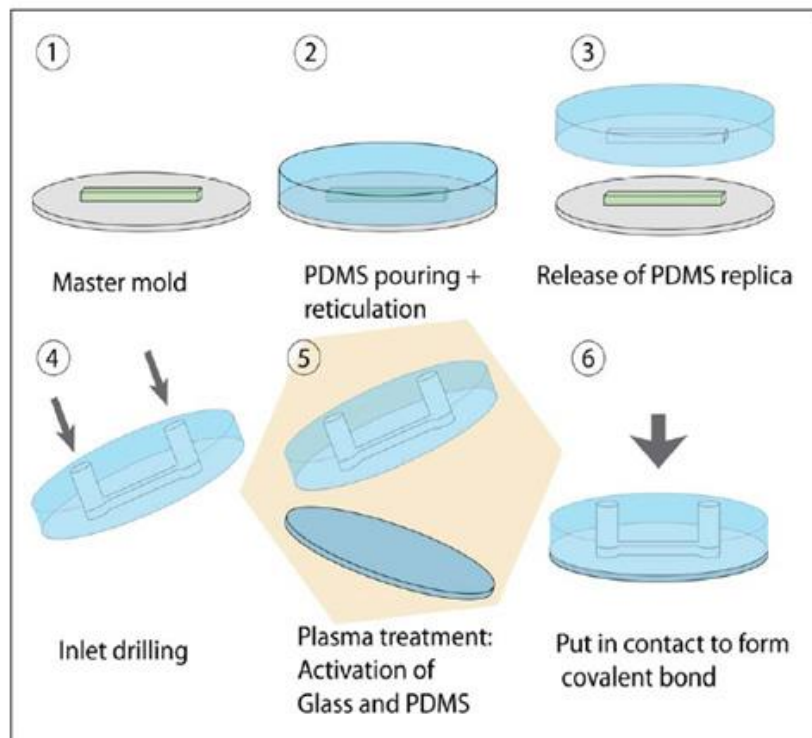


**Figure 3-6:** Sample assembly of PDMS Chips[65]

**Figure 3-7:** Equipment used to create PDMS chips including vacuum chamber (top left), hot plate (top right), plasma asher (bottom left) and vacuum oven (bottom right)

### 3.2. Blood Separation

In the previous chapter, it was determined that because of the cost,

functionality and easier integration, it was determined that the preferred method of

blood separation would be to use filter paper and membranes. However, since there is

a large selection of filter papers, there has to be a way to sort and narrow down the

search for a proper filter paper. There are numerous tests that one can perform that

include measuring the amount of filtrate and seeing how well they keep blood cells

out.  These methods will be implemented in determining a suitable filter for blood cell separation.

One of the companies that produce blood filtration membranes is the Pall Corporation.  Pall has a line of membranes known as the Vivid Plasma and the Cytosep Membranes[68,69].  These are two types of membranes that not only are able to separate a large amount of plasma, but also show no sign of blood cells rupturing or escaping the membrane.  This is based on the characteristics of the membrane.  The membrane consists of two different sides: a rough upper side and a smooth lower side.  Blood is placed upon the upper side and the liquid migrates to the smoother side.  There are pores in the paper that allow the blood to flow through, but the roughness of the sides depends on the size of the pores.  The rough side has larger pores, making it ideal for cells to be captured, while the smooth side has pores small enough for the plasma to seep through, while keeping the cells inside of the membrane[68,69].

It is a highly hydrophilic membrane and unlike some papers which are fibrous, or made from paper, these membranes are made from a polymer known as polysulfone in the case of the Vivid Plasma Membrane[68] and of a blend of natural and synthetic fibers in the case of the Cytosep Membrane[69].  Because of this, it actually can withstand higher temperatures and pressure forces exerted onto it.  Plus, since it is hydrophilic, the blood can travel readily throughout and stay in the membrane until an external pressure is applied.  This allows for an adequate amount of plasma to remain on the filter until it is needed to be exerted onto the chip and the amount of plasma can be high enough to be necessary without fear that blood cells and hemoglobin can enter the chip[68,69].

Since these are a few examples of blood filters, it would be essential to test as to how well each one will fair. There are two main tests that will be used to determine how effective these filters are. The first will be a test based the amount of filtrate has been filtered out. This will be performed by just directly measuring the filtrate volume by using a syringe pump to extract the filtrate until the filter does not allow any more filtrate to pass through. The second test will be to see how well the filters separate the blood cells from the plasma. This can be performed with multiple tests including blood staining and microscopy. An additional test can be performed to see if either plasma or serum is obtained from the filtrate. From these tests, a suitable filter will be found that can be used to separate blood cells from whole blood.

### 3.2.1. Filtrate Volume Tests

The first test involves the volume of the filtrate. The main filtrate that will be going through the chip should be plasma. As stated previously, plasma accounts for approximately 55% of the total blood volume. This means that for every 100 µL of blood, there will be approximately 55 µL of plasma to go along with it. In experiments that require only a small amount of plasma, it is not a big issue that 100 µL of blood is used. The issue occurs when for example 15 µL of blood is filtered to yield approximately 8 µL pf plasma. The reasoning for this that the filter can adsorb a good amount of plasma and that red and white blood cells, known as the filter cake in this experiment, can ultimately block off the channel so that no more filtrate can be pulled into the channel. With this knowledge, it is important to find out if these filters are even functional to begin with.

Tests will be performed with the filter to make sure that these filters are functional. In each experiment, a syringe will be connected via tubing to a PDMS slab. The tubing will be marked at 1 μL intervals, which will be used to measure the filtrate volume. A small piece of filter paper will be placed in the slab, where a drop of blood will be placed. The filter papers being used in these experiments were the Vivid Plasma Membranes GX and GR from the Pall Corporation (Ann Arbor, MI). According to Pall, these filter papers are some of the top of the line papers because of their double-sided nature. The top side has rough openings that can entrap blood cells, while the bottom has a smooth, yet finely holed surface that allows plasma to escape. By use of active transport, fluid motion with the aid of an outside influence, in this case, a syringe pump, the blood would flow through the filter and into the tubing. The remaining plasma will then be collected, measured and analyzed based on techniques that are used to characterize and determine the quality of the plasma.

The syringe pump that is used is from the company SyringePump.com from New Era Pump Systems. The model number of this pump is NE-1010. A picture of this pump is shown on the next page as well as the setup of the experiment in figure 3-8. The reason this experiment focuses on active transport is because it is the easiest method to collect and isolate blood filtrate. The filtrate is collected inside the tubing due to the movement of the syringe and the vacuum pressure created inside of the tubing. This forces the plasma through the membrane into the tubing at a steady rate. The one issue concerning this would be the filter cake that builds up. The cake is defined as the items that did not effectively flow through the filter (RBC, WBC, Platelets). This causes a slowdown in filtrate extraction and a buildup in vacuum

pressure. In addition to this, membrane rupturing and hemolysis can occur. This causes a problem mainly in the antibody-antigen assay, where hemoglobin can interfere with the interactions between antigen and antibody.



**Figure 3-8:** Apparatus of syringe pump (left) and PDMS chip (right) used for plasma extraction

During each experiment, the main focus would be not only how much plasma can be extracted, but also if 5-7 µL can be extracted from a blood volume between 25 and 45 µL. This is important because the reservoir for the antigen has a volume of 5-7 µL and through experiments, it will be shown that it is the ideal volume for the amount of plasma that can be collected, where the risk of hemolysis, or rupturing of the blood cells is minimal. These values will be graphed and compared with each other in order to determine which filter is the most ideal filter to use in the system.

### 3.2.2. Blood Cell Analysis

The second factor with blood filtration involves the clarity of the filters. For this segment, two aspects are taken into account. The first is whether or not red blood cells are filtered from the plasma. This is important because in the immunoassay, only the proteins in blood are important in detection. Since RBC's have an average diameter of 6-8 microns[18,44], they can interfere with the proteins while performing the

immunoassay.  Because there cannot be any trace of blood cells in the channels as the filtrate is added to the serum, it is necessary to see if cells were able to penetrate through the membrane and into the channel.  In order to perform this, microscopy has to be used.  There are two different methods that can be utilized in order to perform the microscopy.  One method is by use of what is known as a Giemsa Blood Staining. In this method Giemsa Stain is applied to a dried sample and by using microscopy, the different specimens can be identified based on the color given.  One example performed in the lab used this testing to find the effectiveness of the Pall Membranes.
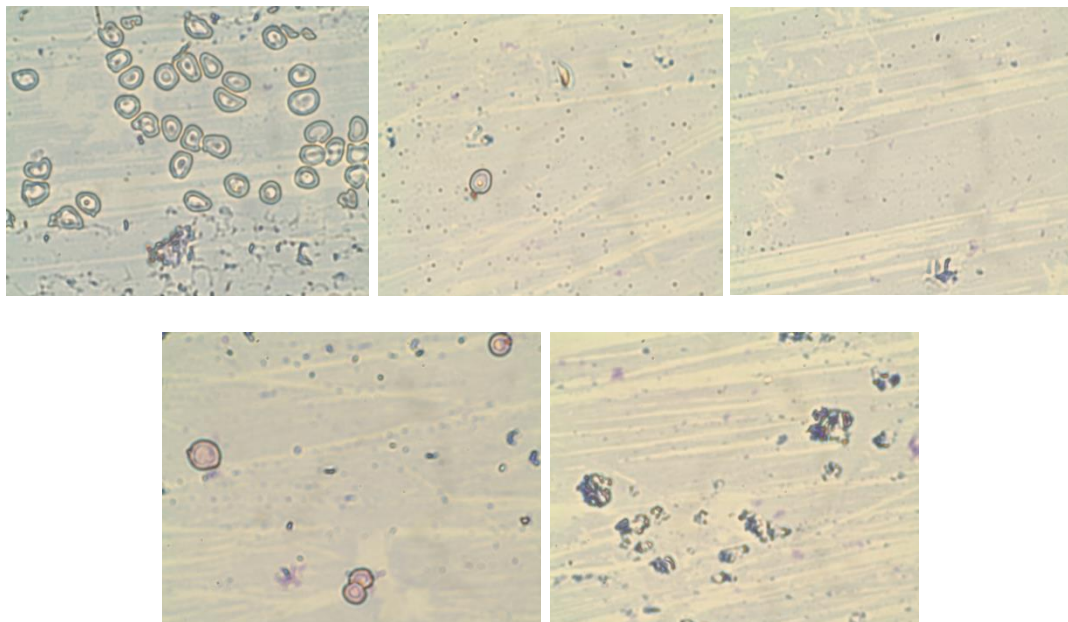


**Figure 3-9:** Giemsa Staining of filtrate from different membranes (top, left to right: whole blood, filtrate from Vivid Plasma Membrane GX, filtrate from Vivid Plasma Membrane GR; bottom, left to right, filtrate from Cytosep 63, filtrate from Cytosep 62)[70]

As shown in figure 3-9 above, this test was performed on both the Vivid Plasma and the Cytosep Membranes.  Both sets of the Vivid Plasma Membranes performed well and did not filter through any blood cells.  The Cytosep Membranes on the other hand not only allowed more blood cells to seep through, but some of the filter paper broke apart in the filtrate.  From these tests, it was shown that the Vivid

Plasma Membranes performed better than the Cytosep Membranes[70]. However, one of the main issues with using Giemsa stain is that it is very oxidative, meaning that if not carefully performed, a stock solution of Giemsa Stain can be ruined with a small amount of oxygen in air and water. Because of this, an alternative method of direct microscopy of filtrate samples will be performed for this project. This involves creating chips that hold the filtrate and place them directly under a microscope. Keep in mind that because of these previous results with the Giemsa staining; only the Vivid Plasma Membranes will be in use for this experiment.

Another factor is based on whether or not hemolysis has occurred. Hemolysis is the process where RBC membranes rupture and hemoglobin is released in the plasma. This can occur either in the filtration process due to a bad filter or a high pressure caused by the syringe pump, or by using old blood. Normally, if there is redness in the filtrate, it is either caused by RBC's or hemolysis. Normally, it can be seen with the naked eye whether or not it occurs, but it is better to check using a microscope if there is noticeable redness. This can involve seeing whether or not blood cells remain in a "reddened" filtrate. This would show whether or not hemolysis has occurred in the blood and the amount can tell whether it was the filter or that old blood was used in the system.

To perform the microscopy aspect of this project, slides will be prepared by using a PDMS chip with 5 microchannels. One microchannel will contain whole blood as a control and to see the relative RBC size. The other microchannels will contain a filtrate based on a specific type of filter paper, the dimensions and area of it and because a syringe pump is used to withdraw the plasma, the rate of extraction. Each

blood sample will be of the same volume based on the average drop size and because the drop needs to soak into the filter paper completely, a common time between dropping the blood onto the paper and the start of the pump. This method will help determine what the ideal method is for blood filtration. During each experiment, various observations will be noted, including total plasma volume, plasma color upon initial inspection, and volume before possible hemolysis. These observations will play a vital role in characterizing the filters and help determining the aforementioned qualities if the filter paper. Microscopy will be performed on a Leica DMLB microscope (Buffalo Grove, IL). This microscope has the ability to vary in resolution size, from 5X to 20X and is able to take a snapshot of the channels by way of a mounted camera connected to the computer. With this camera, pictures can be taken of the microchip to be used for further processing.
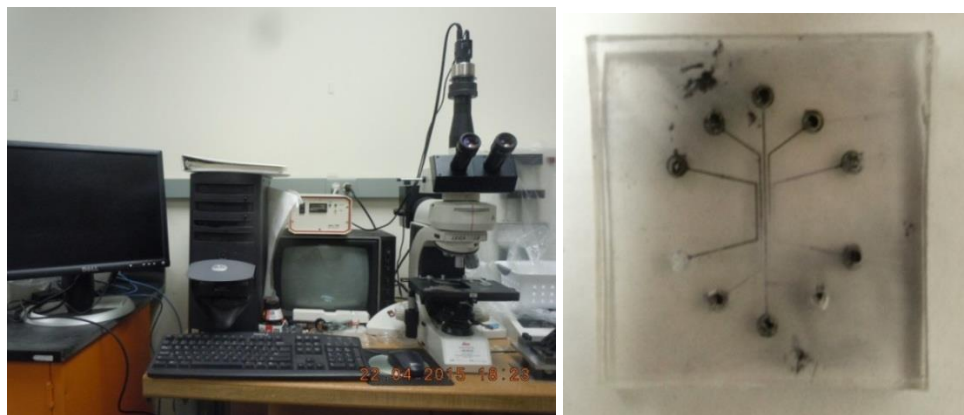


**Figure 3-10:** Microscope and chip used for blood cell analysis

### 3.2.3. Clotting Factors: Plasma vs. Serum

One other factor that has to be taken into account is whether or not the filtrate is plasma or serum. The difference between these two is based on fibrinogen, or clotting proteins present in the filtrate. Serum contains no amounts of fibrinogen, which means that even if there is a small amount of fibrinogen in the filtrate, it will

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still be identified as plasma. Since there is not an ideal method to locate fibrinogen under a microscope, the best method to characterize the filtrate will be to precipitate the fibrinogen out of solution. This is performed by coagulating the fibrinogen, which is soluble, into fibrin, which is not soluble. There are different reagents that are able to perform this, such as thrombin and certain aqueous salts[71]. This project however will focus on using ethanol for fibrinogen aggregation[71]. By adding ethanol into plasma, fibrin will precipitate out of solution, leaving only serum in liquid form. If this experiment does not perform, then it will be clear that fibrinogen is left behind in the filter and serum is yield in the filtrate. However, if even only a small amount of fibrin is recovered in the filtrate, then the only possible way to classify it is to say that these filters will filter out plasma. If this happens, then further research will have to be conducted as to how serum can be extracted using these filter papers.

### 3.2.4.   Design of a Microchip that Incorporates Blood Filtration

Ultimately, this new chip design will incorporate a one-touch system, where the user will use a drop of blood onto the filter and the micropump will draw the plasma into the chip and perform the immunoassay with only the touch of a button. The filter will be embedded inside the microchip to allow for filtration without the need for an external source. Because filters come in all shapes and have different microstructures and properties, they have to be taken into account. From all of this, a microchip that enables both sequential loading and blood microfiltration will be used to perform immunoassays in order to detect various diseases found in the blood.

This chip will encompass the idea of sequential loading, where a reagent is transported into a detection area one at a time by use of a micropump. Along the way,

different projects will encompass the focus of the main project. These projects include finding a suitable method for microfiltration, finding out how much plasma or serum can be extracted by using this method of filtration and checking the quality of the plasma based on the number of blood cells filtered out. From these results, a suitable design for the sequential loading microchip that encompasses microfiltration will be designed.

However, in order to make sure that blood filtration and sequential loading can be easily combined into one chip, a prototype filtering chip has to be introduced. This chip would only involve the direct separation of plasma from blood. This is just to make sure that not only the filter can be embedded inside the microchip, but the plasma can easily flow into the microchannel without the risk of hemolysis or a sudden increase in the vacuum pressure. In order to perform this, a PDMS slab will be used to hold the filter in place. This slab will have an indent, which will act as a mechanism to hold the filter in place. To make sure that this procedure works each time, the filter has to be cut at precisely the exact dimensions to fit in the chamber properly. If the filter is too small, the blood would run over the sides. However, if the filter is too big, the PDMS slabs will not stick together properly. In order to figure out the appropriate size of the filter paper, tests will be performed by dropping different amounts of blood onto different filter paper sizes. This will be based on the design specifications of the filter paper, which was given by the manufacturer. By knowing this and that a drop of blood should be in the volume range of $40 - 50$ µL, the appropriate size filter will be found based on these characteristics.

From these specifications, moldings of the filter will be made in order to place it between two slabs. Ultimately, the new chip will have multiple layers, instead of the two layers that were on it previously. Before the new design, the chip had only two layers: a channel layer and a blank slab. This new design will include one additional layer: to hold the filter in place. In total, the new chip will comprise of three layers, which is shown below.

### 3.3. CRP Detection and HAP Removal

#### 3.3.1. Surface Functionalization

Surface functionalization or modification is when a surface has to be coated in order to change its properties[22]. This can mean by changing the surface of the channels by either making them either more hydrophilic or hydrophobic, or by allowing proteins or other antigens to bind more easily to the surface. For these experiments, because of the surface structure and the hydrophobicity of the PDMS, the antibodies used in the ELISA cannot bind to the surface of the channels well. However, there are certain known proteins that can readily bind to IgG antibodies. One certain protein is known as Protein A, which has a high affinity to IgG antibodies[72]. However, this protein cannot be stand alone in the channel. The reason for this being that even though the antibody would still bind to the protein, the protein could bind to other impurities in the chip or that the antibody would bind to the channel in a way that is not beneficial to the experiment. This type of binding is called non-specific binding. To circumvent this, a blocking agent has to be placed on the surface beforehand. One known protein that is able to do this is bovine serum albumin (BSA). BSA is a non-reactive protein that can be used in order to coat the surface

before protein A can be inserted into the chip. However, since BSA does not react and proteins usually do not bind with each other, there has to be a linker that can be able to connect both proteins. One known linker is glutaraldehyde ($C_5H_8O_2$). Glutaraldehyde is a 5 carbon single chain with two aldehyde (one carbon attached a hydrogen and a double-bonded oxygen). With these two aldehyde groups, it is used as an amine-reactive crosslinker, in which it can easily link two proteins together. This would enable a tight link between the blocking agent BSA and the capturing agent protein A.

### 3.3.2. Method of Surface Functionalization

To functionalize the PDMS, each chip is first checked to make sure that no leaks occur in the chip due to improper sealing. PBS (phosphate buffer saline) (Fisher Scientific) is inserted into the channel to clean out the channels and to make sure leaking does not occur. Once the PBS is removed from the channel, a mixture of BSA (Sigma Aldrich, St. Louis, MO) and PBS (1.5 mg/mL) is inserted in the chip. The chips incubate at room temperature for 4 hours in order for the BSA to attach to the channel. The BSA is removed from the chip and PBS is inserted and removed to wash the chips and remove excess BSA. The channels are aerated in order to remove excess liquid. A mixture of glutaraldehyde (Sigma Aldrich) and water (0.4% v/v) is inserted into the chip. The chip is then incubated at room temperature for 1 hour. The glutaraldehyde is removed and the chip is washed with PBS and aerated a second time. Protein A (Sigma Aldrich) in PBS (50 μg/mL) is added in the chip and incubated at room temperature for 1 hour. The protein A is removed and the chip is washed with PBS and aerated a third time. A storage buffer consisting of 10 mM Tris in distilled water, 0.05% BSA (w/v), 0.05% Proclin 300 (v/v) and 5% Glycerol (v/v) is inserted

into the chip and the chip is stored at 4°C until the chip is ready to be used[22-24].

Below is the finished schematic of the functionalization process. The protocol for this

procedure can be found in appendix A.4. A completed functionalization diagram is

found on the below in figure 3-11
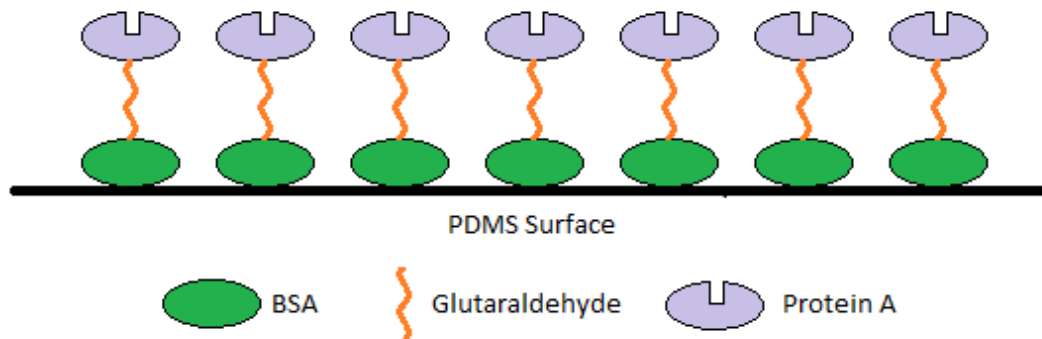


**Figure 3-11:** Diagram of protein A functionalization on the channel surface

### 3.3.3. CRP ELISA and Detection

This section will explain the process of performing the ELISA inside the

PDMS chip. This process will be exclusively for the detection of CRP. Later

modifications, including the addition of the filter and the antibodies for albumin

removal will be explained the upcoming sections. This section will only focus on the

detection area. To begin, the storage buffer is removed from the microchip. PBS is

then inserted inside the chip, which will remove the excess buffer. In the case where

the chip only includes the detection area and not any other channels for protein

removal, an additional aeration step will be required after every wash in order to

remove any excess fluid. In the case of multiple liquids, the fluid will be moving slow

enough so that there is no accumulation of excess fluid on the surface of the channel.

After the PBS has been removed, the captured antibodies are inserted into the

detection area. The capture antibodies being used are Mouse monoclonal anti-human

CRP IgG antibody (Abcam, Cambridge, UK) at a concentration of 200 µg/mL in Protein A/IgG binding buffer (Thermo Scientific, Rockford, IL). These antibodies are incubated in the channel for 7 minutes. The antibodies are removed from the chip and PBS is inserted to wash the channels. The CRP (Fitzgerald Industries, Acton, MA) is then inserted into the chip. In terms of the type of protein, it is unclear on whether or not the CRP is in the pentagonal form, or the monomer based on the Fitzgerald website[73], but it can be assumed it is the pentagonal form because it is the most native form and the CRP has to go through a dissociation process in order break into monomers[74]. The protein is mixed with either a solution of PBS or serum that does not contain any CRP (Fitzgerald). Because of this, different concentrations will have to be added to the solution. For these experiments, the CRP concentration in either PBS or serum will be between 0.7 and 100 µg/mL. Also, depending on the type of experiment, the protein will have various incubation times associated with it. However, for normal detection of CRP, an incubation time of 5 minutes will be used for each experiment. This will not have an effect on whether or not buffer or serum is used.

Once the incubation of the protein has completed, it is removed and washed for a third time with PBS. A mixture of the detection antibodies in PBS (50 µg/mL) is then inserted into the chip. The detection antibodies used are Goat polyclonal anti-human CRP IgG antibody labeled with FIT-C (Abcam). The antibodies will then incubate for 5 minutes. After the incubation, the antibodies are removed and a final was step is required. This time, instead of PBS being used for the wash, PBST (1X PBS + 0.5 µL Tween 20 per 1mL PBS) will be used. This is a stronger wash than PBS

and it will remove more of the improperly bound antigens. After 5 minutes of

incubation, the PBST is removed from the chip and PBS is then inserted in order to aid

in detection.   This is also shown in the protocol in appendix A.4.  A diagram of the

sandwich ELISA is pictured below in figure 3-12[22-24].
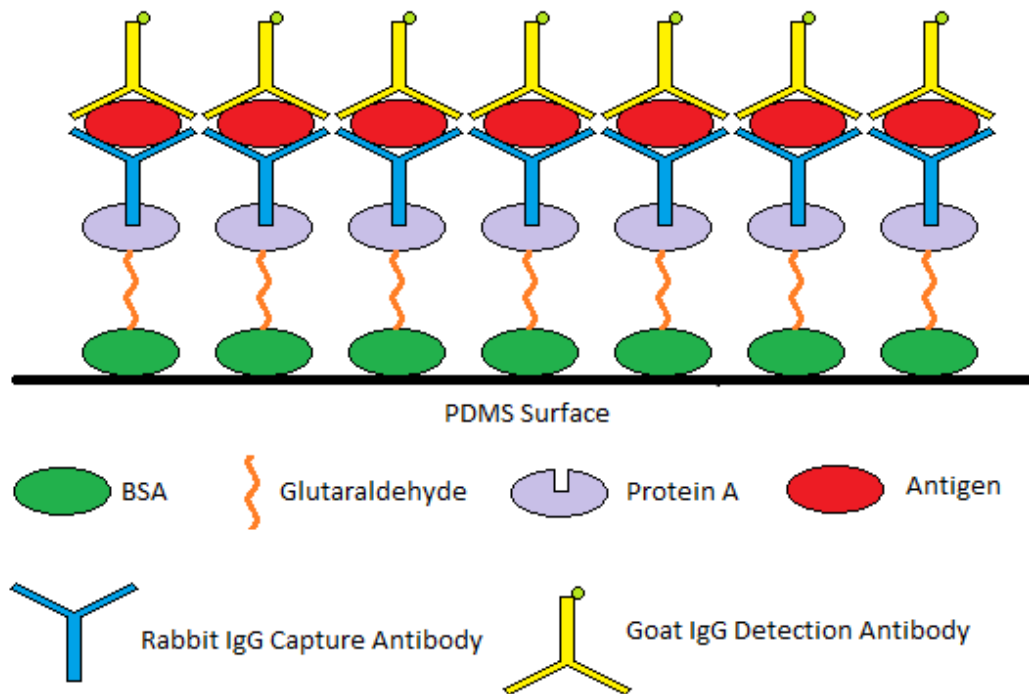


**Figure 3-12:** Diagram of completed sandwich ELISA for CRP detection

Detection is performed by using fiber-optics.   The detection area consists of a

spectrometer (Ocean Optics, USB4000, Dunedin, FL), which is hooked up to a

computer.  To perform the detection, the fiber-optic cables are inserted into the

openings of the chip next to the detection area. The chip with the fiber-optics is placed

inside the detection box. To activate the antibodies, the power is turned on, which

activates an LED (light emitting diode) (Quadica Developments, Brantford, ON,

Canada) in order to excite the FIT-C. The light will travel through a series of bandpass

filters that will filter the incoming light to give off a wavelength of 470 nm (Edmund

Optics, Barrington, NJ). This filter that was used has a bandwidth of 10 nm, a minimum transmission of 45% and an optical density (OD) of 3. This enabled the filter to block excess light that would interfere with the detection area and eliminate most of the background noise in the system. Detection and readings are performed by using the Ocean Optics program Overture. The wavelength is adjusted to 200 nm and the reading will be read at 493 Hz. This is where the spike will appear on the screen. The higher the spike, the more "full" the detection area will be. The absorbance level will be recorded and then plotted using Excel against the concentration of the IgG[22-24]. The experiment setup is shown below in figure 3-13 and example plots for the experiments will be available in appendix A.5.
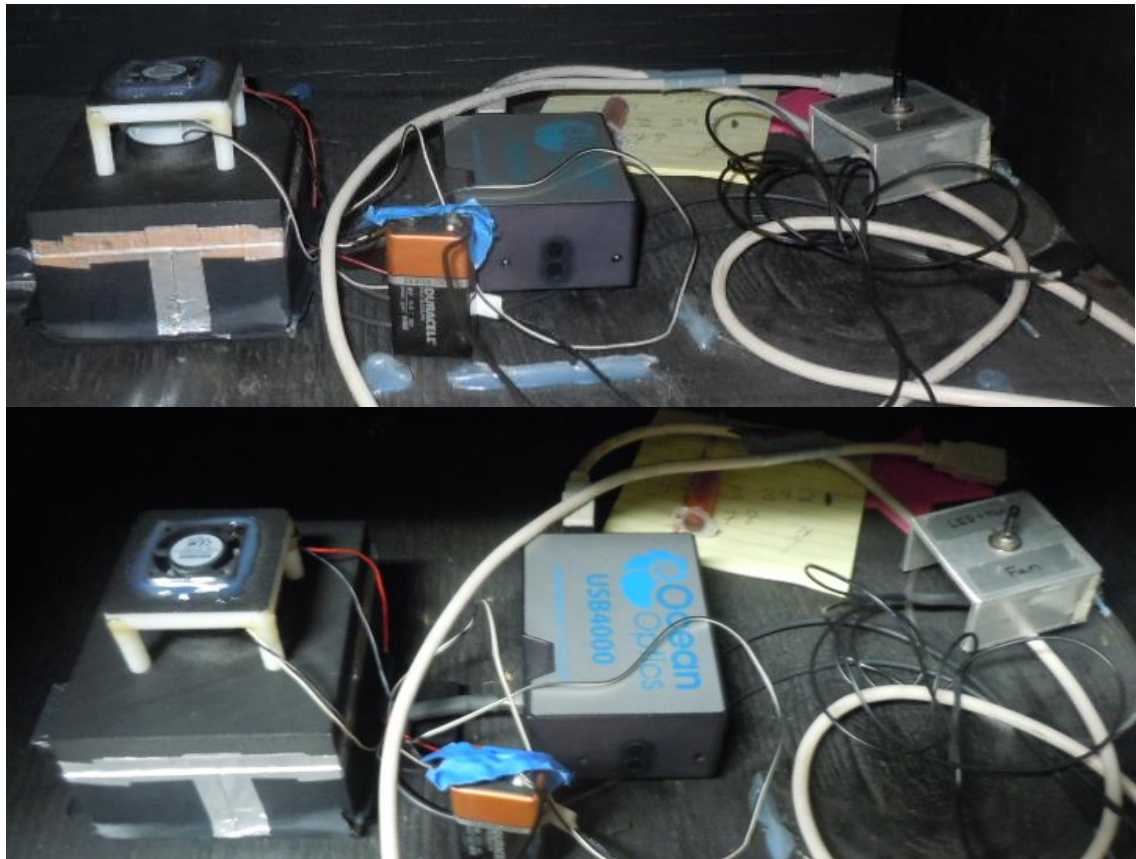


**Figure 3-13:** Front view (top) and top view (bottom) of fiber optic test are for CRP detection

### 3.3.4.   High Abundance Protein Removal

One of the main issues with performing a LOC with using blood plasma or serum is the abundance of proteins in the blood. There are numerous proteins that can be found in plasma that are known to cause interference in an ELISA-type assay. These proteins not only can block the attachment areas in the assay, but also can remove the antibodies that are used to capture CRP. The best way to overcome this would be to remove the high abundance proteins already after the blood cell filtration. In order to do this, it would have to come after the blood cell filtration site and the detection site, since the blood cells can cause havoc with the detection for the same reasons stated above.

Since there are many abundant proteins in serum, it would be most beneficial to focus more on the most abundant proteins: human serum albumin and human IgG. The reason for this being that they are the two most abundant proteins in plasma and serum, they tend to cause the most interference when trying to perform experiments that deal with plasma. There are other proteins that can cause interference, but these two proteins are by far the most abundant and since there are multiple proteins, the chip would have to be restructured in order to integrate multiple protein areas.

The preferable method to remove these proteins would be before the detection area, but after the blood cell filtration. This means that there has to be a method of protein removal in the microchannels inside the chip. There are different methods to remove the proteins, but they involve using different outside sources and expensive methods. These methods include electrophoresis[75], which was explained in the removal of blood cells[34], a mixture of trichloroacetic acid and acetone[76] and

different kits that are designed to remove albumin from serum[77]. While these

processes are effective, in terms of use on a microfluidic scale, it is rather difficult to

integrate due to the size limitations on the LOC system. However, one easy method is

utilized already in the chip: protein A. Protein A already has a high IgG affinity, which

is why it is already used in the detection area as a method to capture the capture

antibody. The same principle can be used in order to effectively capture IgG already in

a mixture like plasma. The channels of the chip can be coated by way of the same

method that was used in the ELISA. Also, since there are also anti-albumin IgG

antibodies on the market, they can be developed the same way as the capture

antibodies are used in the detection area. Except these antibodies will be used to

remove the albumin that is in serum. The antibodies used in these experiments are

Goat polyclonal anti-human albumin IgG antibody (Pierce-Antibodies, Thermo, #

PA1-29338). Two diagrams showing highlighting these methods are demonstrated

below in figure 3-14 and on the next page in figure 3-15.
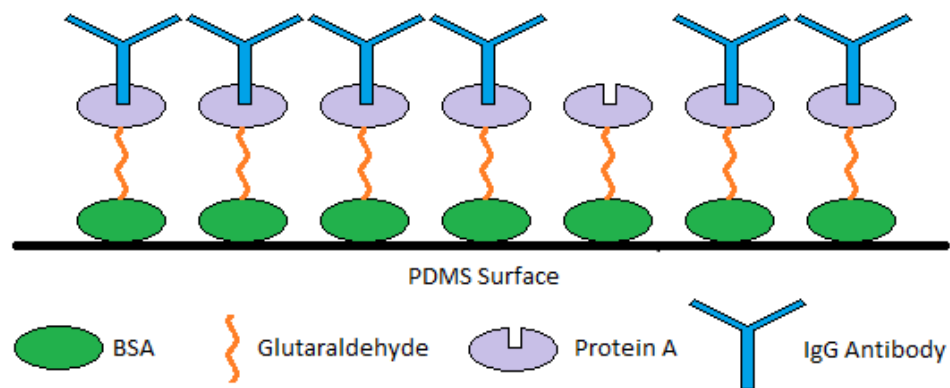


**Figure 3-14:** Schematic of Human IgG removal from Blood with by way of protein A
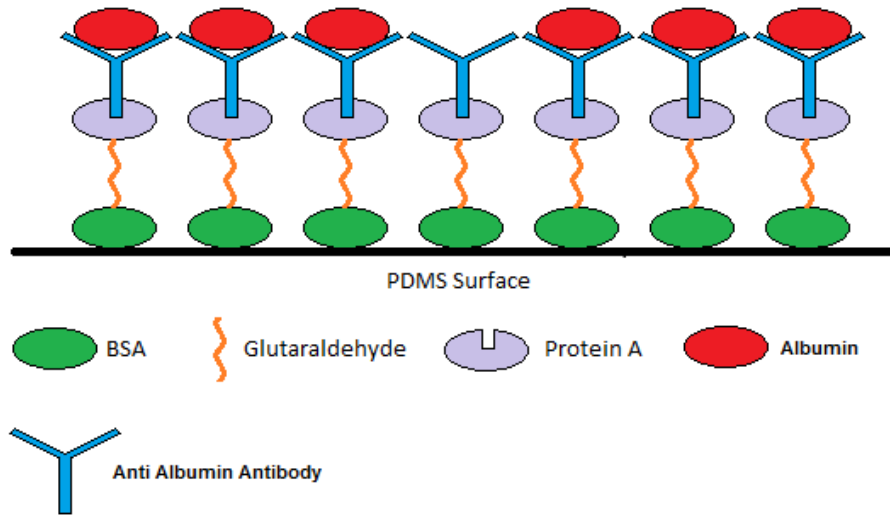
**Figure 3-15:** Schematic of Human Serum Albumin removal from Blood with by way of protein A and anti-albumin IgG

There have been various methods to test in order to see how the removal of albumin and IgG will help in CRP detection and there have been designs and experiments performed that test this theory. However, most tests come to a conclusion of having the serum flow into a removal site, allow IgG to leave the solution, move to an albumin detection site, allow albumin to leave, and then flow to the detection site. While this theory may work well, there is one flaw to this theory: the amount of IgG and albumin is much greater than that of CRP. This creates the problem that even if there is one removal area for each protein, there would still be too much in solution so that it will cause interference. Because of this, a false reading can be taken and it would incorrectly portray the CRP in a solution.

### 3.3.4.1.     Indirect Measurement of HAP Removal

To see how this method can be better develop, one has to look as to how well the protein – antibody interaction is. This can be demonstrated by measuring the concentration before and after each removal process.  This is what is known as an

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indirect measurement.  During an indirect measurement, the serum is sent into the functionalized channel incubated to remove the high abundance proteins, removed from the chip and collected for concentration sampling.  This will help determine not only how much of the HAP still remains in solution, but also with basic subtraction, how much was taken by the protein.  To perform this experiment, a nanodrop specrtrophotometer was used to record the concentration.  The nanodrop used was a NanoDrop 1000 from Thermo Scientific.  This was performed at the INBRE Laboratory, URI College of Pharmacy.  A drop of the reagent (~2 µL) was placed on the center of the sample area.  This works similar to the fiber optic detection system, in which a light of a specific wavelength detects the particles in solution[22].  The experiments will first focus on the removal of a pre-determined amount of IgG in PBS buffer and if that is successful, the removal of albumin in buffer.

### 3.3.4.2.      Direct Measurement of HAP Removal

The other method will be a direct measurement of HAP removal.  This involves the same procedure as the indirect measurement, but instead of measuring the amount of HAPs still remaining in solution, it will measure the amount of HAP captured on the channel.  This method will be performed by using the same fiber optic detection method as with the ELISA.  The solution that will be used is a mixture of IgG labeled with FIT-C (detection antibodies) in PBS buffer.  With this method, it will be easy to see how well the IgG will attach to the protein A and come to a conclusion as to how well this process is.

### 3.3.5. Chip Designs for Protein Removal

In terms of what type of chips will be implemented, there are four main chip designs that will be implemented. The first design is a one channel chip with two ports and a hexagonal detection area. This design will be used to implement not only how well blood can enter the chip, but also how can IgG attach directly to protein A. This will both be performed with the indirect and direct IgG detections. Also, this chip will be used to measure the CRP detection based on the positive (CRP in PBS buffer) and negative (CRP in serum without protein removal) controls.

The second design is a modified first design, where there is a longer end of the channel between the two ports. It still will encompass the detection area, but on the bottom part of the chip. This design is for the tests that will implement the IgG removal. The whole chip will be functionalized in the same manner as in the previous chip, only the upper channels will be used as the IgG removal areas, and the hexagonal area will incorporate the detection area.

The third design will incorporate multiple IgG removal areas. This design expands on the premise of the first design. In this design, there are three separate chambers above the detection area that will incorporate more of the capturing of the IgG antibodies. The bottom area below the detection area incorporates a waste area. No detection / IgG capturing will be performed in this area. All this area serves is that the fluid will just enter this area after it passes through the detection area. All of the channels of the chip will be coated with BSA, while the detection area on up will include the protein A on the channels.

The final design comes from the Franzblau design of the final microchip, since this would eventually be integrated in this system[24]. In this design, the same concept as the third design will be encompassed, but there are noticeable changes. Starting at the top, there are holes that will lead to a channel which head towards the main channels. This is where the inlet for where the blood / plasma / serum will enter the chip. This is due to the fact that all the reagents will be present on the chip before blood plasma is introduced to the chip. Above that junction where the two channels meet will be the storage for the reagents used after the plasma enters the detection zone. In order are, from bottom to top, PBS, detection antibody, PBST, PBS. Below this are the reagents used before the plasma enters the channel. They are, from top to bottom, PBS, anti-albumin capture IgG, anti-CRP capture IgG. Notice that the detection area now is the width of the channel. This is to allow a better signal within the confines of the chip. Below this is the waste area, where channels have been replaces by paper. This is to better the movement of the fluid in the chip, without adding a delay in starting and stopping. Full pictures of the chips will be presented in the figure on the next page.

**Figure 3-16:** Chips used in serum/blood experiments. Clockwise from top left: single-channel detection area, single channel with IgG depletion area, multichannel with IgG and albumin depletion areas, multichannel with multiple IgG depletion areas.

### 3.3.6. Combination of Cell and Protein Removal

Using all of the methods described above the final steps will be to combine all

of this into a LOC device that is able to detect CRP from whole blood more

effectively. All three aspects, blood filtration, high abundant protein removal, and

CRP detection will be implemented in this new chip. With many methods of detecting

CRP in laboratories and with LOC processes, this new chip would not only have a

simple to use design, but also detect CRP with the upmost accuracy. The experiments

will show the most ideal method of removing blood cells, remove the majority of the

high abundance proteins and be able to detect real-world levels of CRP from any

person. With this, testing for the risk of heart disease will be faster, easier to use, more available outside of the lab and more accurate.

### 3.3.7. Limits of Detection and Quantification

Based on the results of the experiments with serum and blood, titration curves will be created which display the comparison between CRP concentration in blood /serum and intensity detected by the fiber optics. This diagram will be useful in that at a specific intensity value, a CRP concentration value can be founded. This is useful in testing and in homecare use, but it would be ideal to find how well this system can effectively detect CRP, especially at low concentrations. In order to see the effectiveness of the system, the limit of detection has to be implied. The limit of detection (LoD) is the amount of, for example CRP, that can be effectively be detected in which there is a distinct signal (in which background noise is not a factor)[78,79]. With the LoD, one can find out the minimum CRP concentration that is allowable for accurate detection.

There are different ways to find the LoD. This project will focus on 3 methods to calculate LoD. The first is what is known as a visual evaluation. This involves creating a titration curve and checking to see where at low concentrations the graph begins to level off to where a constant signal occurs. This is usually good for estimation, but there are two other methods in which LoD can be easily detected.

The second method is what is known as a signal-to-noise calculation[78]. This is a comparison between the amounts of the desired signal compared to the background noise. This means that for a certain amount of signal that is produced, there is an amount of noise that goes along with it. In any detection experiment the

amount of signal detected should be greater than the amount of noise detected.

However, in order to obtain an accurate signal, the signal must be significantly higher that then background noise. In most cases, a 3:1 ratio of signal to noise would be adequate in order to obtain an optimal reading. However, there is a limit known as the limit of quantification (LoQ) in which the obtained signal is adequate without any objection. This is usually a 10:1 ratio. Both of these measurements can be easily calculated using the titration curves[78].

In order to calculate the LoD and LoQ, one has to plot the concentrations and signals for the lower end of the curve, usually near or past the point when the signal is concentrated at the zero point. This plot is ideally linear and will give a slope (SL). The next step will be to obtain the standard deviations (SD) for each signal omitted. This is to see how far from a signal can the reader emit. This is usually about 95% of the mean signal. In Microsoft Excel, the STEYX formula is useful for this because it relates the standard deviations at each intensity for a given concentration and predicts the standard error of all values. With the slope and standard deviation, the following two equations can be used to calculate the LoD and the LoQ respectfully[78]:

$$LoD = \frac{3.3(SD)}{SL} \tag{1}$$

$$LoQ = \frac{10(SD)}{SL} \tag{2}$$

The third method, based from Armbruster and Pry, calculates LoD based on the limit of blank (LoB)[79]. The LoB is the highest expected amount that can be obtained where no antigen (CRP) is present in the system. Even though there is no CRP in the detection area, a signal can still be obtainable. For this, reason, a blank sample has to be taken into account. To calculate the LoB, separate measurements of

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the blank have to be measured. From these measurements, the mean blank

measurement and the blank standard deviation has to be calculated. The LoB can be

found using the following equation:

$$LoB = mean_{blank} + 1.645(SD_{blank}) \tag{3}$$

Once the LoB is found, a sample with a low amount of analyte is measured and

the standard deviation is recorded. The LoD can then be found using the following

equation[79]:

$$LoD = LoB + 1.645(SD_{low\ conc.\ sample}) \tag{4}$$

The LoQ can also be calculated based on the following method by rearranging

and combining the standard deviation / slope ratio[78]:

$$\frac{SD}{SL} = \frac{LoD}{3.3} = \frac{LoQ}{10} \tag{5}$$

Rearranging the right side obtains the approximation:

$$LoQ = 3 \times LoD \tag{6}$$

For this project, the limits of detection and quantification will be examined

using these three methods and compared against each other.

# CHAPTER 4

## FINDINGS

### 4.1.    Blood Cell Filtration

After many tests with the vivid plasma membranes, it was shown that they were effective on removing RBC's from whole blood. During each run, the syringe withdrew the plasma at a constant flow rate into the tubing until either an air gap was present, or the filters became clogged. Plasma was then collected in separate vials and analyzed using a microscope. This provided the basis for the removal of blood cells. Since the pores of the filters effectively allowed plasma to flow through, it was assumed that the proteins also flowed through the filter as well.  This was further exemplified during the experiments for fibrinogen detection.

#### 4.1.1.   Evidence of Blood Filtration

In order to make sure that the following experiments can even be performed it is important that plasma can even be filtered out of solution.  The following pictures give an example as to if this process can even work.  For each test, piece of filter paper was placed on a slab of PDMS connected to tubing which connected to a syringe.  In this example below, a piece of filter paper was sandwiched in between the channeled chip and a PDMS slab designed to hold the filter paper. 45 µL of blood was inserted in an opening that allowed filtration.  A syringe pump was enabled at a pull flow rate of 100 µL/min.  The filter paper used in this example is the Vivid Plasma Membrane GX from the Pall Corporation.
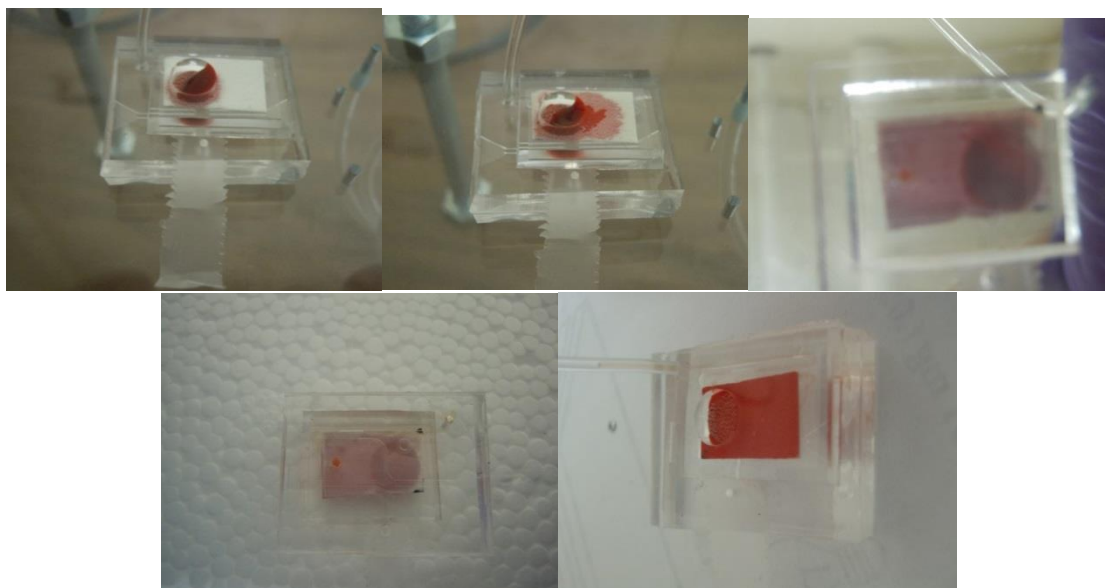
**Figure 4-1:** PDMS chip with Vivid Plasma Membrane GX with blood first inserted onto the paper (top left), spreading throughout the paper (top center), the underside after the blood spreading (top right), plasma extracted into the chip (bottom left) and filtrate inside of the tubing (bottom right)

As seen in figure 4-1, the blood is added onto the chip and is able to migrate through the filter paper. Note that the inlet into the channels is on the opposite side from the inlet to the paper, as to avoid tearing of the paper due to the force of the syringe pulling. After two minutes, the paper is concentrated with blood and there is noticeable separation in the membrane. This is given off by that there is a tannish color on the paper between the edge and the area of red blood cells. Normally, this paper is white as seen in the top left of figure 4-1, but because this area is darker, it is assumed that this would be the filtrate. The top right shows the underside of the chip. Here, the blood has covered the entire area of the filter with it being red, in order to show that there are blood cells that are attached to all points of the filter paper. It is at this point that the syringe pump is turned on in order to effectively draw the filtrate into the chip.

The bottom left shows that chip with the filtrate already extracted into the chip. The filtrate is the light tan fluid in the channels of the chip. It might be a little difficult to see the filtrate inside of the chip, so bottom right includes the filtrate inside of the tubing that is connected to the syringe. It is here that one is able to see very clearly that the filtrate is being pulled into the tubing, which means that the filter paper is very effective in obtaining a good amount of filtrate. Also, it is worth noting that the filtrate has no red in it at all. This means that the filter has both effectively separated the cells not allowed hemolysis to occur. Because of this, it can be stated that the Vivid Plasma Membrane GX is an appropriate filter for separating cells from plasma in small quantities of blood. The analysis of the blood will be investigated in section 4.1.3. The next section will explain how much filtrate can be exacted with the Membranes GX as well as the GR.

### 4.1.2. Filtrate Volume

The two filter papers that this project is concerned with are the Vivid Plasma Membranes GR and GX. The main difference between the two, according to the Pall website, were the amounts of whole blood it can filter out per one square centimeter of paper. The GX can handle roughly 20 – 30 µL of blood, while the GR can handle 40 – 50 µL of blood[66]. This is due to the void volume of the filters, where the GR has a larger pore space than that of the GX. Based on this information, the following graphs detailing the average filtrate volume are shown below (Figure 4-2 for the GX and figure 4-3 for the GR). Each initial blood sample was set at 45 µL. Each test was run until the filtrate stopped moving due to the blockage caused by the blood cells. Four tests were compiled based on the rate of extraction (50 – 100 µL/min) and by shape of

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the filter (100 mm$^2$ square vs 96 mm$^2$ rectangle, based on whether or not the paper

would break during extraction)
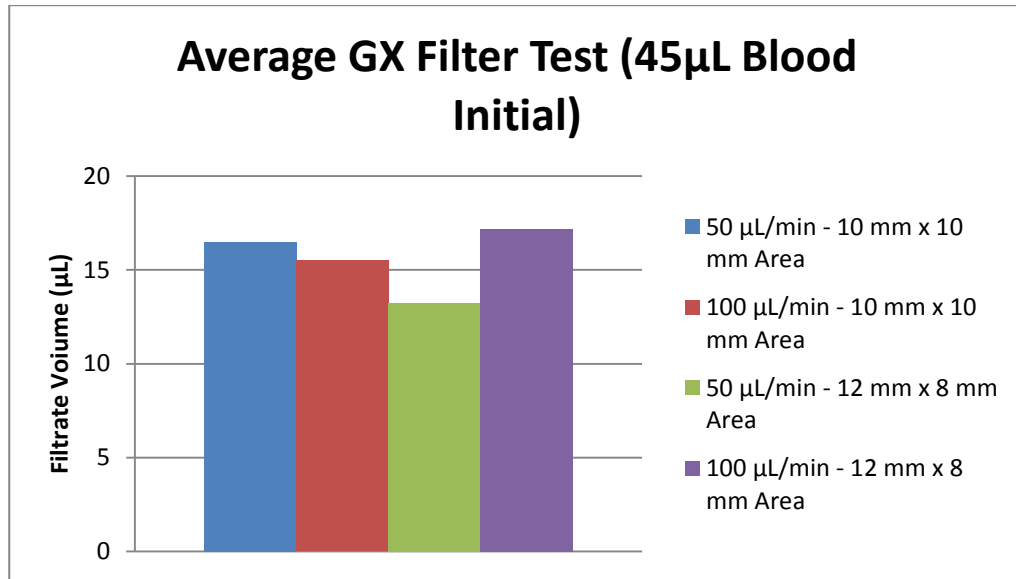


**Figure 4-2:** Average Filtrate Volume from the Vivid Plasma Membrane GX with 45 µL of blood

Based on figure 4-2, the average filtrate volume did not vary significantly.  The values

stayed around 13 – 16 µL filtrate, regardless of the filter shape and flow rate.

However, in figure 4-3 below, a different story applies.
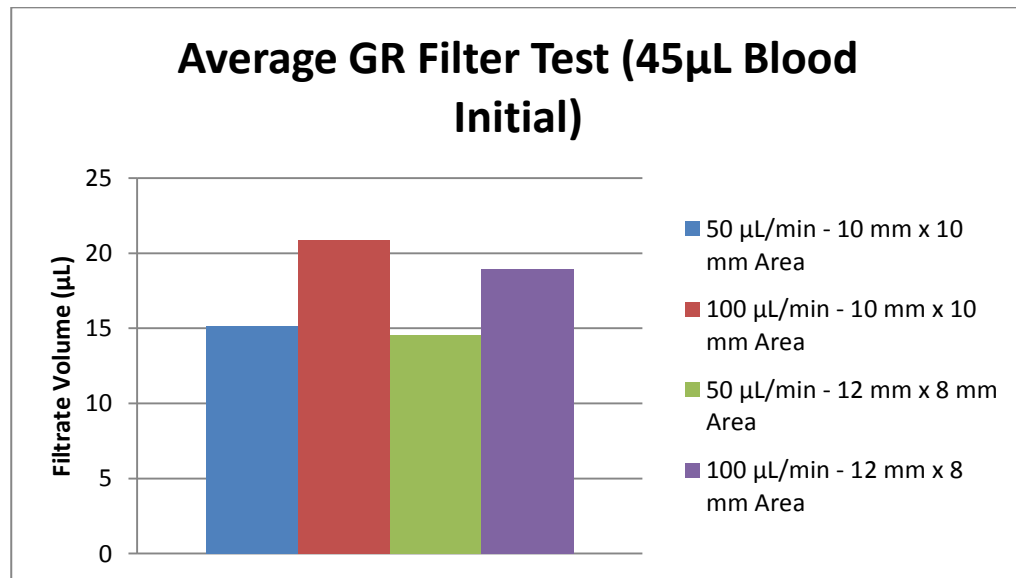


**Figure 4-3:** Average Filtrate Volume from the Vivid Plasma Membrane GR with 45 µL of blood

With the GR filter in place, there is a noticeable 5 µL increase with the higher flow rate.  This is mainly due to the fact that more filtrate can be extracted without environmental factors that include evaporation and the drying of the filter cake.  This did not show up in the GX due to the fact that the GR can handle 25% more liquid than the GX.  But other than this, there is no difference in the filter papers at the lower end of the flow rate.  With both types of filter papers, and both dimensions, it was apparent that up to 15 µL of filtrate can be extracted, or up to 35% (rounding) extracted from the original amount of blood.  Based on the estimation that 55% of whole blood is plasma, this amounts to an estimation of up to 68 – 70% of plasma recovered.  A table of values showing each volume extracted as well as percentages for total blood and plasma volume recovered for both papers is show below.

| Blood Vol. (µL) | Dim. (mm x mm) | Average Vol. (µL) | Blood % Recovered | Plasma % Recovered |
|---|---|---|---|---|
| 50 | 10 x 10 | 16.5 | 36.37 | 67.90 |
| 50 | 12 x 8 | 13.25 | 29.44 | 54.52 |
| 100 | 10 x 10 | 15.5 | 34.44 | 63.79 |
| 100 | 12 x 8 | 17.17 | 38.15 | 70.64 |

**Table 4-1:** Values of filtrate volumes from Vivid Plasma Membrane GX

| Blood Vol. (µL) | Dim. (mm x mm) | Average Vol. (µL) | Blood % Recovered | Plasma % Recovered |
|---|---|---|---|---|
| 50 | 10 x 10 | 15.13 | 33.61 | 62.24 |
| 50 | 12 x 8 | 14.56 | 32.36 | 59.93 |
| 100 | 10 x 10 | 20.88 | 46.39 | 85.91 |
| 100 | 12 x 8 | 18.94 | 42.08 | 77.93 |

**Table 4-2:** Values of filtrate volumes from Vivid Plasma Membrane GR

Based on these findings and the fact that only about 5 - 7 µL is to be extracted, either the Vivid Plasma Membrane GX or GR is acceptable.  However, for the upcoming experiments, based on the amount of blood that can be withstood, a few tests will be conducted with the GX membrane. Even though the GR membrane could

handle more blood, it should be noted that the final chip design calls for two inlets: one for blood and one for the reagents. Because of this, the blood inlet has to be sealed in order for the reagents to flow through the chip. Because only a few microliters of blood are need and that the blood cells have to plug up the filter, it makes more sense to use the GX membranes.

However, it is crucial to find the limit of what these filters can take. The first test that demonstrated this was performed was with a larger sample size of blood (75 µL). Based on the previous experiments, it was assumed that the results would be the most plasma that could be extracted from that size of filter paper, since it was dependent on the recommended size for that specific blood volume. For this experiment, since the amount of blood being used was about 1.5X the original amount, it would be best to use a filter area 1.5X that as well (150 mm$^2$). Also, the opening into the chip was on the opposite end of where the majority of blood cells would be filtered. Based on this experiment, it was shown that a large amount of filtrate was extracted, with more extracted at a higher flow rate. This satisfied the findings on the proportionality of the paper, where about 35 - 37% of the total blood volume was extracted (compared to 33 – 35%), which resulted in an overall percentage of filtrate extracted being between 68 and 70%. The figure and table for this experiment is shown on the next page.
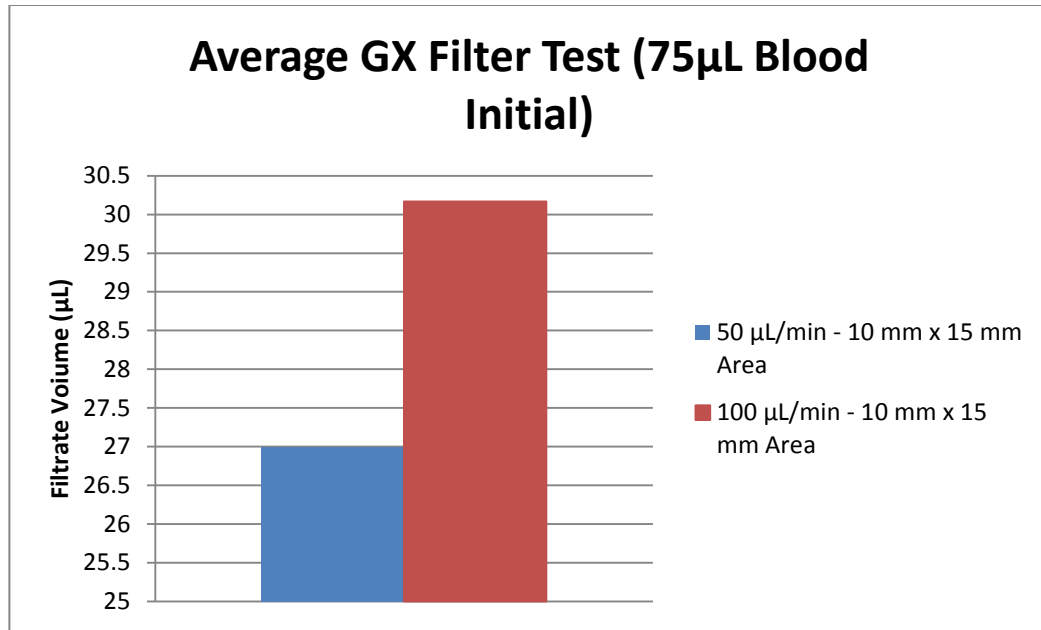
**Figure 4-4:** Average Filtrate Volume from the Vivid Plasma Membrane GX with 75 µL of blood with a filter area of 150 mm$^2$

| Blood Vol. (µL) | Dim. (mm x mm) | Average Vol. (µL) | Blood % | Plasma % |
|---|---|---|---|---|
| 50 | 10 x 15 | 27.00 | 36.00 | 66.67 |
| 100 | 10 x 15 | 30.17 | 40.22 | 74.49 |

**Table 4-3:** Values of filtrate volumes from Vivid Plasma Membrane GX with 75 µL of blood with a filter area of 150 mm$^2$

However, with the same filter dimensions, but with the original starting amount of blood, it was shown that less filtrate could be extracted. In the first experiments with the GX membrane, about 15 µL out of a total of 45 µL could be extracted. Here on the other hand, only an average between 8 µL at 50 µL/min and 12 µL at 100 µL/min could be extracted. This relates to 30% of the total blood yield and about 58% of the total plasma yield. It was possible to extract 15 µL, but only at a rate of 150 µL/min. It should also come to be known that at 10 µL/min, only 4 µL of plasma could be extracted. The graph and table explaining this is located on the next page.
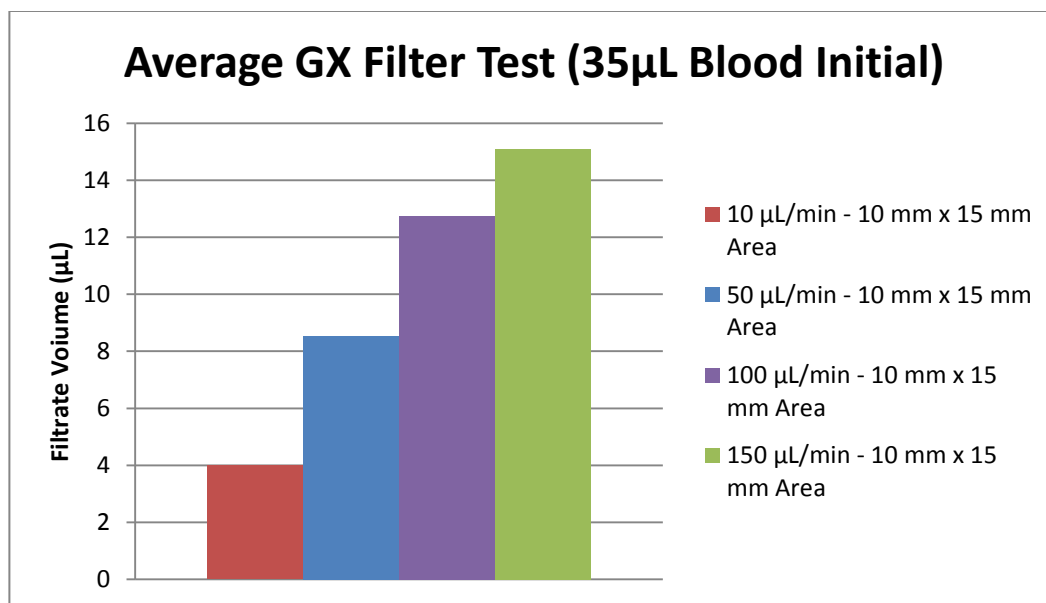
**Figure 4-5:** Average Filtrate Volume from the Vivid Plasma Membrane GX with 45 µL of blood with a filter area of 150 mm$^2$

| Blood Vol. (µL) | Dim. (mm x mm) | Average Vol. (µL) | Blood % | Plasma % |
|---|---|---|---|---|
| 10 | 10 x 15 | 4.00 | 8.89 | 16.46 |
| 50 | 10 x 15 | 8.54 | 18.98 | 35.15 |
| 100 | 10 x 15 | 12.75 | 28.33 | 52.47 |
| 150 | 10 x 15 | 15.08 | 33.52 | 62.07 |

**Table 4-4:** Values of filtrate volumes from Vivid Plasma Membrane GX with 45 µL of blood with a filter area of 150 mm$^2$

Due to these findings, it is important to relate the amount of blood being used and the dimensions of the filter paper. Since the assays would likely be handling plasma or serum volumes less than 10 µL, it should be noted that coming from a starting blood volume between 40 and 50 µL, a filter area of 100 mm$^2$ should be sufficient in not only extracting that amount of filtrate, but making sure that the port to allow the filtrate to enter the chip is sealed due to the filter cake. For this, either the Vivid Plasma Membranes GX or GR are suitable with a total filter area of 100 mm$^2$ based on concerns for the filtrate volume.

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### 4.1.3. Blood Cell Observations
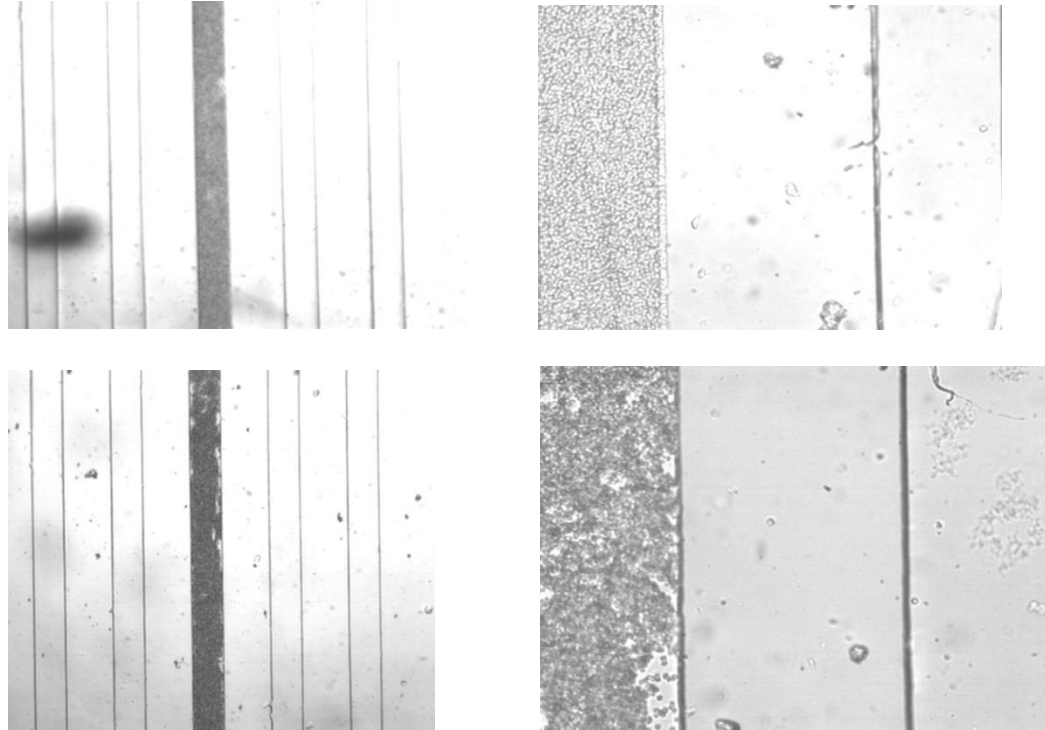


**Figure 4-6:** Microscopic view of using Vivid Plasma Membranes for blood filtration (top left: membrane GX – 5X, top right: membrane GX – 20X, bottom left: membrane GR – 5X, bottom right: membrane GR – 20X).  The dark channel represents whole blood and the four light channels represent filtrate

Based on microscopic observations, it has also shown that there were no amounts of RBC in all but two of the filtrate samples. The reason for the amounts of RBC in the filtrate was based on sample mixing due to a bad microchip. Other than that, it was shown that there were hardly any traces of RBC in the filtrate. The redness in the samples explained above was caused by hemolysis either due to the age of the blood, or the RBC during filtration. As stated previously, even though there were trace amounts of redness in each filtrate, due to the force trying to obtain another 2 μL after

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about half the possible plasma was recovered, there was no sign of RBC's anywhere after filtration. This satisfies the second focus of the study.

To show that there was filtrate in these samples, figure 4-7 shows a microscopic sample of what happens when there is a tear in the membrane. Blood cells do rush into the channel, but not at the amount found in the control channel with whole blood.
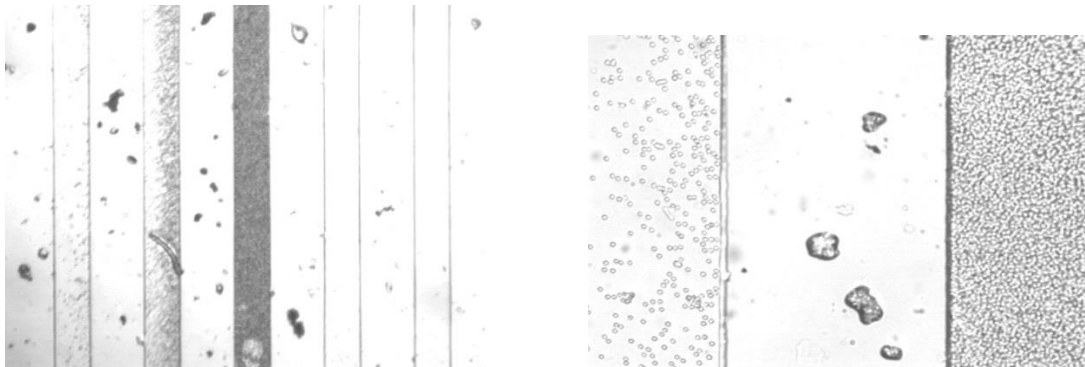


**Figure 4-7:** Microscopic view of using Vivid Plasma Membranes for blood filtration with blood cells entering the channel (left: membrane GX – 5X, right: membrane GX – 20X)

### 4.1.4. Plasma vs. Serum Tests
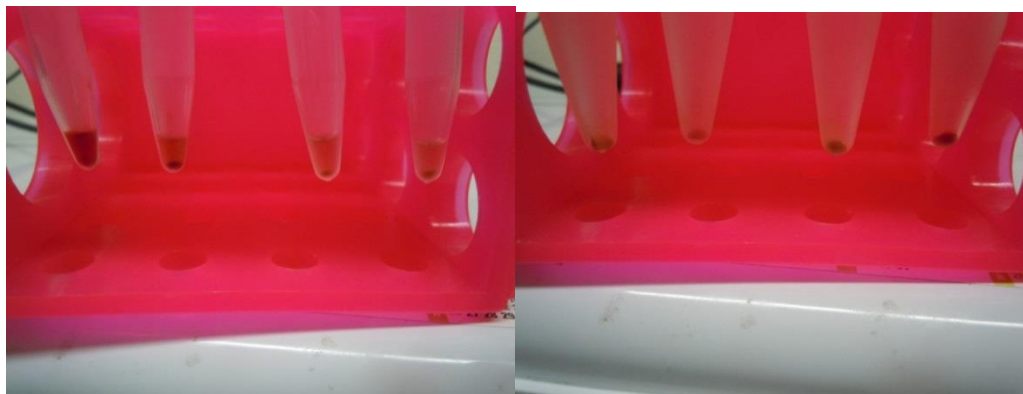


**Figure 4-8:** Centrifuged extracted filtrate with ethanol for plasma/serum tests (left), fibrin remaining after serum extraction (right)

**Figure 4-9:** Centrifuged serum with ethanol for plasma/serum tests.  Note that the middle vial had no ethanol added to it

Concerning the presence of fibrinogen, 10% ethanol by volume was added to each filtrate sample and frozen overnight to allow for coagulation. Each sample was then centrifuged for a half hour to see if there was sediment. The results of this shown in figure 4-8 has shown that there was significant coagulation and sediment for each individual filtrate. In comparison, figure 4-9 shows the same experiment performed in serum.  In figure 4-8, there is a more noticeable amount of precipitate and it is more tannish-red than white, as shown in figure 4-9.  Furthermore, in figure 4-9, the middle vial is serum without any ethanol added and it is similar to the serum with ethanol added.  Therefore, it can be said that each sample was plasma, not serum. In order to make it so that these filters release serum instead of plasma, further research has to be conducted in order to design a filter that separates serum from plasma.

## 4.1.5. Creation of a Blood Separation Chip
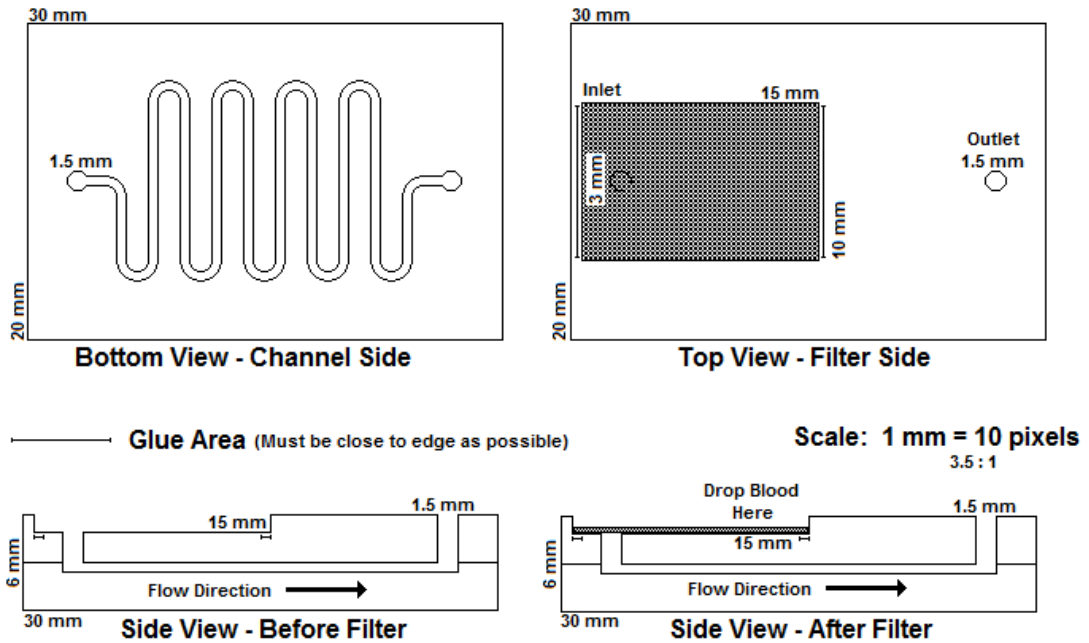


**Figure 4-10:** First Prototype of blood-separation chip

In terms of the chip design, the first design, pictured above in figure 4-10, was to be a two layer chip. The bottom layer consisted of the channels used to direct the fluid, while the top layer consisted of an open filter at the top. The dimensions for this chip are shown in the schematic. The filter was to be held into place using a thin layer of glue. While testing, this prototype, there was one main fault with this design. This was caused by the glue on the filter. The glue seeped throughout the filter, which made it so that the blood could not flow through the filter and into the chip. Because of this, a new prototype had to be created.
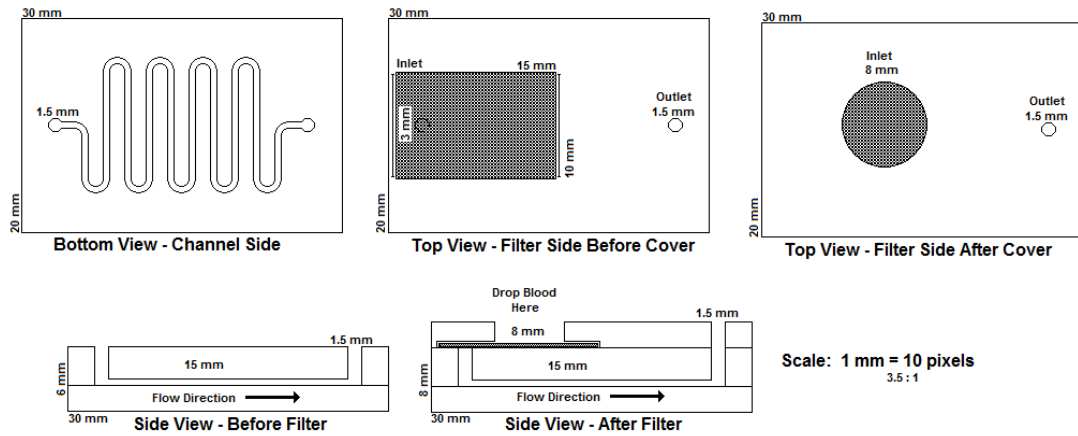
**Figure 4-11:** Second Prototype of blood-separation chip

This new design consisted of three layers.  The bottom layer is just a flat

PDMS layer, the middle layer is where all the channels are located and the top layer us

where the filter is held.  The plasma asher is used to bind all three layers.  The

channels will be bound as normal, but the filter holder will be bound to the top of the

chip, with the filter in between the both of them.  In this scenario, the filter holds up

pretty well and blood flow is not obstructed.  An example of the prototype can be

found in section 4.1.1 with the figures depicting the blood separation.
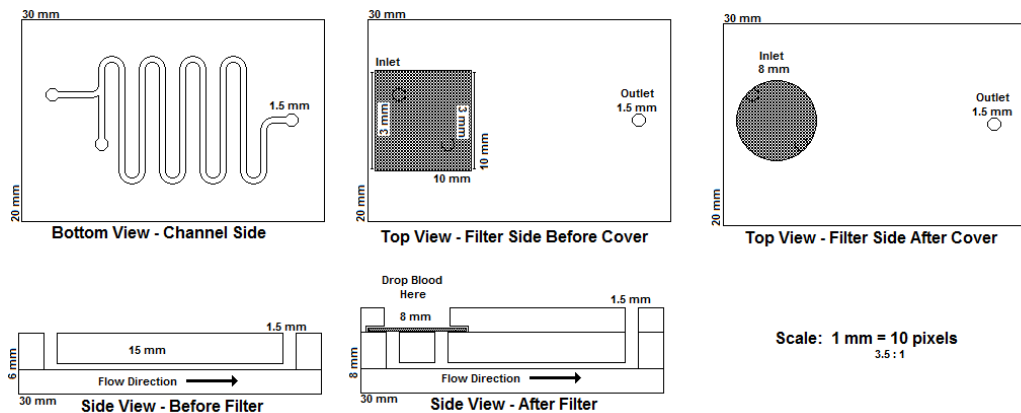


**Figure 4-12:** Modification of second prototype of blood-separation chip

Over the course of this project, this design has been modified in order to

maximize the effects of blood separation.  For example, in the first update, a second

inlet channel was added in order to effectively filter out more blood from both sides of

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the filter paper[23,24].  A second update makes the filter paper circular in order to not allow white area and to cover the filter completely with blood[23,24].  These two adages will be implemented with the new sequential loading chip being used to detect CRP with whole blood.

### 4.2.    Removal of IgG

#### 4.2.1.    Indirect Measurement of IgG
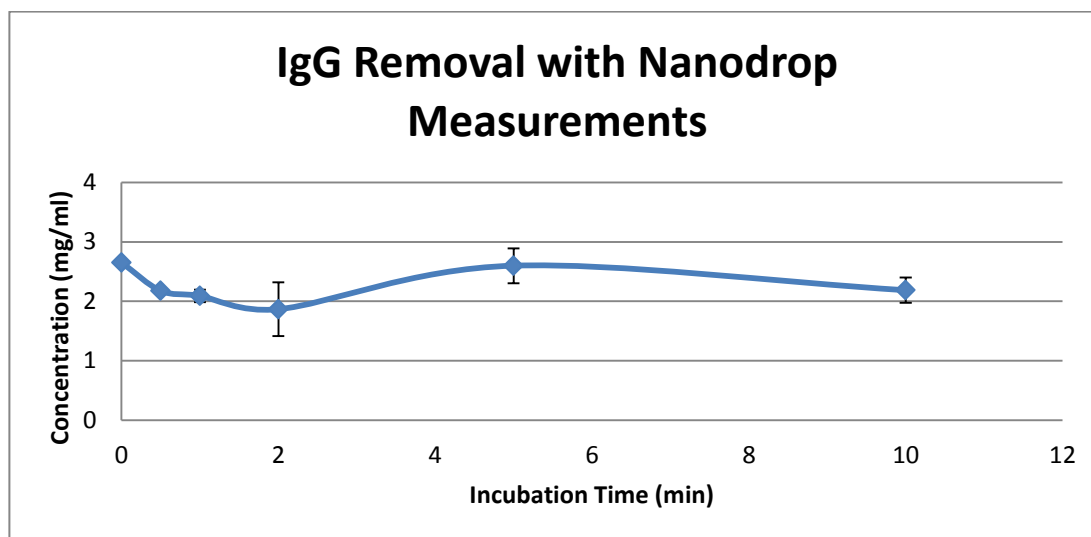


**Figure 4-13:** Representation of indirect IgG capturing with varying incubation times

The first method used in the removal of IgG was to detect IgG that remained in the plasma. Based on the results of these tests, it was determined that the evidence in finding the amount of depleted IgG was insufficient. There was a good amount of IgG depletion during the early stages with shorter incubation times; however, the concentration was shown to be rising as the incubation time elapsed. There not a large amount of change between 0 and 2 minutes, but around 5 minutes, there was a huge spike in the amount detected and in the opposite direction. There are numerous factors that can explain this phenomena happening, which include improper mixing of the antibody before the tests and additional proteins/antibodies in the solution. This can be

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caused by improper binding either to the protein A or the channel and during the wash, where improper binding can wash into the solution. Another issue is at larger incubation times, protein A can become over-saturated with IgG that it cannot handle all the binding and can break off from the glutaraldehyde. The nanodrop does not include these factors in its measurement and therefore, will count these rogue agents as a part of its total concentration. Because of these reasons, this method of indirectly measuring IgG is not useful. A direct measurement of IgG has to be helpful as to how well IgG can attach to protein A. Because detection antibodies labeled with FIT-C are used in the ELISA, and since protein A has a high affinity to IgG, these antibodies are able to attach to protein A and one can be able to use these antibodies to see how well they attach to protein.

### 4.2.2. Capturing and Direct Measurement of IgG

**Protein A Absorption of IgG**
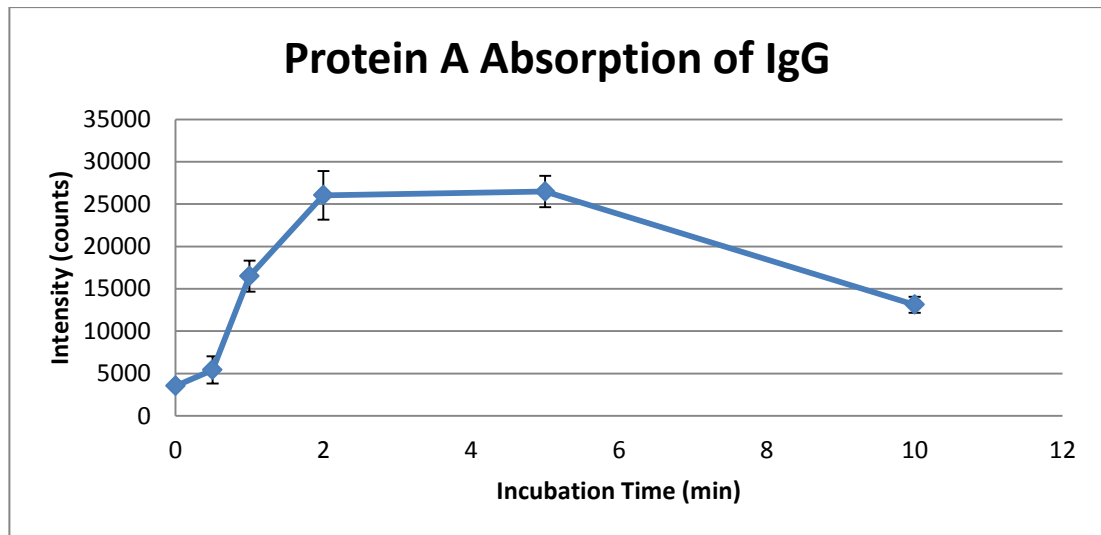


**Figure 4-14:** Representation of direct IgG capturing with varying incubation times

In these experiments, the capturing of IgG was performed with the focus how effectively IgG binds to protein A. In the following graphs, a similar trend occurs. From the start of the experiment to about 2 to 3 minutes, there is a steady increase in

the signal of the IgG. However, after this time frame, the signal stays constant. However, after 5 – 6 minutes, the signal declines. From the data, this is what can be made from it. First, the protein has a rapid rate of capture and it can readily capture IgG. However, as expected, there is a limit as to how much antibody can be on the protein. This is apparent between 2 and 5 minutes, where the detection is relatively level and the same within that time frame. One other thing to keep aware of is that the longer the incubation time is (> 6 minutes), the signal decreases. The main reason for this being that there the protein can become over-saturated when there are too many antibodies on the protein that the protein can likely fall off the glutaraldehyde and out of the detection area.

This is extremely important because once the experiments use serum or plasma, it is necessary to remove a great amount of IgG and later on, albumin, so knowing how long it takes in order for the protein to become saturated is important. Based on these experiments, the optimal time to remove IgG successfully up to saturation would be between 3-5 minutes based on the layout of this chip. To be on the safe side, it will be the most ideal to have the incubation time set at 5 minutes, which would make sure that the majority of the IgG that will be able to attach to the protein can be achieved without fear of it breaking away and causing a disruption in the detection area.

Based on this information, one can see how well IgG can readily attach to protein A. But how does this help in CRP detection? Both antigens are found in serum so it would be best to see how well they can CRP be detected in a buffer that also contains IgG. To do this, two variables will have to be considered: First, how

incubation time can affect detection. For these trials, multiple incubation times were used, which ranged from 0 minutes to 5 minutes. Second, how IgG concentration can affect detection. For these trials, IgG concentration varied between 0X to 4X the concentration of CRP in solution. This should give an idea as to how well CRP can be detected before serum, which contains a larger amount of IgG, can be used.

### 4.2.3. CRP Detection with IgG in Buffer
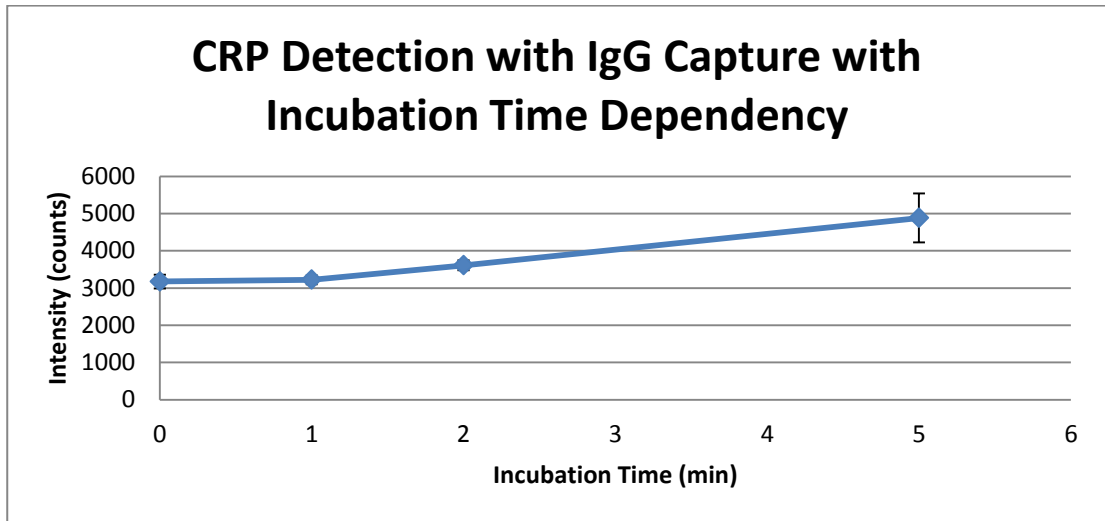


**Figure 4-15:** Representation of CRP detection with IgG present with dependence on incubation time
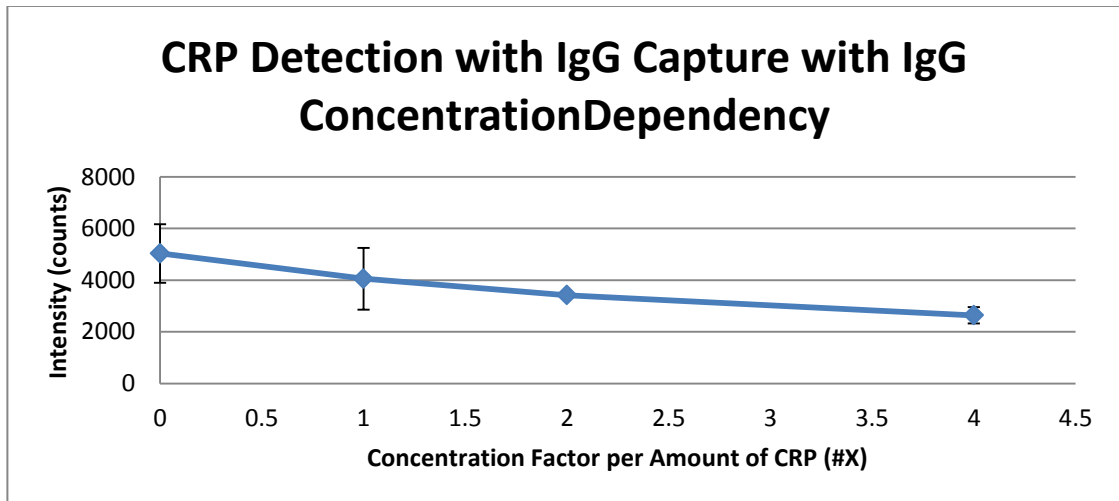


**Figure 4-16:** Representation of CRP detection with IgG present with dependence on IgG concentration per CRP concentration

In these experiments, the capturing of IgG was performed with the focus of two variables: the change in incubation time and the change in IgG concentration. Based on the results of these graphs, two things can be said. First, as incubation time is increased with the IgG concentration the same, the signal also increases. This is to be expected since as less IgG is in the detection area, the less interference it causes, which means that the CRP signal will be greater. Second, as IgG concentration increases, with the incubation time kept constant, the signal also decreases. This is based on the fact that there is so much IgG in the system and a limited amount of protein A on the channels. There is a lot more interference in the detection area, which attributes to the drop in absorbance. This is helpful because in these experiments, the ratio to the amount of IgG to CRP varied between 0:1 and 4:1. In human blood however, this ratio is much higher than that of the buffer. Because of this, there has to be a way to increase the amount of signal that is comparable to that of buffer.

There are various ways to try to increase the signal such as increasing the concentration of protein A and the surface area of the incubation channels. However, it is assumed that the channels are fully concentrated in protein A and in order to increase the surface area, the chip would have to be redesigned completely. However, based on the structure of the chip and the dimensions, there is a large amount of space utilized for the storage and movement of the fluid compared to the amount of fluid used. Because of this, multiple incubation areas can be utilized for the capture of even more IgG. The reasoning for this being that up until a certain incubation time (5-7 minutes), the incubation area will become saturated with IgG that there will still be a

significant amount of antibody in the detection area that interference will occur. To make it so that this does not happen, multiple IgG capture areas will be integrated in order to see if there is a rise in protein A detection. The amount of detection areas will vary between 0 and 3, based on the limitations of the design of the chip.

### 4.2.4.  CRP Detection in Buffer with Multiple Incubation Areas
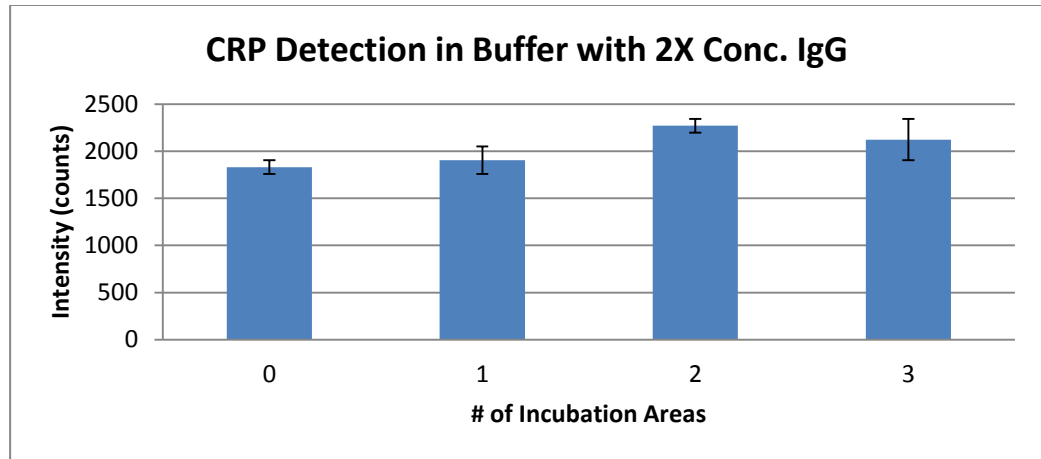


**Figure 4-17:** Bar graph of 10 µg/mL CRP detection in Buffer with 2X the concentration of IgG mixed with 0 – 3 incubation areas
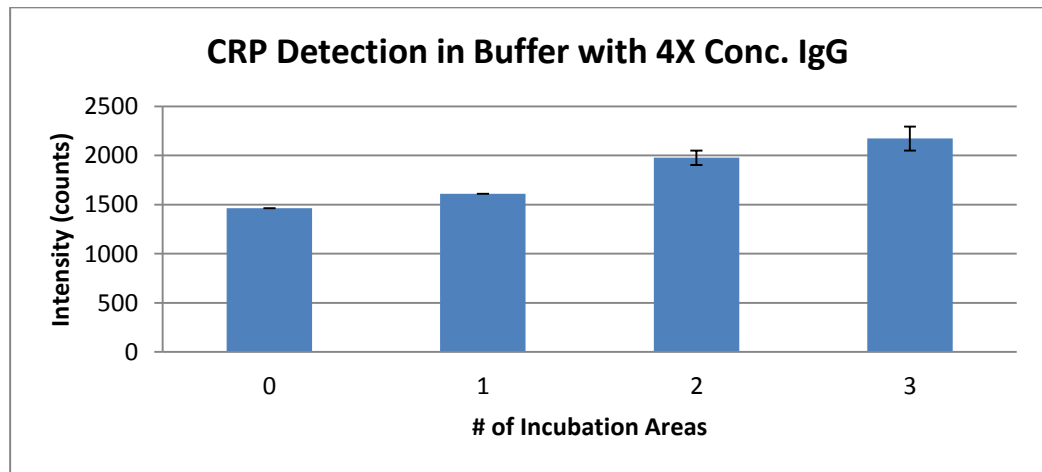


**Figure 4-18:** Bar graphs of 10 µg/mL CRP detection in Buffer with and 4X the concentration of IgG mixed with 0 – 3 incubation areas

The data above shows the detection of CRP with multiple incubation areas. Note that only 3 incubation areas are used due to the amount of liquid and the limitation on the chip design. Based on these results, it shows that as the number of

incubation areas increase, the intensity detected will also increase. This is to be expected since the amount of IgG has been depleted due to the multiple incubation areas, which allows for less interference in the detection phase. One thing to be aware is that the highest IgG:CRP ratio used here is 4:1. One has to take into account the amount of IgG is a lot greater in that of serum and that albumin will also cause interference. So even though this data is high, it still cannot compare to a real world use as with serum, as shown in the upcoming experiments.

### 4.2.5. CRP Detection in Serum with Multiple Incubation Areas
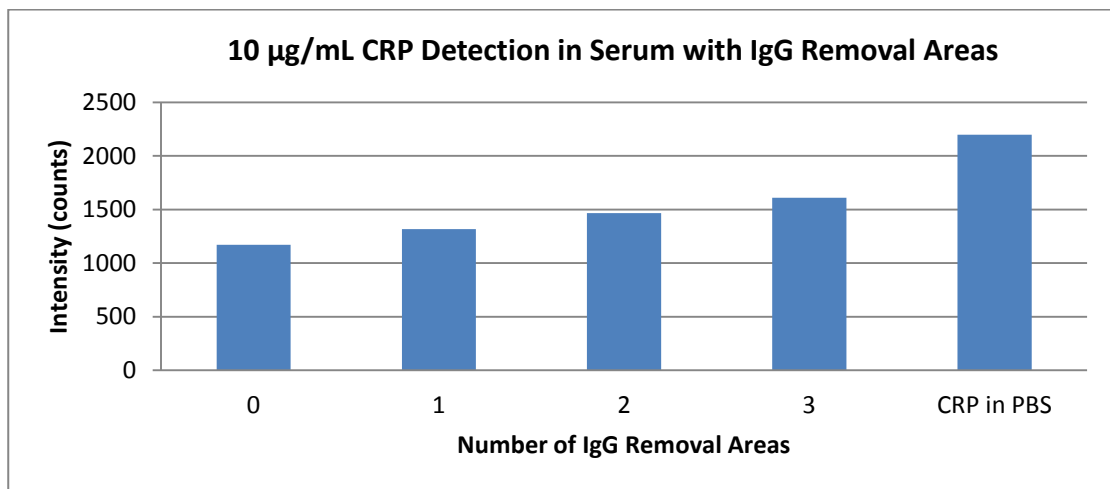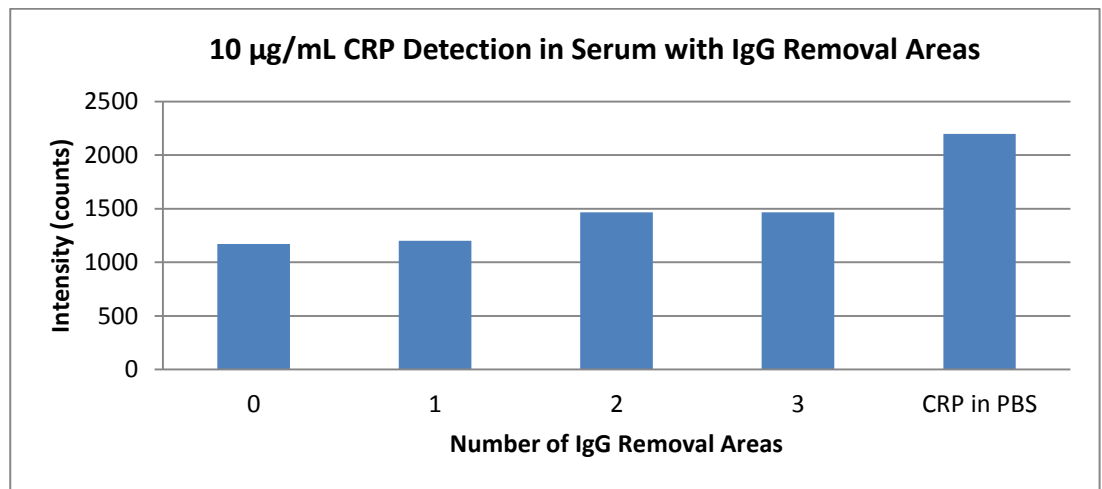


**Figure 4-19:** Bar graphs of 10 µg/mL CRP detection with IgG removal in specified removal areas

The graphs on the previous page show the same experiments above, but with serum instead of buffer. Note that the light source runs on batteries, which explains the low CRP in PBS signal. The above chart shows two experiments with serum with a CRP concentration of 10 µg/mL and with incubation areas that vary between 0 and 3. For the most part, there is a noticeable increase in intensity as the number of removal areas increase. However, once the third incubation area is added, there is no noticeable rise in intensity. It is not as easy to see with the second chart, as that there is an increase with the additional incubation area, but in the first chart, it is noticeable as that these two values are identical. This may stem from the fact that because the amount of IgG is decreasing, the fact that there could be just too little that it either makes no difference in the actual assay, or that at every 5 minute incubation areas, there is just too little IgG left in solution that grabs on to the protein. Regardless of this, it would be beneficial to observe this with other concentrations.

**100 µg/mL CRP Detection in Serum with IgG Removal Areas**
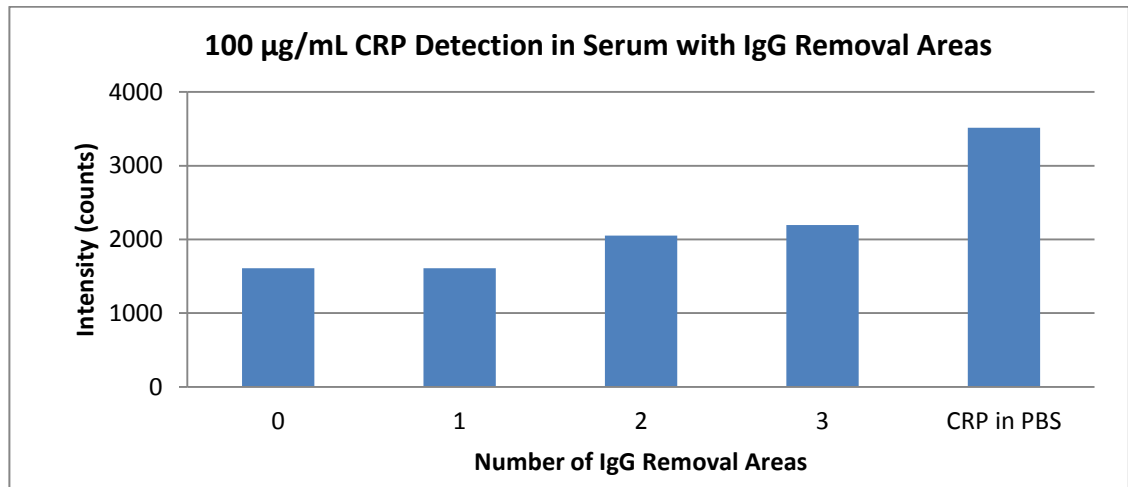
**Figure 4-20:** Bar graph of 100 µg/mL CRP detection with IgG removal in specified removal areas
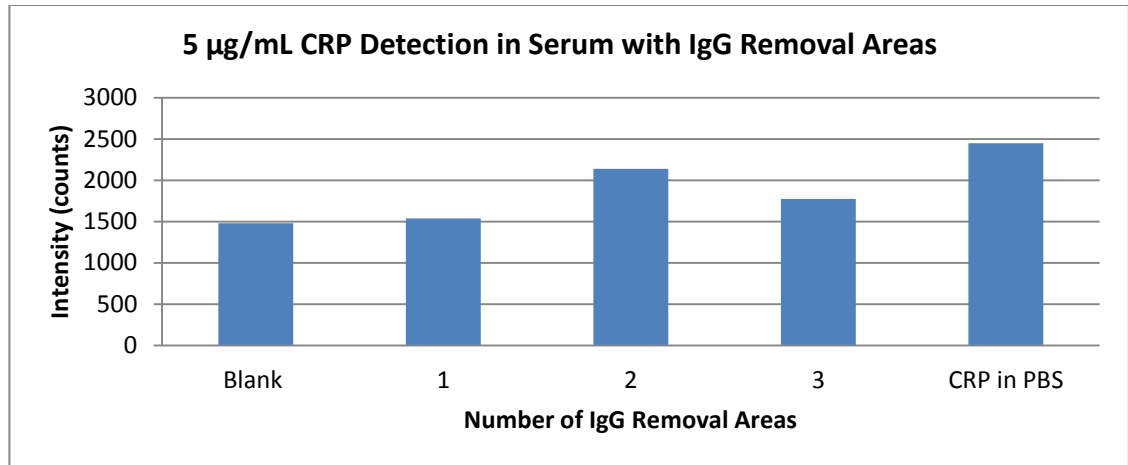
**Figure 4-21:** Bar graph of 5 µg/mL CRP detection with IgG removal in specified removal areas

In the case with 100 µg/mL concentration of CRP in serum, this follows suit as previously stated. However, with 5 µg/mL concentration of CRP in serum, there is a different trend that shows. The experiment follows the same pattern up to 2 IgG removal areas, but once the third removal area is reached, there is an issue because this value is a lot lower than that of 2 areas. This can be from the fiber optics not receiving a signal, or that something happened to the detection area that did not allow the ELISA to function properly. Regardless of this, it seems that the highest levels of detection occur with two IgG removal areas. The following graphs give a comparison to how well CRP is detected at these three concentrations, and would allow seeing the difference in intensity for all three concentrations.
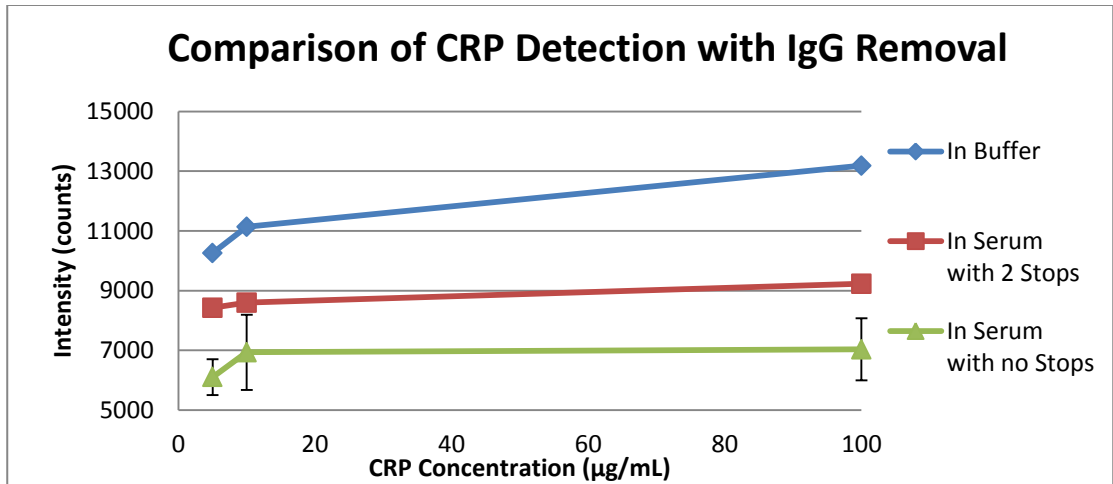
**Figure 4-22:** Line plot of CRP detection comparison with 2 IgG removal areas
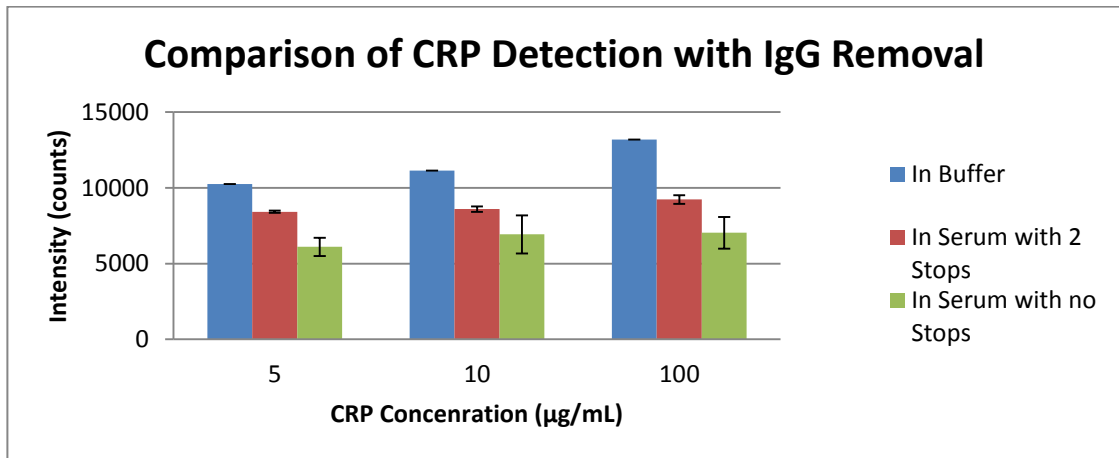


**Figure 4-23:** Bar graph of CRP detection comparison with 2 IgG removal areas

As shown in the graphs, blue represents detection of CRP in PBS, green represents CRP detection in serum without any IgG removal and red represents CRP detection in serum with two IgG incubation areas. This was performed with 3 CRP concentrations: 5, 10 and 100 µg/mL. In terms of both the bar graph and the line plot, there was a significant increase across all concentrations with IgG removal. However, the amount detected was still considerably low compared to the amount found in PBS. This means that there is interference caused by albumin in the sample. For this reason, it is safe to assume that the removal of IgG is prevalent, however, the fact that albumin still has to be removed has to be brought to attention.

## 4.3. Introduction of Albumin Removal

### 4.3.1. Order of Incubations
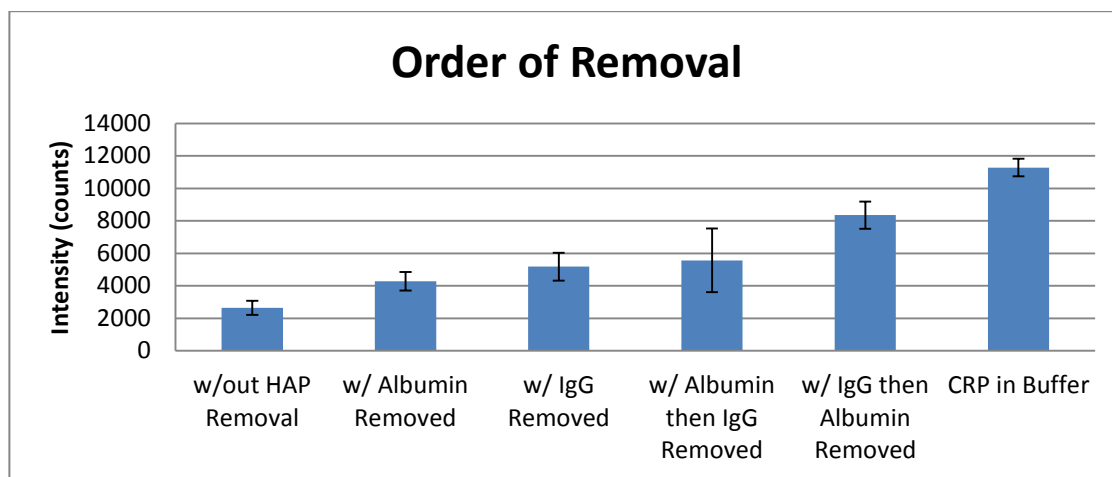


**Figure 4-24:** Bar graph representing how order of removal is affected

Once it was discovered that the removal of IgG was effective, the integration of anti-albumin IgG had to be considered. The first issue of this is in what order should the removal be (albumin → IgG or IgG → albumin). The reasoning for having the removal of albumin first is that there is more of it in serum than IgG, whereas the reasoning for the removal of IgG first is that IgG in serum can cause interference and can break away the IgG already used in capturing albumin. To perform this task, two runs were produced where CRP was detected in its normal fashion and the integration of protein removal was used. Each run was different as to the order of the removal of the protein. The first run consisted of the albumin removal first, followed by IgG removal, whereas the second run was the opposite: IgG removal followed by albumin removal. Intensity measurements were performed after each removal stage to see how well the CRP can bind in the detection area. A negative control which contained no removals and a positive control containing CRP in buffer were also used as a reference.

The results on the previous page show that when the albumin was removed first, there was as slight increase in signal. There was also a slight increase when the IgG removal came later. However, when the IgG was removed first, a higher signal was present, that basically was about the same as the signal obtained when albumin was removed, followed by IgG. A stronger signal was emitted when the albumin was removed after the IgG. Compared to the CRP in PBS detection, it was still not up to that level, but a stronger signal was found. The results from this experiment show that the preferred order for HAP removal would be the removal of IgG, followed by the removal of albumin. This follows the same reason as in Godfrin and Franzblau[23,24]. Figure 4-30 on the next page shows the completed chip with the areas highlighted for each fluid and where the areas for IgG and albumin removal would be. Keep in mind that even though one area is highlighted for each removal process, the number of incubation steps in each area will vary based on the experiment.
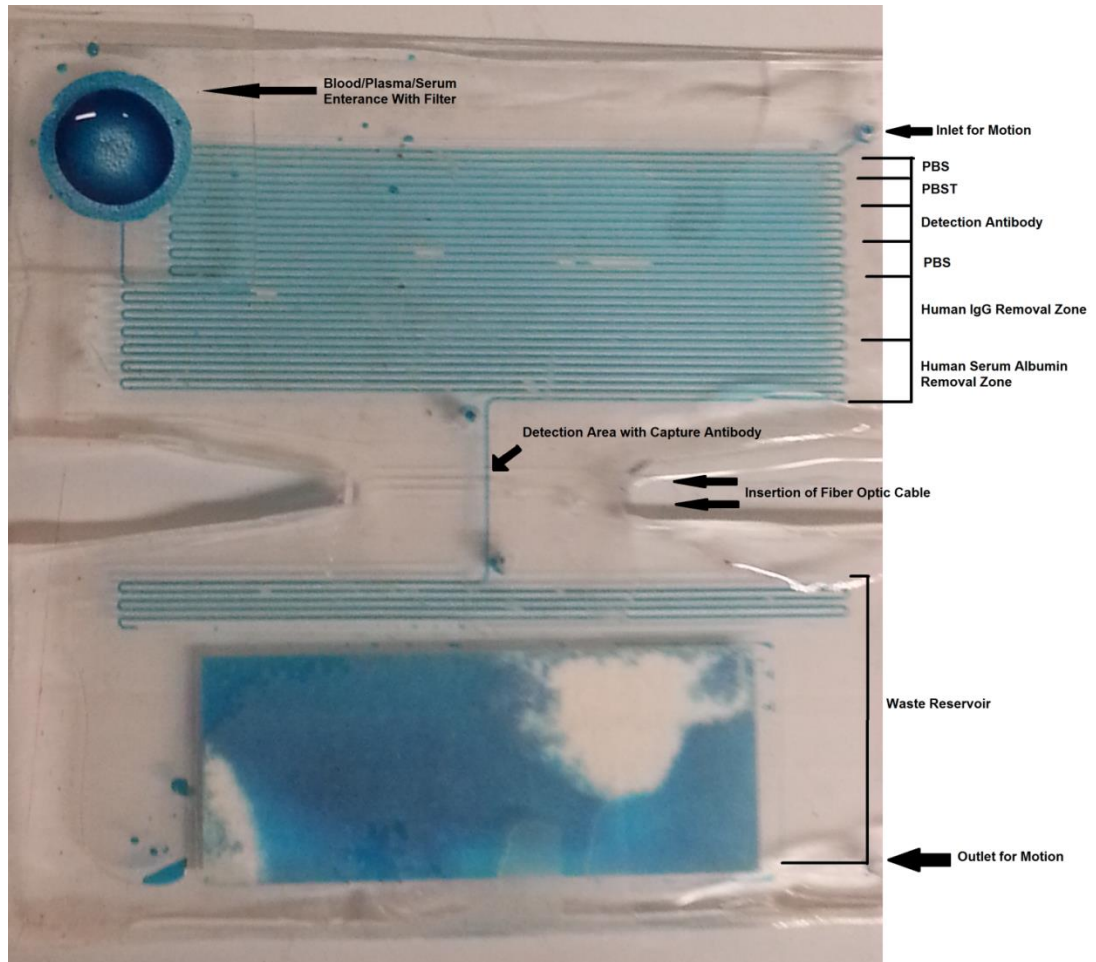
**Figure 4-25:** Microfluidic chip including blood separation and areas where IgG and albumin removal zones are located

### 4.3.2. Introduction of Albumin Removal Areas
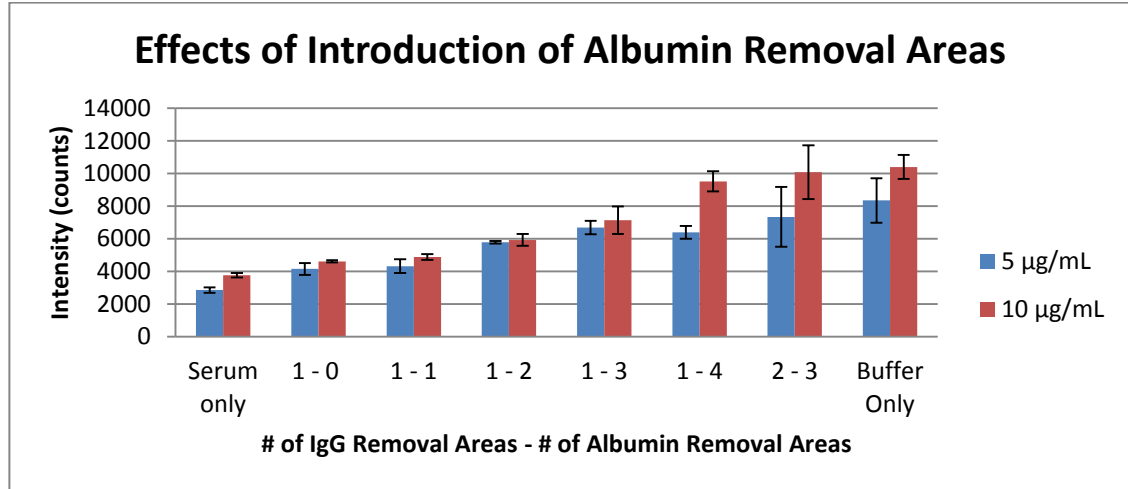


**Figure 4-26:** Bar Graph representing the addition of albumin removal areas

The graph above shows the comparison as more albumin removal areas are in place. The first experiments were performed with 1 IgG removal site, followed by an increasing amount of albumin removal areas, from zero to three, including one with just serum without any removal and one with CRP in PBS. The first experiments were performed with a CRP concentration of 10 µg/mL, represented the red bars. The initial response was that this performed well because the signal increased with the number of detection areas. However, a second experiment was performed with 5 µg/mL concentration of CRP and even though the signal also increased, the difference between the two concentrations was too small. This means that if one was to measure a CRP in this manner, for a specific intensity, the intensity range for those two concentrations would be too small to tell. Compared to the example of CRP in serum, there needs to be a wide range of intensity values for these two concentrations

To solve this, two more experiments were performed to see the reasoning behind this. Based on the highest experimental results (1 IgG and 3 albumin areas), an additional removal area was added to both experiments (one had 1 IgG and 4 albumin,

and the other had 2 IgG and 3 albumin).  Based on these experiments, there now was a noticeable difference between the two concentrations.  Each produced a high signal and each concentration was close to that of the buffer.  This can be tied in with the previous experiments, where even with one round of IgG removal; there was still a large amount of IgG remaining in the solution.  Because of this reason, and that the intensity increased during this round of experiments, the new flow method for the removal of IgG and albumin will be 2 IgG removal areas, followed by 3 albumin removal areas.

### 4.3.3.  CRP Detection in Serum
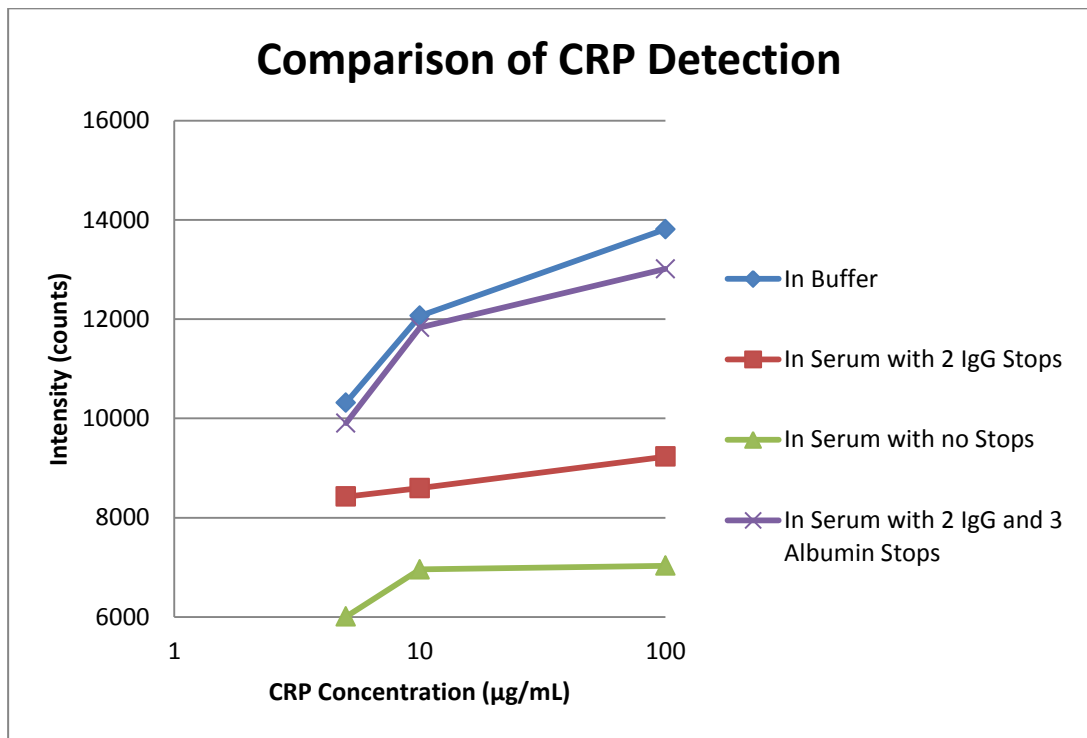


**Figure 4-27:** Linear comparison of CRP removal with 2 IgG and 3 Albumin removal areas for 5, 10 and 100 µg/mL
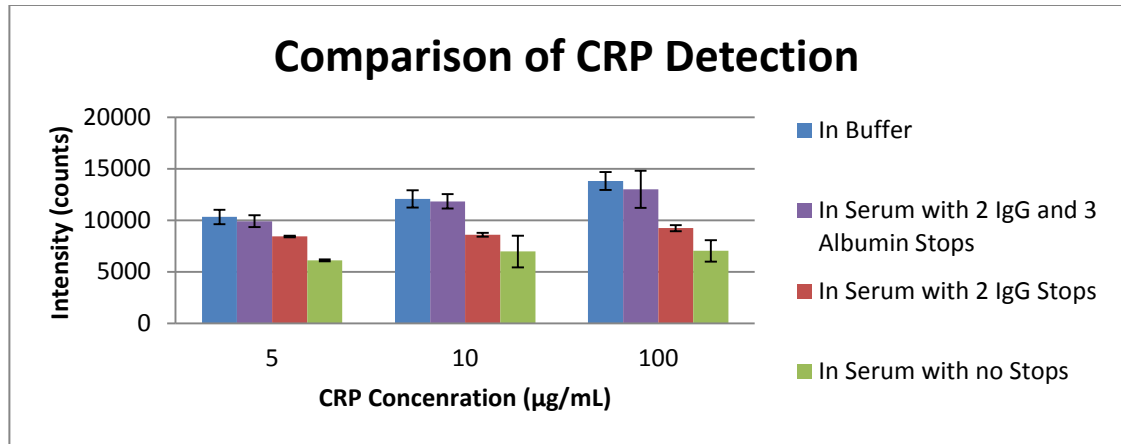
**Figure 4-28:** Bar comparison of CRP removal with 2 IgG and 3 Albumin removal areas for 5, 10 and 100 µg/mL

The above graphs show the results of the serum tests with this new protocol. In this run, 3 different concentrations of CRP were added to the CRP free serum: 5, 10 and 100 µg/mL. Each of the graphs show that a different amount of CRP can be easily identified based on whether or not serum or buffer is used. This is shown based on the different color lines on the graph. From here, we can see that a different value/range can be associated with each amount of CRP that is added.

One other thing to note is that the difference in adsorption between 5 and 10 is steeper than that of 10 and 100. This means there is a greater difference in whether or not serum has 5 or 10 versus whether or not serum has 20, 50 or 100. For this reason, it is essential to see if values can be associated with values between 10 and 100.
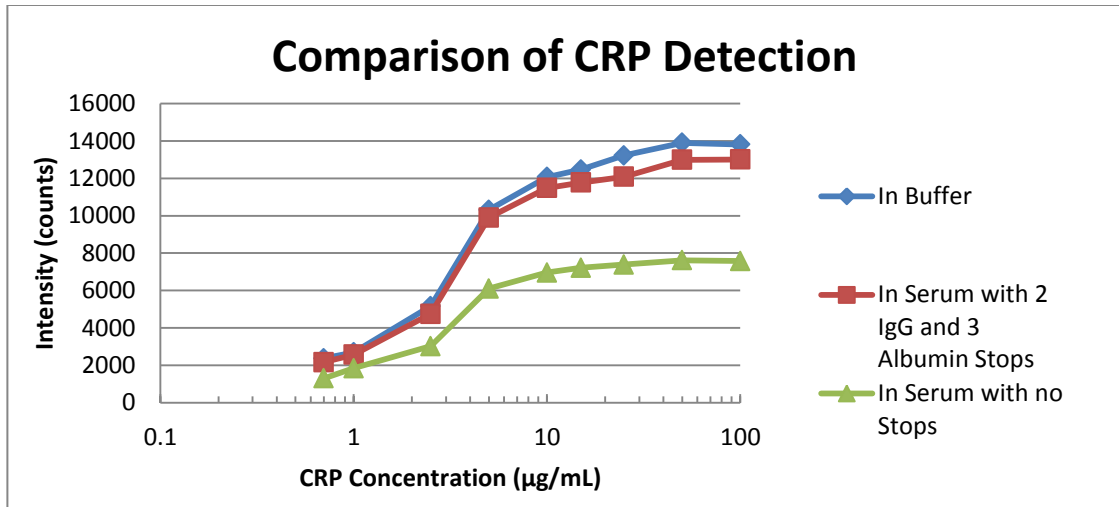
**Figure 4-29:** Linear representation of CRP detection in Buffer, Serum with high abundant protein removal and Serum without High abundant protein removal
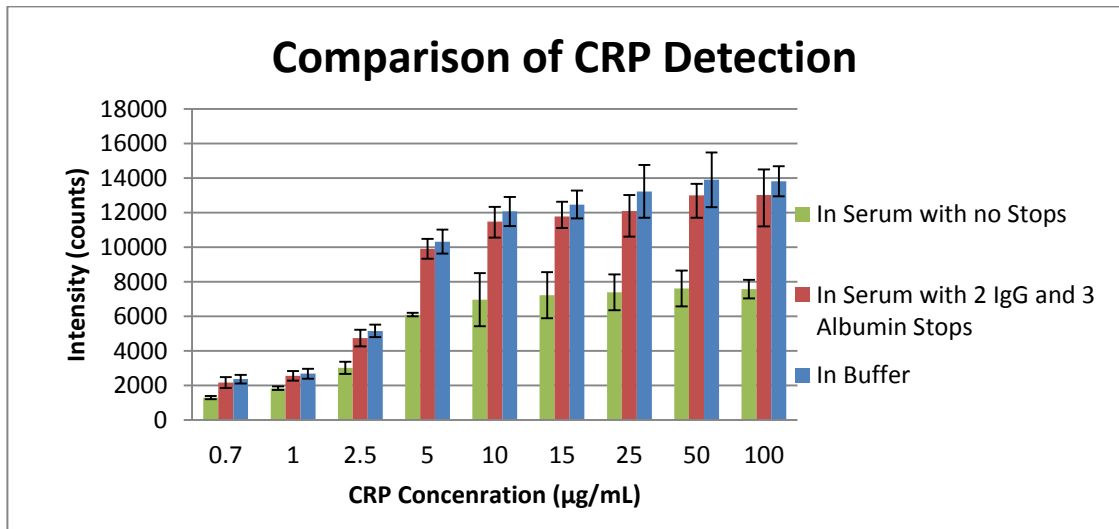


**Figure 4-30:** Bar representation of CRP detection in Buffer, Serum with high abundant protein removal and Serum without High abundant protein removal

Based on the diagrams above, there is still no change in intensity at 50 and even at 25. However, there is a slight drop when the 15μg/mL level is reached. It is at this point where an accurate measurement of CRP can be determined. It can also be seen that the detection area is fully concentrated, which means that no more CRP can be added to increase the signal. Because of this, one can see that the CRP detection area is idea for identifying samples of CRP at low concentrations, but not ones with

high concentrations. However, his can signify a good yes-no marker on whether or not a person has a high risk of cardiovascular diseases, but not an extent as to how the risk factors are.

| CRP Concentration (µg/mL) | Signal in Serum (no removal) | Signal in Serum (IgG and Alb. Rem) | Signal in Buffer |
|---|---|---|---|
| 0.7 | 1300 | 2166.4 | 2373 |
| 1 | 1840 | 2563.5 | 2685 |
| 2.5 | 3017.6 | 4747 | 5156.4 |
| 5 | 6007 | 9004.2 | 10316.43 |
| 10 | 6959 | 11486.4 | 12067.53 |
| 15 | 7215.75 | 11779.2 | 12467.53 |
| 25 | 7384 | 12088.6 | 13223.25 |
| 50 | 7618.5 | 12989.4 | 13894.83 |
| 100 | 7570 | 13010.6 | 13811.9 |

**Table 4-5:** Signals of varying concentrations of CRP in Serum (with and without IgG and albumin removal) and in Buffer

The following table depicts values obtained when the assays are performed with different combinations. The second column represents the signal in serum with no albumin or IgG removed (represented by the green line in the graph), the third column represents the signal in serum with IgG and albumin removed (represented by the red line in the graph) and the fourth column represents the signal in buffer (represented by the blue line in the graph). These numbers represent the signal emitted at specific concentrations. These vales show what has previously been stated, but in a numerical format. This shows the rise in signal compared to the rise in CRP concentration and how it varies between the three solutions. This still shows the same issue that once higher CRP concentrations are met, the signal becomes more linear due to the design of the detection area and the incubation time in said area.

Also, it shows where this change from a rise to a linear plot likely occurs. This was shown to be around $10 - 15$ µg/mL for all three curves. From this, a table can be

made where these two areas should meet.  This was based on dividing the graph into

two sections and adding a quadratic trend line on the left side and a linear trend line on

the right.  The graph showing one example is below and the table with the list of

values is also below.
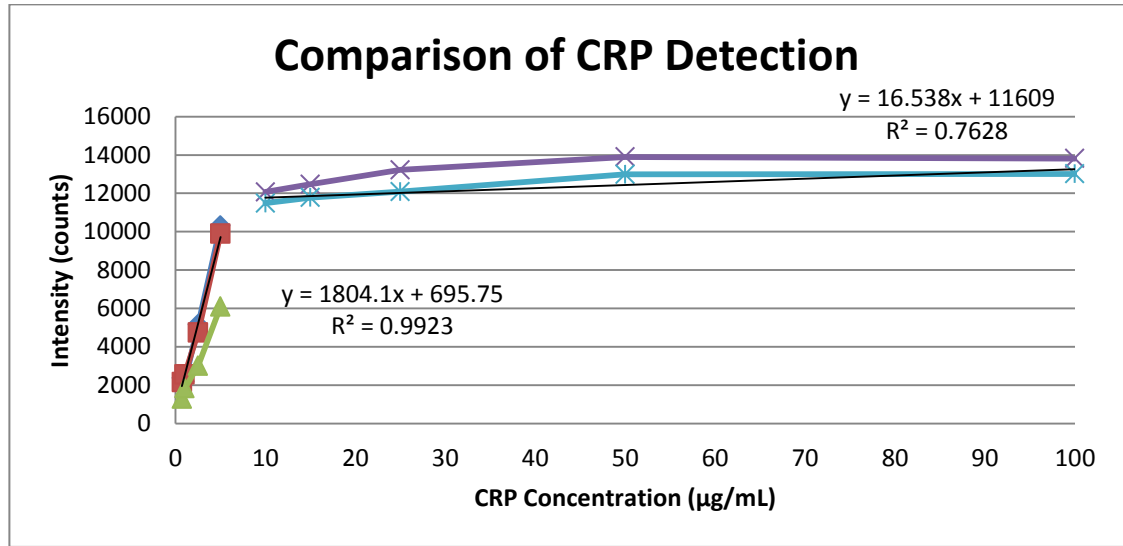


**Figure 4-31:** Plot representing where the change of signal in concentration occurs
(trend lines for serum with stops)

| Type of Curve | Eq. for Left Side | Eq. for Right Side | Concentration (@ Intensity) |
|---|---|---|---|
| Serum w/out Stops | Y = 1809x + 560.51 | Y = 5.5865x + 7126.1 | 6.224 µg/mL (@7161) |
| Serum w/ Stops | Y = 1804.1x + 695.75 | Y = 16.538x + 11609 | 6.122 µg/mL (@11710) |
| Buffer | Y = 1870.3x + 824.5 | Y = 17.541x + 12391 | 6.243 µg/mL (@12500) |

**Table 4-6:** Concentrations indicated where a rising signal turns more linear

This was based on the fact that the signal started to become concentrated

around 10 µg/mL.  As a result of this, it was shown that with the increase of

concentration, the signal started to become stagnant around 12000.  This was easily

associated with CRP concentrations less than 10 µg/mL.  This was not due to the

removal of HAPs, but more to do with the detection area itself.  The time taken to

capture CRP in the detection area was five minutes.  This was based on the previous experiments where at this time, there surface was already concentrated with CRP that no further binding could occur.  This method worked well for CRP concentrations under 10 μg/mL but over 10 produced this linear correlation whereas the concentration of CRP increased, the signal would either increase slightly, or not at all.  Perhaps the signal would be better if the incubation time was increased, but with the fact that the detection site can become saturated with CRP, this would be unlikely.  The best solution to see that there can be an increase in signal would be to increase the surface area of the detection area to accommodate this problem.  This method could be investigated further in order to see if this holds true.  In the meantime, it can be said that at CRP concentrations under 10 μg/mL, this method is preferred, but at or greater than 10 μg/mL, there is a high signal, but no clear and concise differences between concentrations, especially at concentrations over 25 μg/mL.

### 4.3.4.  CRP Detection in Blood
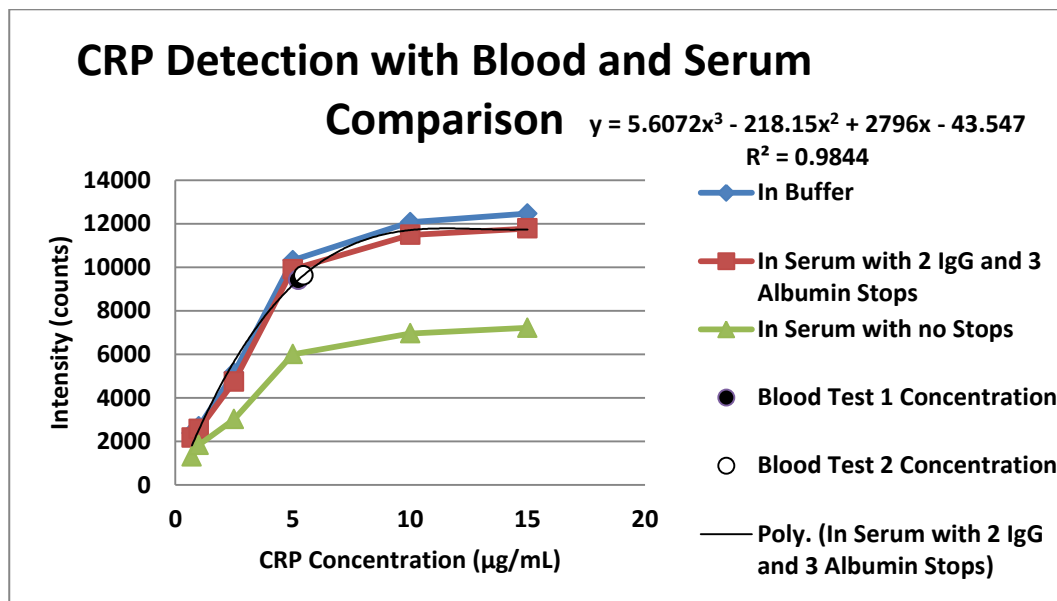


**Figure 4-32:** Graphical Analysis of CRP detection in whole blood compared to the serum results

After a few experiments in which a normal blood sample was used, a number of conclusions can be reached. First, the filter filtered of the plasma well. This meant that there was adequate amount of plasma in the channel during the experiment. Also, there was an absence of not only red blood cells, but also hemoglobin. This allowed the plasma to be a clear liquid, or a light tan liquid, which eliminates the obstruction caused by the blood cells.

Also, the points above on the graph were based on a trend line with the red line as the basis for the trend line. This line is a rough estimate as to how far outside the previous experiments were. This graph however does not take into account a point where no signal occurs. For this, a second plot was created at a CRP concentration that is close to 0 µg/mL (around 800 for intensity).
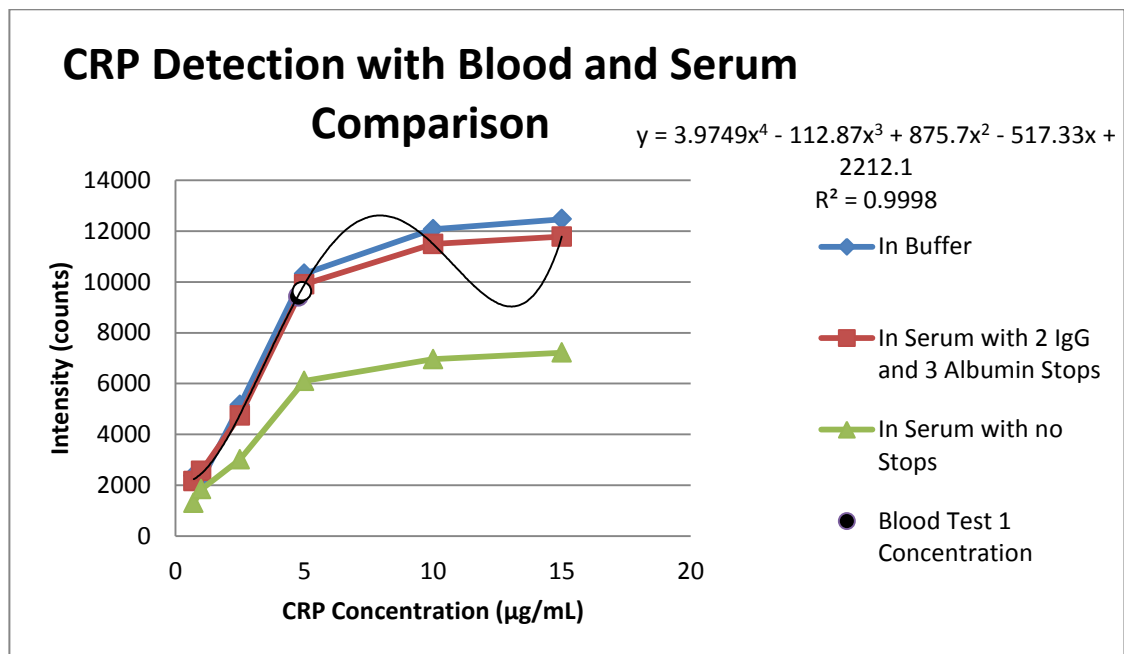


**Figure 4-33:** Graphical Analysis of CRP detection in whole blood compared to the serum results with an estimate point at a CRP concentration of 0 µg/mL

In this graph, there are two considerable differences in the trend lines. The first is that two different concentrations of CRP in blood are given. The table on the next page highlights the two differences.

| Graph | Signal Emitted | CRP Plasma Concentration | CRP Blood Concentration |
|-------|----------------|--------------------------|-------------------------|
| 4-37 | 9416.875 | 5.2323 µg/mL | 2.8254 µg/mL |
| 4-37 | 9629.125 | 5.4570 µg/mL | 2.9468 µg/mL |
| 4-38 | 9416.875 | 4.7377 µg/mL | 2.5584 µg/mL |
| 4-38 | 9629.125 | 4.8526 µg/mL | 2.6204 µg/mL |

**Table 4-7:** Values of CRP in whole blood based on experiments and figures 4-32 & 4-33

Even though the values represented in figure 4-33 are less than that of figure 4-32, they do represent the curve better since they are more likely to be found on the red line than in the figure 4-32. This makes this second figure more ideal in estimating the value of CRP in blood. However, the first graph is better at estimating values greater than that of 5 µg/mL concentration. For this reason, in order to accurately see what kind of plot can be obtained from these experiments, the two equations created from the trend lines were set in order to solve for the intersecting point that was accurately depicted on the plots. From this, it was shown that the two trend lines intersected at a concentration of 3.856 µg/mL at an intensity of 7816. Any values lower than this was fitted with the trend line form figure 4-33 and values greater than this was fitted with the trend line from figure 4-32.
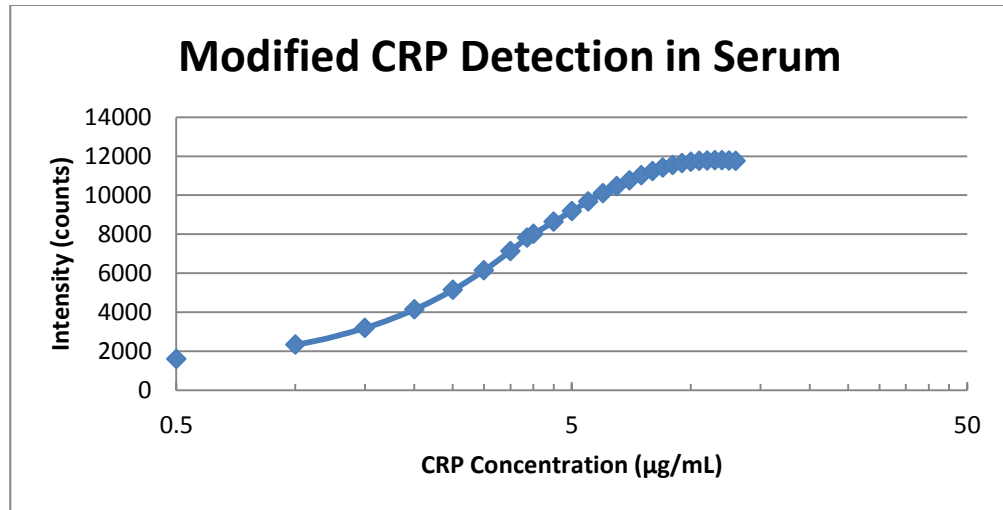
**Figure 4-34:** Representation of possible CRP detection in Serum

The graph above shows the possibilities of the detection of CRP as each specific concentration is associated with an intensity value. However, these values are for serum alone, which was in the detection area. In order to see the values of CRP in blood, all the serum values would have to be multiplied by 0.54 since 54% of the total volume of blood is serum. The graph for the blood is shown below, which is nearly identical to that of serum, but with a different X-axis. The two black dots represent the two experiments with blood being used as a fluid.
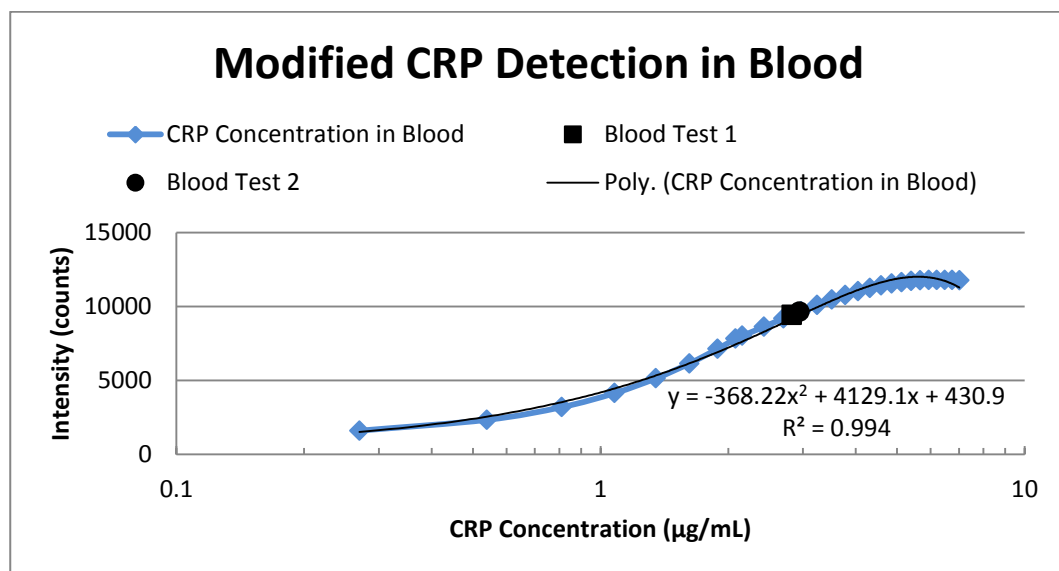


**Figure 4-35:** Representation of possible CRP detection in Blood

The following table on the next page represents the intensity values associated with the graph at various CRP concentrations.

| Intensity | CRP in Serum (µg/mL) | CRP in Blood (µg/mL) | Intensity | CRP in Serum (µg/mL) | CRP in Blood (µg/mL) |
|---|---|---|---|---|---|
| 1605.66 | 0.5 | 0.27 | 9668.31 | 5.5 | 2.97 |
| 2337.37 | 1.0 | 0.54 | 10090.20 | 6.0 | 3.24 |
| 3197.06 | 1.5 | 0.81 | 10453.49 | 6.5 | 3.51 |
| 4142.88 | 2.0 | 1.08 | 10762.37 | 7.0 | 3.78 |
| 5136.75 | 2.5 | 1.35 | 11021.05 | 7.5 | 4.05 |
| 6144.37 | 3.0 | 1.62 | 11233.74 | 8.0 | 4.32 |
| 7135.25 | 3.5 | 1.89 | 11404.64 | 8.5 | 4.59 |
| 8008.91 | 4.0 | 2.16 | 11537.95 | 9.0 | 4.86 |
| 8631.87 | 4.5 | 2.43 | 11637.89 | 9.5 | 5.13 |
| 9183.60 | 5.0 | 2.70 | 11708.65 | 10.0 | 5.40 |

**Table 4-8:** Values of CRP in serum and whole blood based figure 4-40

On the graph, it is worth noting that all CRP concentrations in blood greater than 5.5 tend to converge at 12000. Based on the previous experiments with serum only, the signal can get over 14000, but this requires a blood CRP concentration greater than 10 µg/mL. However, from the introduction and the literature review, a high risk of cardiovascular disease occurs at 3.0 µg/mL concentration of CRP in blood (5.56 µg/mL in serum), this chart works well in the acceptable range of an individual with either a low or a moderate risk of cardiovascular disease. For more extreme cases however, it is still functional, but there is known error due to the small spacing between the intensity values and CRP concentration. Therefore, according to the table and chart, a reading of under 4000 means that a patient has a low risk of cardiovascular disease, a reading between 4000 and 10000 signifies a moderate risk and a reading over 10000 signifies a high risk. According to figure 4-40, the blood samples gave off a reading of about 9500, which translates to a CRP blood level of

2.87 µg/mL concentration of CRP in blood (5.3 µg/mL concentration of CRP in serum), which translates to a moderate risk of cardiovascular disease.

Since there is no definitive method of checking to see if removal of IgG and albumin has occurred in the channel, it would be best to detect the amount of CRP in the plasma and then compare it to the amount taken by the graph made in the previous experiments. In this case, the fiber optics recorded a signal for two measure measurements of blood of 9416 and 9629, which correlates to a CRP plasma concentration of 4.78 µg/mL and 4.91 µg/mL, respectively. In order to obtain the blood concentration, since the mass of CRP is constant and adding cells increases the volume, a good estimate would to take the concentrations and divide by the amount of plasma in blood (54%). This will give CRP blood concentrations of 2.58 µg/mL and 2.65 µg/mL respectively.

This shows that it is in the normal range of serum as in the previous tests. However, one thing of noting is that fibrinogen could still be in the plasma. This could still cause some interference within the detection area and/or the incubation areas. In order to see if this experiment is performed well, this has to be taken into account. This will be talked about in more detail in the upcoming section. Other than this, it was shown that the plasma was filtered well and that a respectable signal could be found with the addition of blood in the detection of CRP.

### 4.3.5. Limit of Detection

This section will explain the different methods for limit of detection that were used in this thesis. The first method implemented was the use of direct observation. Using the experimental curve with the IgG and albumin removal, it was found that the

lowest limits in which CRP that can be detected in which there was a persistent increase in intensity was at 0.7 and 1.0 µg/mL. This was shown in figure 4-34. Averaging these two values together gave a limit of detection of 0.85 µg/mL CRP in serum, or 0.46 µg/mL CRP in blood.

The second method implemented was the signal to noise method of determining limit of detection. The four lowest values that were detectable were plotted and a linear trend line was also plotted in order to show the correlation between these values. The standard deviation was also calculated for all the points and this was placed with the slope in equation 1 in chapter 3. The result of this was a limit of detection of 0.7 µg/mL CRP in serum and 0.39 µg/mL in blood. Using equation 2, the limits of quantification for both serum and blood respectfully were 2.12 and 1.17 µg/mL. The graph displaying the slope is shown below in figure 4-36.
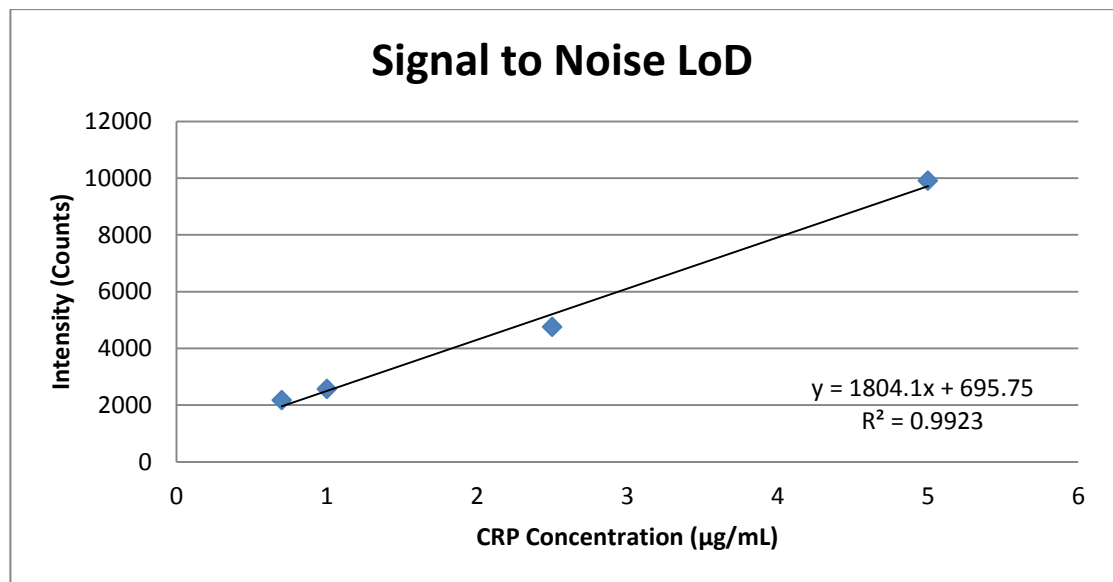


**Figure 4-36:** Trend line for limit of detection using signal-to-noise

The final method implemented was performed using the limit of blank. In this method, assay tests were recorded with no CRP or antibody signal. The mean and standard deviation was recorded and placed into equation 3. The result of this was a

limit of blank of 0.82 µg/mL.  This was placed into equation 4 with the standard

deviation of the lowest measurement (0.7) and a limit of detection was found to be

1.10 µg/mL for serum and 0.61 µg/mL for blood.  The limit of quantification was

found using equation 6 to be 3.31 µg/mL for serum and 1.82 µg/mL for blood.  Table

4-9 summarizes the methods and values obtained for LoD and LoQ.  The discussion

about the limit of detection will be addressed in chapter 5.

| Method | LoD Serum | LoD Blood | LoQ Serum | LoQ Blood |
|---|---|---|---|---|
| Observation | 0.85 µg/mL | 0.46 µg/mL | 2.55 µg/mL | 1.38 µg/mL |
| Signal:Noise | 0.70 µg/mL | 0.39 µg/mL | 2.12 µg/mL | 1.17 µg/mL |
| Limit of Blank | 1.10 µg/mL | 0.61 µg/mL | 3.31 µg/mL | 1.82 µg/mL |

**Table 4-9:** Summary of limits of detection and quantification for blood and serum

# CHAPTER 5

## CONCLUSION

### 5.1.    CRP Detection

Based on the results of the previous experiments, there are a number of conclusions that can be made. The first conclusion is that minute amounts of blood be effectively filtered and have no trace of blood cells anywhere.  This is due to the construction of the filter membrane in that it is very hydrophilic and has pores large enough to allow plasma to pass through and keep blood cells out.  Also, there was no sign of either blood cells escaping or hemolysis occurring.  This means that all of the filtrate entering the chip was plasma.  This by itself eliminates the majority of objects that can cause obstruction with the ELISA in the detection area.  Because of this, a chip was created that was able to incorporate blood filtration by using separation membranes.

In addition to this, it is possible to detect CRP in any medium, including buffer, serum and whole blood. It is even possible to detect it without the extraction of small proteins. However, because of the interference between the capture antibodies and high abundance proteins, it was shown not only to be more difficult, but there is no variance between different amounts, as compared to detecting CRP with buffer. It is also worth noting that protein A can effectively capture human IgG in serum. One thing though that has to be pointed out is that there is a limit as to how well protein A can hold on to IgG. Usually over 5 minutes of incubation time, the protein becomes

too saturated with antibody that the antibodies start to fall off the protein or the protein breaks off completely from the channel. This should explain why the signal is lower at incubation times over 5 minutes. This was one of the reasons why multiple incubation areas had to be included on the chip. The other reason being that the amount of IgG in serum is far greater than that of CRP (coupled with the amount of human albumin). In order to obtain a better signal, a method of removal of more proteins/antibodies has to be used.

Based on the design of the chip, two incubation areas were originally created: one for IgG removal and one for albumin removal. The channels in the chip between the filter and the detection area can hold about 50 microliters of fluid at one time. Since 5 microliters of serum are only needed for each experiment, it would be best if multiple incubation areas were introduced. This would be performed for both the IgG and albumin incubation areas. In the following experiments, it was shown that the signal for CRP did increase with each additional incubation area, but it is also important to know that the rise in signal between 2 and 3 areas is lower than between 1 and 2. It is best to say that there should be at least two incubation areas just for IgG removal. Also, before any experiments are performed with albumin, it is safe to say that there should be at least 2 incubation areas for albumin based on the fact there is a greater concentration of albumin than there is of IgG.

The next experiments involved seeing whether or not removal of IgG or removal of albumin would come first, based on this assessment, it was determined that the removal of IgG would come first because there would not be any interference between the IgG in the serum and the IgG already on the protein A. This is based on

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the graphical data, where there was a higher final signal with IgG then albumin than the other way. From the previous experiments, it was then time to test the effects of albumin with one base sample (10 µg/mL concentration of CRP). This first experiment increased the amount of albumin detection spots, which came after 1 IgG incubation area. The results showed that as the amount of incubation areas increased, the signal in the system also increased. The same theory applied when a lower concentration was used (5 µg/mL). The experiment revealed that the signal also increased. However, when the two results were compared there was no clear separation in the intensities. There was a difference in the intensities, but it would not be considered great. In order to make sure that this is functional, there has to be a large enough gap between 5 and 10 µg/mL of CRP. One of the reasons for this being that there is only one IgG incubation area. This causes interference not only in the detection area, but also with the anti-albumin IgG. Because of this, a second experiment was performed, with 2 IgG and 3 albumin incubation areas, and 1 IgG and 4 albumin areas.

It was also shown that with concentrations of 5 and 10 µg/mL of CRP, there was a distinct difference in the levels in intensity. This difference in intensity was between 1500 and 2500 for 5 and 10 µg/mL. This was provided that there had to be 2 IgG removal areas, followed by 3 albumin removal areas. Because of this, it would be essential to remove as much IgG in serum before albumin can be removed. For this to occur, at least two IgG incubation areas have to be established in order for this interference to be mitigated.

As a comparison between the two previous tests, a higher concentration was performed (100 µg/mL). The results of this test showed that there was a rise in signal with the increase in concentration. However, the rise in concentration was not as high as compared to the lower concentrations. The reason for this being either the incubation time in the detection area is too low or the detection area is too concentrated with CRP. Because of this, it would be ideal to see what the maximum concentration that the detection area can hold. As it turns out, with different dilutions, it was shown that a decrease in the signal occurred at15 µg/mL in serum. This is not very high considering that this is around the lowest concentration in which the CRP level is in the "danger zone". Because of this, this detection system can inform the user whether or not his CRP level is too high, but not to the extent of how high it can go. However, it can give the level of CRP in a healthy person accurately.

The results of the previous experiments resulted in a curve that can be used as a correlation between intensity and CRP concentration. This is shown as the red line in the graphs in chapter between the blue line representing CRP in buffer (positive control) and the green representing CRP in serum without protein removal (negative control).  As shown in the graph, the red line is very close to the blue line, which means that the process of IgG and albumin removal before the detection of CRP worked well. Compared to the line for CRP in serum without any removal, it is on a level about 2 times that. Is it possible to achieve a higher signal compared to the previous experiments, yes, but that will require another incubation area (either a third for IgG or a fourth for albumin), and even then, this does not factor in other proteins in serum. Even with the extra incubation area, the intensity would only go up by a few

hundred. The important thing is that there is a reasonable curve that can be used to detect an amount of CRP in serum, even though it can only accurately detect concentrations less than 15 µg/mL.

As for the tests with whole blood, the experiments worked well from top to bottom. There was an ample amount of blood that was filtered well into the chip without any blood cells entering or rupturing. The plasma did go through all 5 incubation areas in the chip and CRP was detected (there was a detection signal emitted) in the detection area. One thing to point out is that the signal shows a concentration, compared with a trend-line that was less than 5 µg/mL concentration. The addition of a 2.5 µg/mL test has shown that there was a decrease in intensity by about 3000. However, with tests at 1 µg/mL, the decrease was around 1200, with an addition 500 decrease around 0.7.  This shows that while it is possible to detect low amounts of CRP, at concentrations under 1 µg/mL, it becomes more difficult due to the relative amount of CRP compared to IgG and albumin.  While this test shows that it is possible to detect CRP in whole blood, there are still three issues that have to be dealt with. First, the blood that was used was from one source. If multiple sources were tested, one could see that there could be varying patterns between the different blood types. It is not as easy as inserting a fixed concentration of CRP because one would have to account for the CRP that is currently in the blood. Second, this is CRP detection from a planned detection system. If it could be possible, an actual measurement of CRP would have to be performed to see if this method would hold water. Finally, the previous tests used serum as a medium for CRP. This new test used plasma as a medium. The main difference between the two is the presence of

fibrinogen. Fibrinogen is not as abundant as albumin, but it is a larger protein and it can cause blood to coagulate when it is outside of the human body. One set of future experiments could be to insert a sixth incubation area that removes fibrinogen and see if the CRP detection changes.

From the results of these experiments, a few advantages and disadvantages can be pointed out. The first advantage is that not only is CRP detectable in serum and blood, but it is within the area of using CRP in a buffer solution with the same detection. This means that a wide range of signals that are normal can be found and there can be different absorbance values associated with them. The second advantage is that blood that was either not de-coagulated with EDTA or diluted worked in the system. This means that the system can be used outside of a laboratory setting where a prick on the finger for blood extraction can be performed. One final advantage is that this can qualify as an LOC – POCT device, in which patient care can be performed on the spot and the amount of blood that can be obtained is less than 50 μL per patient.

However, there are a few disadvantages to this process. The first of which being the time taken to perform the assay. It takes on average between 50 and 60 minutes to perform the assay, with the majority of the time spent on IgG and albumin removal. It is true that the majority of the IgG and albumin can be removed within 2 - 3 minutes per incubation, but this experiment used 5 minutes as a time in order to effectively fill the channel surface effectively with IgG or albumin. If a 2 – 3 minute incubation time is used, it might make the time elapsed half of what this experiment calls for (20 – 30 minutes). A second disadvantage is the detection area. As

performed in the experiment, a titration curved was created for the detection of CRP in serum and blood. Unfortunately, any blood CRP concentration greater than $5 - 6$ μg/mL kept the curve stationary, meaning that the same signal was still going off. Because of this, there is no way to determine the extent of detection for high CRP patients without a simple yes or no. In order to fix this, either a better detection area has to be created, or another incubation area time has to be utilized. One final disadvantage is that multiple tests still have to be performed on a patient. This is due not only due to the fiber-optic signal, but also that CRP levels can vary from person to person within a few days. Because of this, multiple readings have to still be implemented in order to obtain a good reading. Despite these disadvantages, this method has shown that for normal CRP levels, this method of CRP detection from whole blood is ideal.

In terms of the limit of detection, it has been shown that this system is able to detect minute amounts of CRP in whole blood ($< 0.5$ μg/mL in whole blood). However, both the signal-to-noise and the limit of blank methods display two different values. This is due in part to the signal achieved due to the blank. Without a chip in place in the system, the level of intensity is around 800. This is fine for the method of signal-to-noise because it assumes a intensity of 700 at no CRP concentration (see figure 4-41), but in the case of limit of blank, it has been shown that with a blank, there is still an intensity level of 1900 that has been detected, with a standard deviation of around 150. The lowest experimental level is at 0.7 μg/mL and the intensity values have shown have a value of around 2100 and a standard deviation of around 300. With the calculations, it was shown that the limit of detection was doubled using the

limit of blank.  As a comparison, a second signal-to-noise ratio was conducted, as seen in figure 5-1.
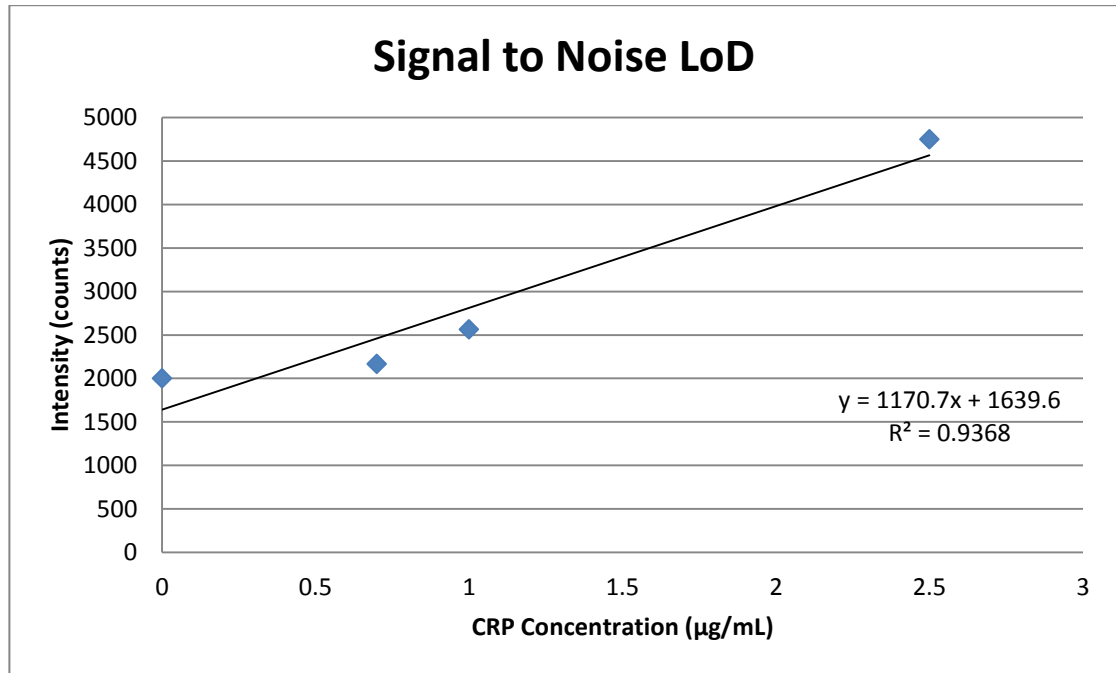
**Signal to Noise LoD**



**Figure 5-1:** Signal-to-noise LoD calculation with emphasis on the blank point

This time, this calculation was based on the blank measurement.  The result showed that LoD for serum and blood were the same as with the LoB method (1.11 µg/mL & 0.61 µg/mL respectfully).  Because of the blank intensity being that high, this was shown in the LoD calculations.

As a comparison for this project, it would wise to take a look at the results from Godfrin[23].  In his set up, only there was only one incubation area for each HAP removal.  These incubations was only for 7 minutes each, whereas the incubations for this report were for only 5 minutes based on the protein A experiments.  Also, his included a blocking step where BSA was used to eliminate any non-specific binding, whereas this project did not incorporate it, since it relied more on the protocol from Li[22] and Franzblau[24].  Furthermore, the CRP incubation time was longer than the

one used in this report (10 min as compared to 5 minutes). Because of this, the

majority of time used in CRP detection in Godfrin[23] whereas the majority of the time

was used in HAP extraction. The table below highlights the times needed to perform

each step.

| Incubation Step | Time via Godfrin (min) | Time via Pytka (min) |
|---|---|---|
| Capture Antibody | 7 | 7 |
| Blocking w/ BSA | 5 | 0 |
| IgG Removal | 7 (1 @ 7min) | 10 (2 @ 5 min) |
| Albumin Removal | 7 (1 @ 7 min) | 15 (3 @ 5 min) |
| Sample (CRP) | 10 | 5 |
| Detection Antibody | 5 | 5 |
| PBST | 5 | 5 |
| Rinse, Pumping & Detection | 4 | 8 |
| Total | 50 | 55 |

**Table 5-1:** Comparison between CRP detections based on the Godfrin[23] and Pytka systems

Even though these differences apply, the system used in this report was only 5

minutes longer than the one used by Godfrin. Knowing that less than 5 minutes was

an acceptable time for the incubation, if this was implemented, it amount of time for

the process would decrease by about 20%. Also, it would be ideal to look at the

comparisons graphically, which are shown in figures 5-2 to 5-4.
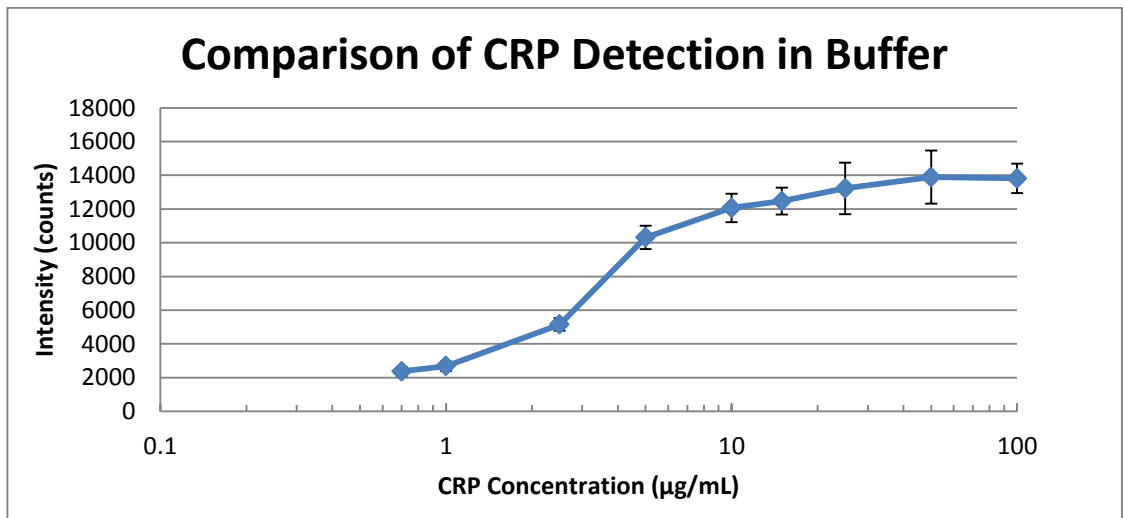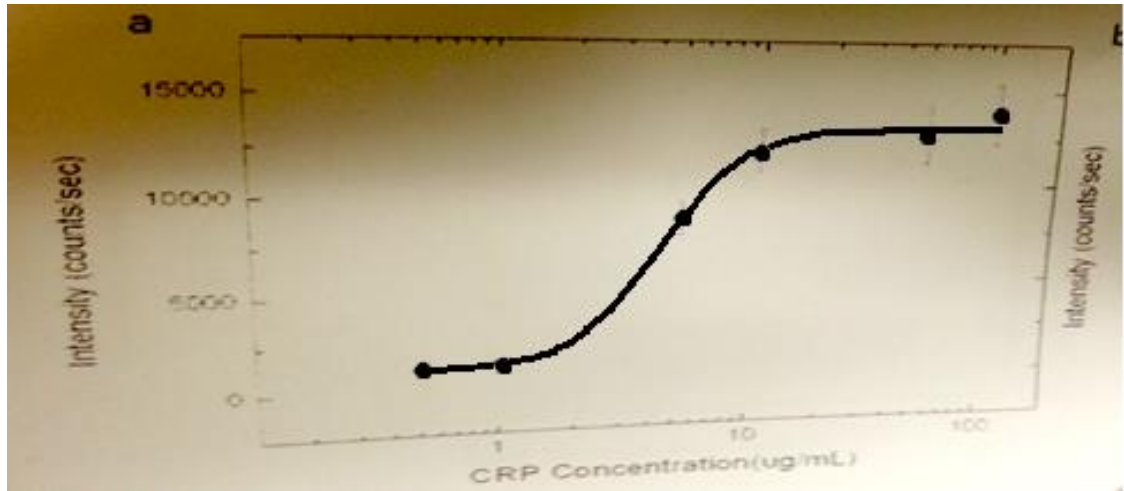
**Figure 5-2:** Comparison of CRP detection in buffer graphs between the Godfrin[23] project (top) and this project (bottom)

The first comparison is the CRP detection in PBS buffer. Based on the results of these graphs, it was shown that both graphs are similar not only in shape, but also in the intensity values. Based on this, both graphs can be used as a bench mark for a comparison with serum and blood.
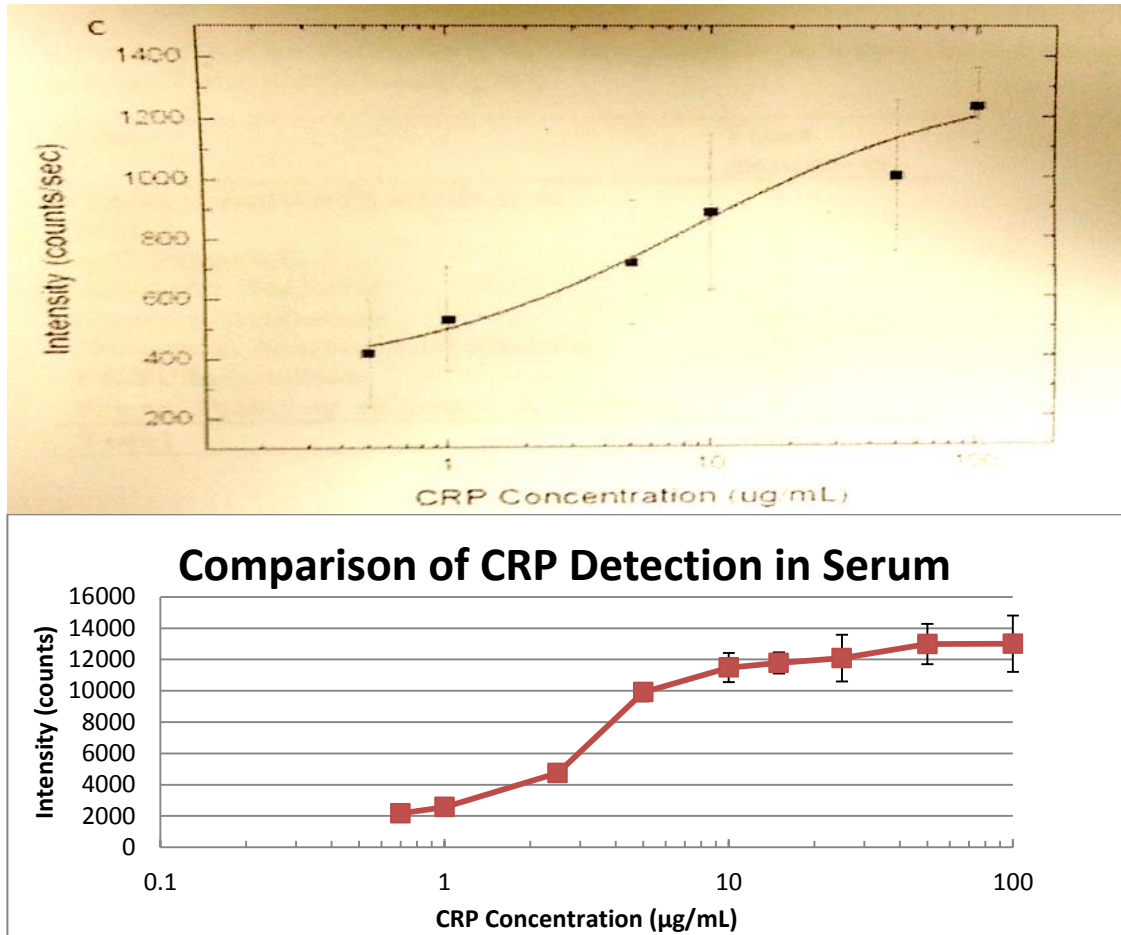
**Figure 5-3:** Comparison of CRP detection in serum graphs between the Godfrin[23] project (top) and this project (bottom)

In the serum tests, there are a few key factors to point out. First of all, the Godfrin test used a diluted serum (10:1 PBS:Serum) in order to test the functionality of the system[23]. This report, on the other hand, did not use diluted serum, which means that the normal IgG and albumin concentrations in serum were kept intact. Even with this modification, it was still shown that the rise in intensity in the Godfrin experiments were not as great as the experiments in this report. This could be partially based on the amount of HAPs still in the serum. There was a rise in the intensity as concentration increased, but compared to the values to the bottom graph, it was not high enough and did not compare to the increase in intensity based on the CRP in

buffer curves. The bottom graph, on the other hand, matched almost exactly the buffer curves. Table 5-2 on page 118 shows the percentages of CRP detected in serum compared to the buffer curves for both experiments.
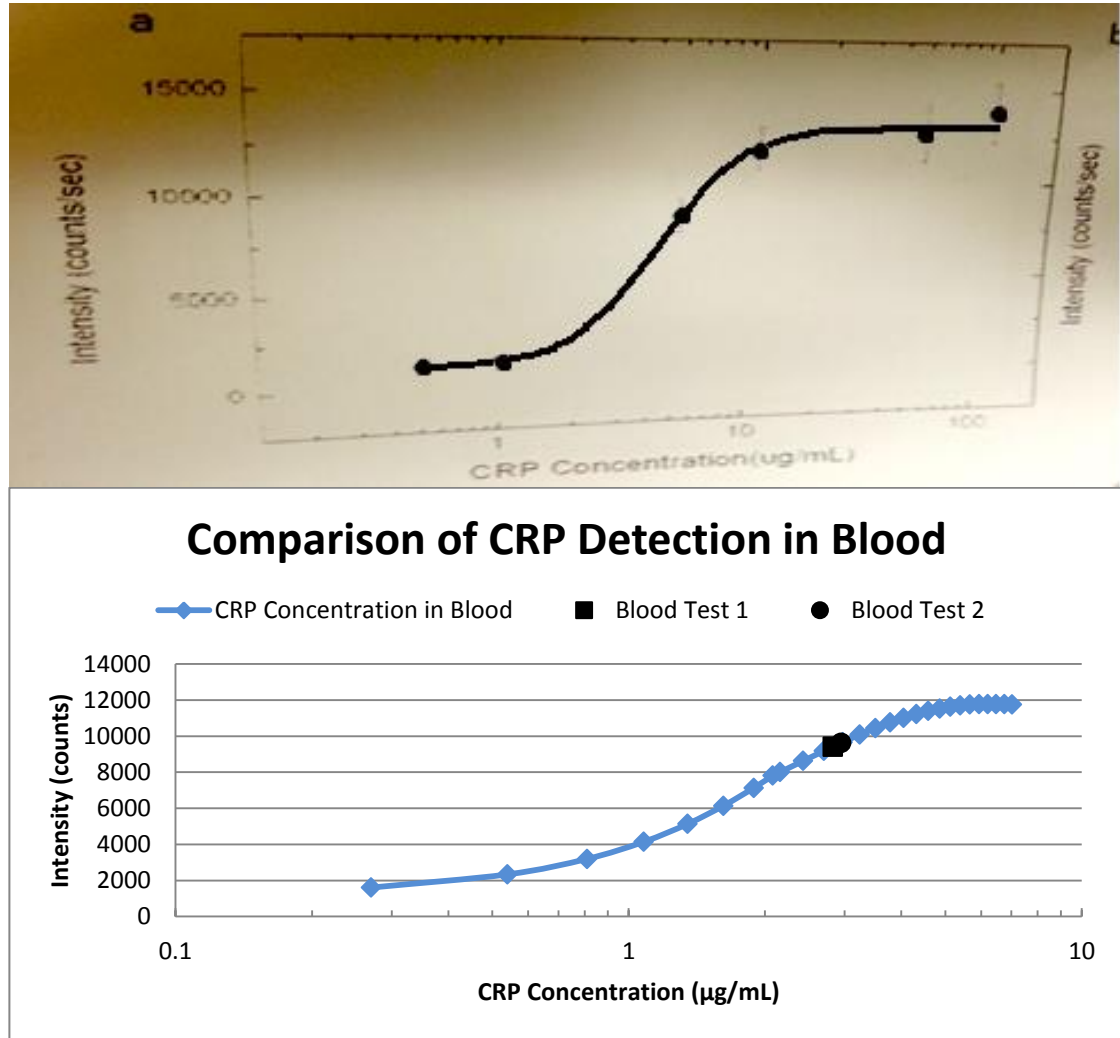


**Figure 5-4:** Comparison of CRP detection in blood graphs between the Godfrin[23] project (top) and this project (bottom)

For the results based on whole blood tests, keep in mind that the CRP concentration in serum is larger than that of whole blood. This is due to the removal of cells that contribute to 45% of the total volume in blood. This is important because the Godfrin tests used blood doped with a certain CRP concentration[23], whereas this project estimated the CRP concentration by multiplying the concentration CRP in

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serum by 0.55.  Because of this, only the lower end values for the Godfrin graph apply

here.  The Godfrin graph shows a rise in intensity based on the CRP concentration, but

it still was smaller than that in diluted serum (see figure 5-2) for each concentration.

The blood values obtained in this project used the assumption the only difference

between the serum and blood graphs were the loss of 45% of the blood volume.  Thus,

taking a look at both graphs, up to 5 µg/mL concentration, there is a substantial rise in

concentration in both graphs, but the Godfrin plot is too low compared to the plots in

this project.  Tables 5-2 and 5-3 shows the specific CRP concentrations of both

projects and compares them to that of the concentrations for each one in buffer.

| Godfrin Project | | | | Pytka Project | | |
|---|---|---|---|---|---|---|
| Conc. | C (Se) | C (Bu) | % Det. | C (Se) | C (Bu) | % Det. |
| 0.5 | 405 | 2200 | 18.4% | 2166 | 2373 | 91.3% |
| 1.0 | 510 | 2250 | 22.7% | 2564 | 2686 | 95.5% |
| 5.0 | 700 | 7500 | 9.33% | 9904 | 10316 | 96.0% |
| 10.0 | 930 | 14000 | 6.64% | 11486 | 12067 | 95.2% |

**Table 5-2:** Comparison of detectable CRP in serum amounts and percentages detected
for both projects

| Godfrin Project | | | | Pytka Project | | |
|---|---|---|---|---|---|---|
| Conc. | C (Blood) | C (Buffer) | % Det. | C (Blood) | C (Buffer) | % Det. |
| 0.5 | 490 | 2200 | 22.3% | 2337 | 2373 | 98.5% |
| 1.0 | 450 | 2250 | 20.0% | 4100 | 4300 | 95.3% |
| 5.0 | 590 | 7500 | 7.87% | 11600 | 12000 | 96.7% |

**Table 5-3:** Comparison of detectable CRP in blood amounts and percentages detected
for both projects

The Godfrin plots can only detect normal CRP in the intensity range of 450 –

600.  This system on the other hand, can detect CRP in the intensity range of 1600 -

12000.  This means that because of the high range, there is a small window of error

between two distinct concentrations (for example, a 1.0 µg/mL difference in

concentration).  Even though there is still error in this new system, it only varies about

0.5 µg/mL compared to 1.0 µg/mL. Even though the CRP could be detected accurately with a concentration less than 5.5 µg/mL concentration in blood, this is still within the range of normal CRP levels in blood. This difference is due to the amount of HAPs still in the serum during CRP detection. This was shown in the amount of CRP recovered in each of the experiments. The Godfrin experiments only recovered about 20% of the available CRP, and then decreased for each additional concentration. Whereas this project saw the percentage of captured CRP did not drop below 90%. This was due to the addition of multiple incubation areas in the chip. Because of this, a better method of accurate detection of CRP in whole blood was possible.

One final thing to point out is the comparisons of the limit of detection. The method used in the Godfrin experiments was a variation of the limit of blank. In this case, the LoD was found by taking the average signal at the blank and adding it to three times the standard deviation of the blank. Using this method, this LoDs in this thesis were 0.93 µg/mL and 0.50 µg/mL for serum and blood respectfully. In the Godfrin thesis, the same LoDs were at were 0.14 µg/mL and 4.54[23]. The reasoning for the smaller LoD in serum is that, again, diluted serum was used for the experiments, and that the zero concentration intensities were a quarter of the experiments in this thesis. As for the blood LoD, because of the amount of HAPs still in the system, it was not accurate in low concentration areas. This is the reason for the high LoD for blood compared to the method in this thesis. Because of this, it was shown that the addition of multiple HAP incubation areas not only could accurately capture and detect low amounts of CRP in blood, but also detect amounts less than 0.7 µg/mL in blood.

### 5.2. Further Work

Even though the previous experiments performed well and had shown that CRP can be detected with whole blood, there are a few more experiments that can be performed in order to make the system better. The first experiment could be the addition of a fibrinogen incubation area. As discussed previously, the blood plasma tests were assumed that the fluid behaved similarly to serum, when in actuality, the main difference is that serum does not include fibrinogen. Fibrinogen is a relatively large protein that is used in clotting and solidification of blood. The reason that this is an issue is that fibrinogen can act like albumin in that it can cause interference in the detection area. Even though there is not a large concentration of it, in comparison to albumin, it is a larger protein and it can cause more havoc. If there was any interference due to fibrinogen in the chip, the intensity would be a lot lower than what it should be. In order to get a better estimate as to the level of CRP in a sample of blood, it might be essential to include a fibrinogen incubation area either before or after the albumin incubation area.

The next experiment would be the addition of various samples of blood. Each individual has a different level of CRP in their blood and the previously experiments only used one type of blood. With multiple samples, it would be efficient to see where these samples lie on the curve in order to show how well various CRP levels can be detected in blood. One other issue is the lack of an official CRP measurement. The previous experiments were an estimate as to how much CRP is in the blood, but the accuracy of this system cannot be verified until an official measurement can be obtained for comparison.

Even with those experiments being performed, there is a possibility for future work. The first possibility is the integration for automation. The previous experiments were done manually with either a syringe or a syringe pump. There have been attempts to integrate this into an automated system. A previous Master's and PhD project included developing machines that perform this automation[22,24]. This new method would involve rewriting a code that would enable the fluid to move automatically and account for the multiple incubation areas.

The second possibility would integrate a better detection system for CRP detection. The main system used for detection is by way of fiber-optics. While it is a good method for detection, there are various short comings associated with using fiber-optics. These include polishing the optic wires for better detection, the fact that they can easily break and be expensive to repair and replace, and are bulky for a portable system. One good detection system would be the use of a charge-coupled device (CCD). Previously, CCDs were tested as a measurement for detection when it comes to immunoassays[60]. A CCD acts much like a camera where a snapshot of the detection area is taken and that photo is analyzed using imaging software processing[80]. This method is less bulky and cheaper to maintain than fiber-optic cables. The help of a CCD can be beneficial for more accurate and more sensitive detection.

A third possibly would be a new method of chip design and creation. Typically, chips take on average $1-3$ days to create, with an additional $1-2$ weeks to create the molds. Also, with the proximity of volatile chemicals and the fact that the molds could be damaged, a better chip could be constructed. One method is the use of

paper as a substitute for PDMS. Channels can be made in the paper which would allow fluid to travel through it[81]. Also, special areas can be set up for incubation and detection[81]. It would be cheaper to produce the chips on paper and in a shorter time. Finally, no fiber optics will be used in detection. The main source of detection will come in the form of image processing. The only downside is that fluids cannot be stored on a paper chip like a PDMS chip can. But small vials can be used for each reagent. With that aside, a paper-based chip would be useful for the next step in better tuning this current chip.

One final future project would be the detection of other antigens in the blood. There are many different proteins found in human blood and each protein has a specific function associated with it. In addition, various antibodies have been created for capturing and detecting these antigens. They can be integrated in the chip in order to determine how much of a specific antigen does this person possess. An example of an antibody that has been created to detect and attach to a specific protein will be discussed in the next section. With these advancements, it would be possible not only to better detect CRP in blood, but also other proteins and viruses that could be detrimental to human life. In conclusion, a better method of detection C – reactive protein from whole blood using lab-on-a-chip methods was found by using filter membranes for blood filtration and the introduction of protein A and multiple incubation areas for the removal of high abundance plasma proteins. This method not only produced a high array of signals, but was also accurate in detection.

CREATION OF A RECOMBINANT ANTIBODY AGAINST TAU PROTEIN

CHAPTER 6

INTRODUCTION

### 6.1.　Alzheimer's Disease

One of the most common ailments that can affect a large percentage of the older generation is Alzheimer's disease. Alzheimer's is a neuron-degenerative disorder characterized by memory and cognitive loss, dementia and a rapid reduction in brain matter[82]. According to the Alzheimer's Association, there are currently more than 5 million Americans with a form of Alzheimer's and a new case developing every 67 seconds. It is the 6th leading cause of death in the United States with approximately 500,000 individuals dying each year[83]. Currently, there is no cure for Alzheimer's and no method of either prevention or of slowing the disease. If trends like this continue, by 2050, the amount of patients with Alzheimer's would triple from 5 million to just over 13.8 million[83].

The basic methods of detection of Alzheimer's include determination of memory loss, difficulty completing ordinary tasks, problems with speaking and writing, misplacement of goods and mood changes, to name a few[84]. But this only helps in determining whether or not someone has Alzheimer's, not a method of early

detection. This has to be performed by various tests in the blood and in the spinal cavity[85]. While there is no set method to detect Alzheimer's early on, there are relationships in body functions that can relate if whether or not a patient could be diagnosed with Alzheimer's early on.

### 6.2. Tau Protein

One such relationship is the interaction of Tau protein. Tau is a rod-like protein found in the brain and the spinal column that helps to stabilize other proteins known as microtubules[82] and by relaying messages throughout the body. Tau performs these tasks by undergoing a process known as phosphorylation, which allows the protein to interact with phosphates and break up and send messages to other parts of the brain[86,87]. However, if too much of this occurs, the brain area can start to degenerate and exhibit symptoms of Alzheimer's based on CAT scans of different patients[88]. It has recently been discovered that tau may be the culprit of the main effects of Alzheimer's disease[85]. Because of this, there have been new developments as to how tau can be detected.

### 6.3. Antibodies against Tau

One method of the detection of tau protein would be the use of antibodies that attach to tau[89,90]. This was explained in the first section where CRP was detected using antibodies. These antibodies can be used for immunoassays in order to determine the risk of Alzheimer's based on the concentration of phosphorylated tau (pTau)[89]. However, tau is a long protein with little known antibodies, especially in places where phosphorylation can occur[91]. Because of this, it would be ideal to create antibodies specifically to bind to those areas of tau. The most common method

to create antibodies is to inject antigens from multicellular organisms and extracting them[92]. However, since using multicellular organisms is a huge liability, there has to be another method in which antibodies against tau can be extracted. Fortunately, there is a method called phage display which uses a known antibody library packed into phage, which is a virus, which can attach to tau antigens. In this method, the phage injects the antibody library into bacteria and from there, antibody fragments can be produced in the bacteria[93]. Even though these fragments are more basic than IgG, they still contain the necessary parts that can bind to tau. The upcoming sections will explain why an antibody for Tau has to be found and how using bacteria and phage display, these antibody fragments can be created.

### 6.4.    Phage Display

Antibody phage display allows the generation of e.g. human antibodies in vitro. In antibody phage display, an antibody fragment, in this work the single chain fragment variable (scFv), is displayed on the phage surface and the corresponding genetic information for this scFv is encoded on a phagemid which is packaged in the phage particles. Therefore, the genotype and genotype are coupled. In the phagemid, the scFv gene fragment is fused to the phage gene III (gIII), encoding phage protein III (pIII). This allows the expression of a fusion protein: scFv::pIII. During phage assembly in E. coli, the fusion protein will be incorporated in the phage particle and the phagemid will be packaged in the phage resulting in an antibody phage[92-95]. Antibody fragments can be selected by a process called panning. An antibody phage library (HAL7/8 in this work) can be incubated with the desired target molecules (Tau peptides in this work). The non-binding antibody phage will be washed away and the

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bound antibody phage particles will be eluted. E. coli will be infected with the eluted phage particles and with the help of helper phage; new antibody phage particles will be produced. These amplified phage particles will be used in a next panning on the desired target to further increase the fraction of target specific antibodies. These panning rounds will be performed 3-4 times[94,95].

Single bacteria colonies, infected with the antibody phage particles of the last panning round, will be cultivated in micro titer plates and soluble monoclonal antibodies will be produced. These monoclonal antibodies fragments will be tested by ELISA to identify monoclonal binders. Subsequently, the scFv encoding gene fragments can be analyzed by DNA sequencing to identify unique binders and for further analysis. Finally, these antibody fragments can be recloned in any antibody format (e.g. IgG) for further production and biochemical analysis[94,95].

### 6.5.    ELISA

In order to test if these antibodies are beneficial, assays have to be completed in order to make sure that these antibodies bind to the specific proteins. The tests performed are known as Enzyme-linked Immunoassays (ELISA). As explained in chapter 2, these tests use a series of antibodies to capture proteins and antigens and a second set of antibodies to detect said proteins and antigens. A substrate is then used to detect these antibodies[12]. Multiple types of ELISAs exist, which, as explained in chapter 2, include direct (where a protein is unknown and antibodies can detect it), indirect (where the antibodies are unknown, and the protein is known), sandwich (where an antibody specific to a certain antigen is first placed onto the bottom of a well), and competition (where competing proteins/antibodies are used to "compete"

with the main ELISA).  These types can be integrated into two main ELISA experiments: screening ELISAs, where different clones can be found, and titration ELISAs, where the overall strength of detection is found[92].  These two experiments can be integrated in order to create an antibody that can easily detect tau protein.

### 6.6.    Objective

The focus of this investigation is the creation of two recombinant antibody fragments that attach to two specific types of tau as a biomarker for Alzheimer's: phosphorylated tau (tau with phosphate groups attached (pTau)) and non-phosphorylated tau (tau without phosphate groups attached (Tau)).  These two antibody fragments are beneficial in that they would be able to attach not only to healthy tau, but also tau that may be a factor in Alzheimer's disease.

The first task of the project is the creation of the fragments.  This will be performed by the use of phage display.  This method will reveal the proper fragments that will attach to the protein.  Even though only a small antibody fragment would be obtained, it is a cost effective and safer procedure than obtaining IgG (immunoglobulin G) from a multicellular organism.  Plus, these same fragments have all the proper sequencing that is normally found in IgG in which it attaches to the antigen.  This sequencing is what allows the antibody to attach to the protein.  Using phage display, one antibody fragment can be obtained from a library of different antibody possibilities by injecting DNA into bacteria and creating multiple colonies.  After three or four panning rounds, one antibody fragment for both Tau and pTau will be obtained.

The second task of the project is the detection and strength of the antibody

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fragments. This is where the ELISAs will come into play. Using ELISA, these extracted antibody fragments will be detected and checked if they are able to bind to the antigens. This process is crucial because it has to be made certain that different antibodies can be created. Also, there will be many clones that can attach to the protein. Therefore, tests will have to be performed to check if these fragments are identical and are able to bind well. This will be performed again by ELISA in order to see the limits of concentration that these antibodies can be detected. Also, since there are many clones, these fragments will have to be sequenced. This means that the DNA structure has to be checked to see if either multiple clones have the same structure, or that multiple different clones are able to attach to the same protein. This is important because there can be an instance where two antibodies, with different sequences can still attach to the same protein.

The end result for this investigation will be the creation of two distinct antibody fragments that will be able to attach to a section of tau: one that attaches to pTau and one that attaches to Tau. These two antibody fragments will be useful to diagnose an early onset of Alzheimer's. Since tau is a long protein (350-450 amino acids)[91], and since there are antibodies that can already attach to parts of tau, this project will focus on one section of tau that is prone to phosphorylation and does not have an antibody associated with it. With this method, it can be possible to in the future, use this antibody as a diagnostic tool and to map out the entire protein in a cost-effective manner, in order to see which antibodies are able to attach to it.

# CHAPTER 7

# REVIEW OF LITERATURE

## 7.1.    Alzheimer's Disease

One of the most debilitating diseases today is Alzheimer's disease.  Alzheimer's disease is classified as a dementia usually associated with problems with memory and behavior[82].  Dementia is described as a neurological ailment that is associated with memory lost.  While older patients do experience some sort of memory loss, it can only be described as dementia if this memory loss affects normal everyday activities.  This not only includes loss of memory, but also problems in communication, the ability to stay focused and a lower sense of judgment and reasoning[84].  Alzheimer's is the most common form of dementia, with roughly 5 million affected people and a new case every 67 seconds.   Each year, more than 500,000 Alzheimer's patients die and at current trends, the number of Alzheimer's patients will triple to approximately 13 million by 2050[83].  With these numbers, it is important to see how large of a factor Alzheimer's will be in the upcoming decades.

There are various known symptoms for Alzheimer's that affect patients.  According to the Alzheimer's Association, there are 10 warning signs as to check whether or not a person has Alzheimer's.  These 10 signs are listed on the next page[84].

| | |
|---|---|
| 1: Memory loss that disrupts daily life | 6: New problems with words in speaking or writing |
| 2: Challenges in planning or solving problems | 7: Misplacing things and losing the ability to retrace steps |
| 3: Difficulty completing familiar tasks at home, work or leisure | 8: Decreased or poor judgement |
| 4: Confusion with time or place | 9: Withdrawal from work or social activities |
| 5: Trouble understanding visual images and spatial relationships | 10: Changes in mood or personality |

**Table 7-1:** The 10 signs of Alzheimer's disease[84]

One known issue that is important is that currently, there is no known cure for Alzheimer's. Most patients that have been diagnosed with Alzheimer's will die due to complications with Alzheimer's. Not only that, but there is no known method to either prevent or to slow down the process[96]. This means that at this time, once a patient is diagnosed with Alzheimer's there is no efficient method to help slow down the time length from diagnosis to death. Due to this issue and the fact that more people will develop Alzheimer's in the upcoming years, it is important that there is a method to detect and try to treat Alzheimer's early on before these 10 factors quickly come into play.

## 7.2. Known Alzheimer's Factors
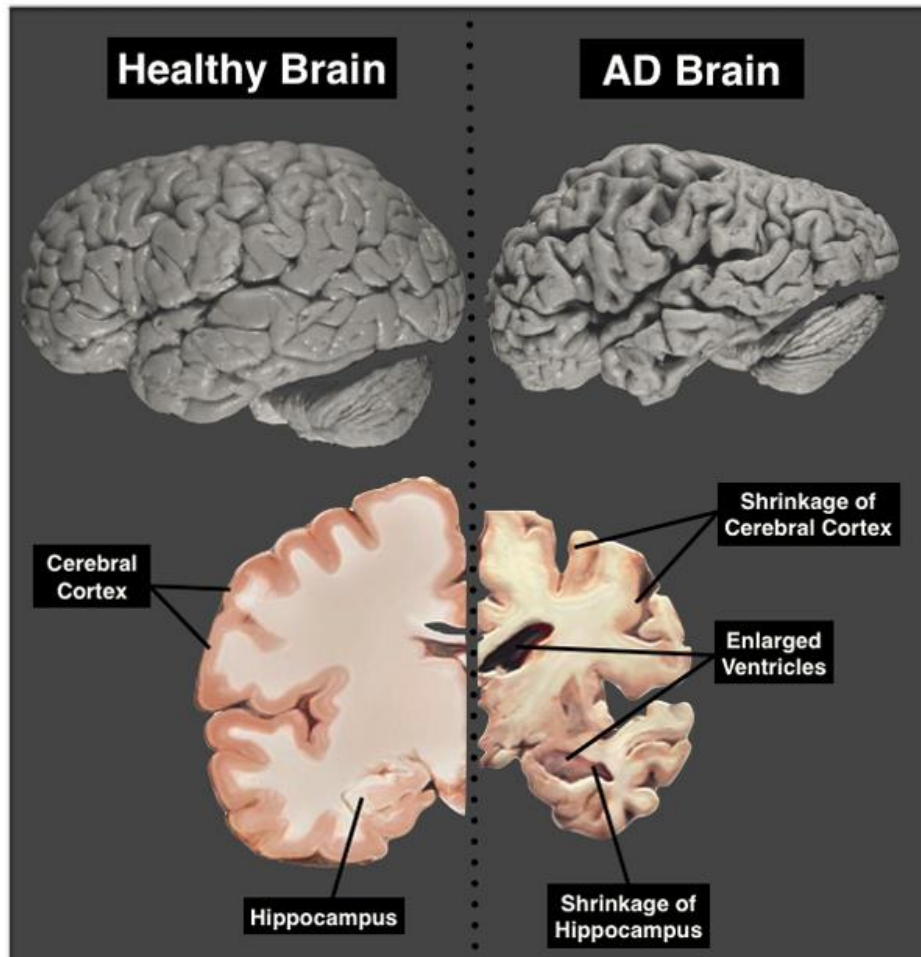


**Figure 7-1:** Comparison between a normal and Alzheimer's affected brain [88]

The figure above shows a sketch of a human brain.  The left side shows a normal fully functioning brain, while the right side shows a brain based on the effects of Alzheimer's.  The most noticeable trait is the difference in size and in structure. The Alzheimer's brain is noticeably smaller in size and shows more rapid decay.  This was demonstrated back in 1906, when Dr. Alois Alzheimer noticed that one of his patients was suffering from dementia and had issues with rapid memory loss, unpredictable behavior and the need to be depended on others for normal everyday activities.  When this patient died, he examined this person's brain and found that the

overall size had reduced and, especially in the cerebral cortex and hippocampus, where learning and memory takes place and large gaps or ventricles[88]. This can be seen in the cat scans below in figure 7-2.
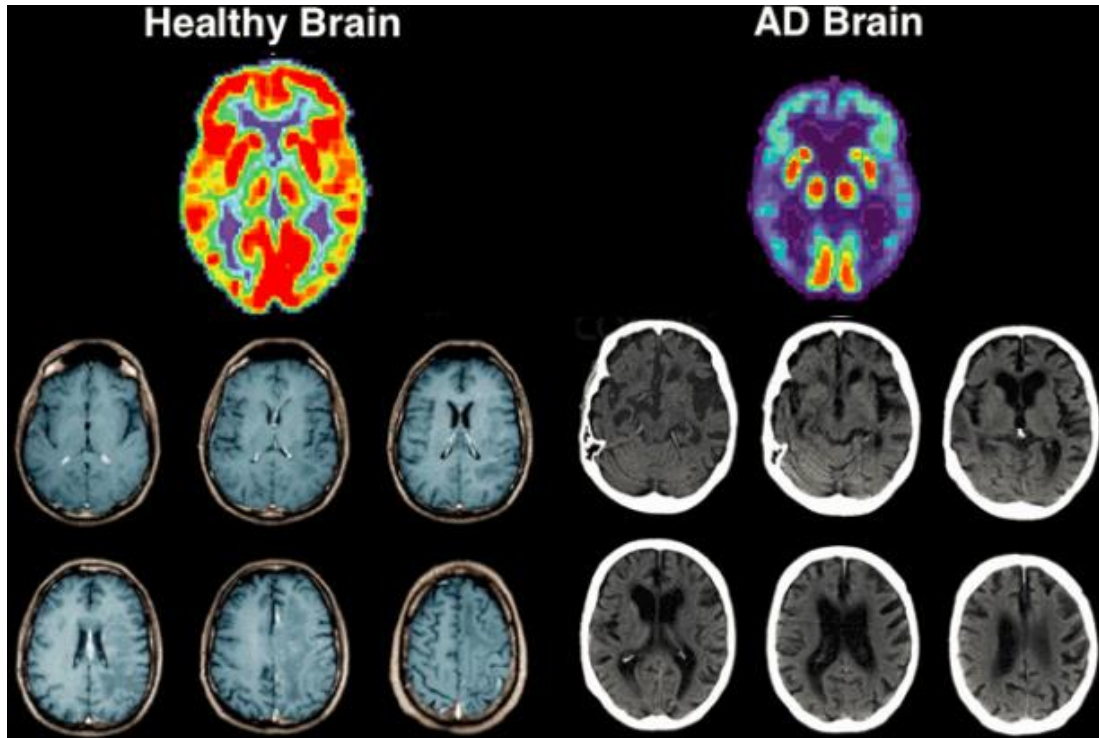


**Figure 7-2:** CAT scans of a healthy and Alzheimer's affected brain[88]

Currently, it is unknown as to what causes this decay in the brain, but there are several factors that associate with this. Some of these factors include beta amyloid, a protein found in plaque areas around the brain[97,98], and tau protein, known tubules, in which fragments can be found the blood and spinal fluids of patients[99,100]. Even though there is no known concrete evidence that these two proteins cause Alzheimer's, they are still regarded as a links into how the disease is formed and what is happening in the brain area. The upcoming sections will explain the proposed roles of both beta amyloid and tau in Alzheimer's.

### 7.3. Beta Amyloid

Beta Amyloid (aβ) is a protein that commonly shows in patients that have Alzheimer's. This protein is a relatively short protein (consisting of about 40 amino acids) and is often the result of proteolysis, or protein breakdown, of the large Amyloid precursor protein (APP)[101,102]. There is little known about the function of APP, but there has been research to suggest that to bind proteins and/or cells together, and that it helps to direct the migration of neurons during early development[103]. Other theories suggest that APP is used in the formation and repair of synapses, which join nerve cells and transmit neurons[104,105]. Whatever the function of APP has in the body, it breaks down to form aβ. However, in patients that exhibit all the symptoms of Alzheimer's, one noticeable trait is that plaques of aβ are found in the brain regions. This is from the result of the breakdown of APP and that certain forms of aβ proteins can coagulate into obscene proportions[102,106]. Below is a diagram that shows this result.
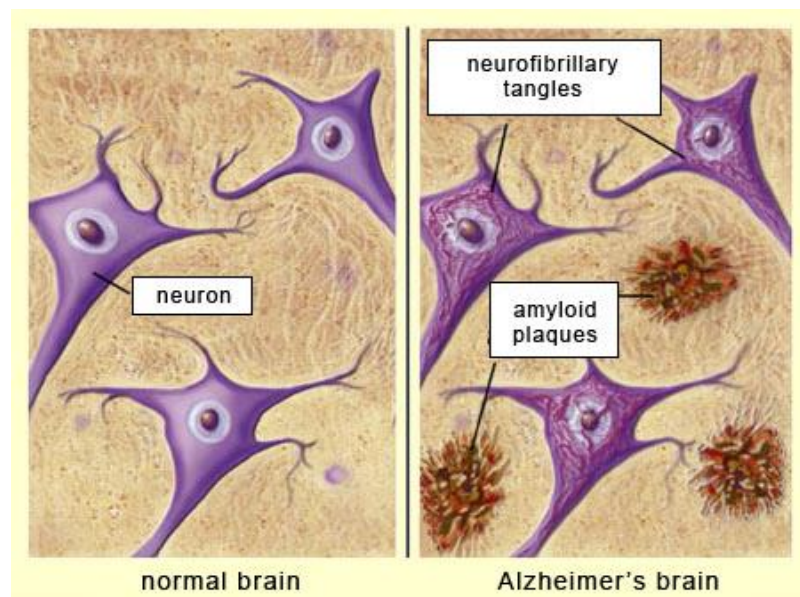


**Figure 7-3:** Sketches of neurons in a healthy brain vs. neurons in an Alzheimer's brain. Note the abundance of aβ plaques in the brain affected by Alzheimer's[107]

The breakdown of APP to aβ performed formed by cleaving a portion of the APP into smaller structures around 40 amino acids in length[102]. There are two common forms of aβ that APP can break down into: $a\beta_{40}$, the most common form and $a\beta_{42}$, the form most prevalent in Alzheimer's. The 40 and 42 recognition only corresponds to the length, or number of amino acids. Also, aβ is made up of a chain of hydrphobic and hydrophilic areas in both the middle and ends of the protein. Aβ attaches readily to each other in its hydrophobic areas but since $a\beta_{40}$ is a smaller molecule, there is a maximum amount copies it can attach. $A\beta_{42}$ has two more amino acids on it, which are hydrophobic. While there can be mutations, where some hydrophilicity can occur in this area, for regular $a\beta_{42}$, these are all hydrophobic. Because of this hydrophobicity, more $a\beta_{42}$ can aggregate and form larger clusters, which can send the brain into a "repair mode". This can explain the aβ plaques found on Alzheimer's patients as well as lessening of the brain matter. These reasons show how important aβ is to the study of Alzheimer's[106].
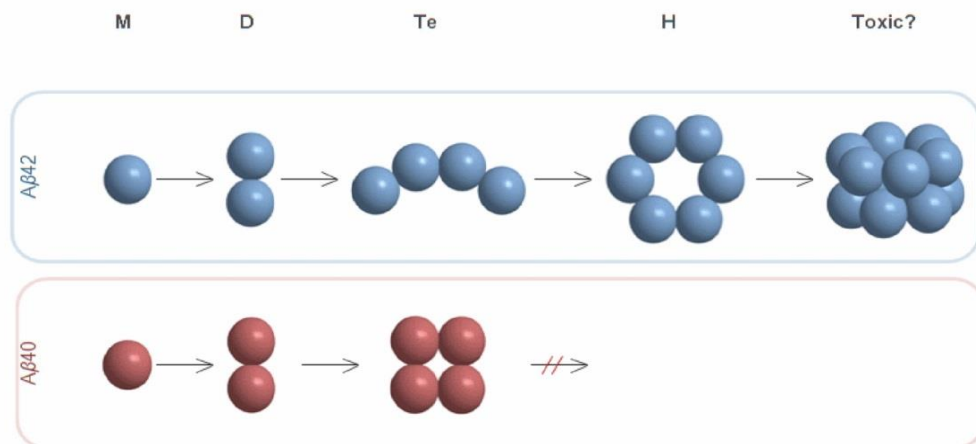


**Figure 7-4:** Differences between aβ40 and aβ42 aggregation. Notice that aβ40, bottom, cannot aggregate more than four times its size, while aβ42, top, can aggregate to greater proportions[108]

### 7.4.    Tau Protein

However, there is another protein that is now factoring into the possibility of a probable cause of Alzheimer's: Tau Protein.  According to Stoothoff, "tau was first discovered in 1975… as a protein that co-purified with microtubules through cycles of assembly and disassembly and was called tau for its ability to induce tubule formation. [109]" The role of tau protein will be explained later on in section 7.5.  Right now, here is general description of the structure of tau.  Tau is a long rod-like protein that is located in the brain and nervous system, mostly found in the neurons[91,100].  There are 6 main isoforms of Tau based on the number of binding domains and inserts.  The binding domains, known as repeats, are located in the C- (carboxyl) terminal of the protein and are positively charged, which allows it to bind to any microtubule, which are negatively charged.  The number of domains can vary between 3 (3R) or 4 (4R), where the second domain is missing from the 3R.  In terms of location on the protein, this report will explain in terms of length based on the number of amino acids.  These domains are located at a length at amino acid 166 in the case of the shortest isoform and at amino acid 244, in the case of the longest isoform.  The more domains the protein has, the better it is at stabilizing microtubules.  Also, there are inserts located at the N- (amino) terminal, which help send messages to the C-Terminal.  The number of inserts can range from zero to two (0N, 1N and 2N). These inserts are located at amino acid 45[91,100].  A diagram of an isoform of tau, the longest, and a table explaining all 6 isoforms of tau can be found on the next page.
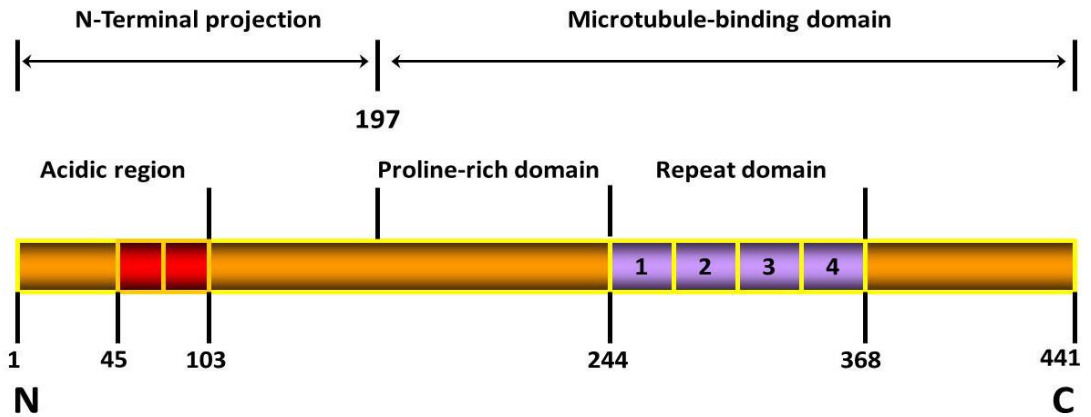
**Figure 7-5:** Tau Protein showing the locations of the domains (pink) and inserts (red)[91]

| Tau Isoform | # of Domains | # of Inserts | Protein Length (# of Amino Acids) |
|---|---|---|---|
| Tau 0N/3R | 3 | 0 | 352 |
| Tau 0N/4R | 4 | 0 | 383 |
| Tau 1N/3R | 3 | 1 | 381 |
| Tau 1N/4R | 4 | 1 | 412 |
| Tau 2N/3R | 3 | 2 | 410 |
| Tau 2N/4R | 4 | 2 | 441 |

**Table 7-2:** Different tau isoforms[91]

Based on this table, the lengths for each tau isoform vary between 352 amino acids for the shortest isoform to 441 amino acids for the longest isoform. Also, it can be seen that each binding domain consists of 31 amino acids and each insert consists of 39 amino acids[91]. Furthermore, it is worth noting that all forms of tau contain the same 352 amino acids that are on the shortest isoform. The only variation is the amount of domains and inserts[91]. The diagram on the next page shows all six isoforms of tau and the locations of these variations. The inserts are located at the 2nd and 3rd exons, or sections. The orange represents the first insert and the green represents the second insert. The black bars on the right represent the domains. The pink is where this change in the number of domains occurs. In this case, all changes

occur at the second domain (from the left).
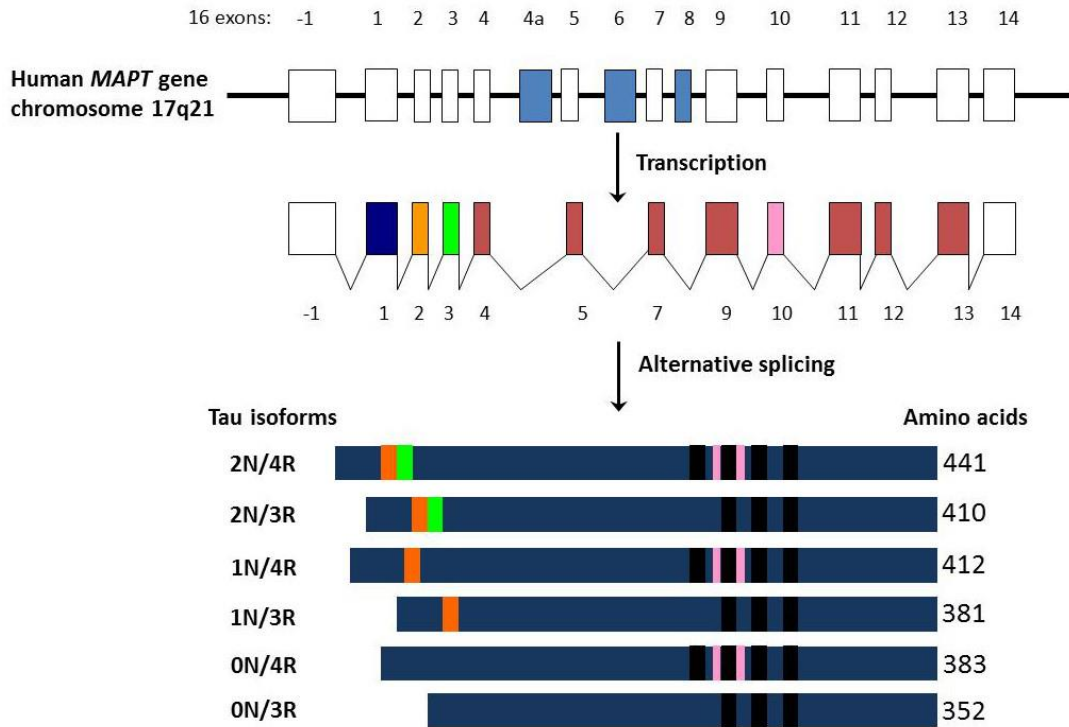


**Figure 7-6:** Differences in the tau isoforms[91]

Tau protein is an interactive protein, in which it regulates and stabilizes microtubules. It does this by interacting with tubulin, which forms microtubules in the brain[91,110]. To clarify, tubulin is a group of globular proteins, which are globe-like in appearance, that come together to form microtubules. There are 5 known types found in the human body: alpha-, beta-, gamma-, delta- and epsilon tubulins, with the most common being the alpha and beta types[111]. The process in which this happens is known as phosphorylation and it will be explained below.

### 7.5. Phosphorylation

Phosphorylation occurs when phosphate groups ($PO_4$) attach a protein, which can alter the specific function or role of the protein. In this method, certain biological processes can be altered without needing to create or destroy more proteins[112].

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Enzymes or molecules known as kinases are responsible for phosphorylation of proteins, which can change the entire structure and function of the protein. Kinases only add phosphate groups to three amino acids: serine (S) ($HO_2C_2H(NH_2)CH_2OH$), threonine (T) ($HO_2C_2H(NH_2)CH(OH)CH_3$) and tyrosine (Y) ($HO_2C_2H(NH_2)CH_2(C_6H_4)OH$)[113].



Phospho Serine, Threonine and Tyrosine amino acids
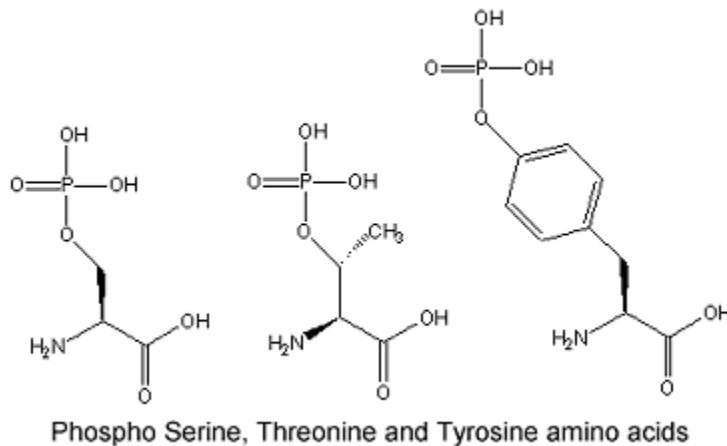
**Figure 7-7:** Phosphorylation occurring at serine (left), threonine (center) and tyrosine (right)[113]

Phosphorylation is what is known as a regulatory process, in which multiple vital actions can occur in the body[87,114,115]. This also include the removal of phosphate groups, known as dephosphorylation[115]. Some examples of where protein phosphorylation is essential in the human body include the regulation of the activities of enzymes and the interactions between different proteins[115]. In the larger scale, these regulations and interactions include various metabolic processes, gene regulation, cell cycle control, transport in the cell and cell adhesion[115]. In short, protein phosphorylation is an important regulatory process in the human body.

In the case with tau protein, phosphorylation is performed in order to promote the assembly of and regulate the stabilization of microtubules[91,109]. Microtubules are hollow cylinders that have a diameter of about 24 nm made up of tubulin[110,116,117].

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They are involved in various processes, which include cell division, and are utilized in the structure and transport throughout the cell[118]. Because microtubules are essential to the human body, in order to help stabilize these tubes, tau is brought in and how it is stabilized depends on the phosphorylation of tau[91]. The amount of phosphate groups on Tau depend on how stable the microtubule is. The more phosphate groups on the protein, the less stable the tubule is[91,109]. This process is a relatively common process in the body and it actually allows the formation of new protein tubes. However, one of the main factors that Tau is a link to Alzheimer's is known hyper-phosphorylation, which will be explained in the upcoming section. Below is the process in which tau helps assemble and stabilize microtubules.
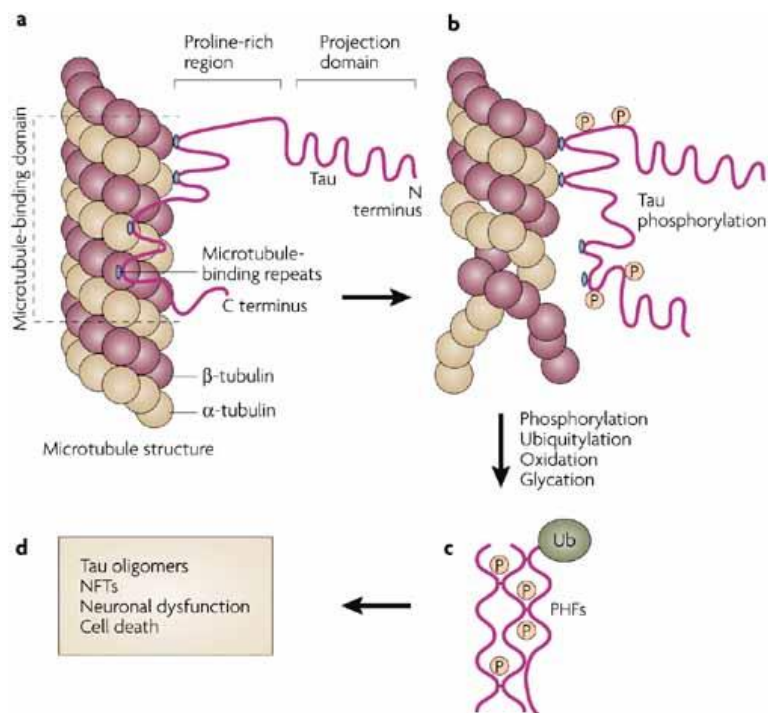


**Figure 7-8:** How unphosphorylated tau (left) and phosphorylated tau (right) help stabilize molecules[118]

### 7.6. Hyperphosphorylation and the Focus on Tau Protein for Alzheimer's

One of the main reasons why tau protein is a link to Alzheimer's is the process of hyperphosphorylation. This is when phosphate groups attach to the Tau at a faster pace and break down microtubules at a quicker pace. This in turn causes aggregation of filaments which in turn forms neurofibrillary tangles, one of the key components to the disease[91,118-120]. This breakdown can be so rapid that, because of the inability to stabilize microtubules, this can result in the decrease of brain matter, as attributed to figure 7-1. This theory of the hyperphosphorylation of tau has only been researched in the last twenty years and it has only been since recently that more evidence suggests this[121]. This can be better shown in figure 7-9. Figure 7-9 displays tau with the known phosphorylation sites. There are about 100 known sites, with 1/3 to 1/2 of them being sites for normal brain activity (green). The remaining sites show where phosphorylation and Alzheimer's have a connection with tau (red and blue)[91].
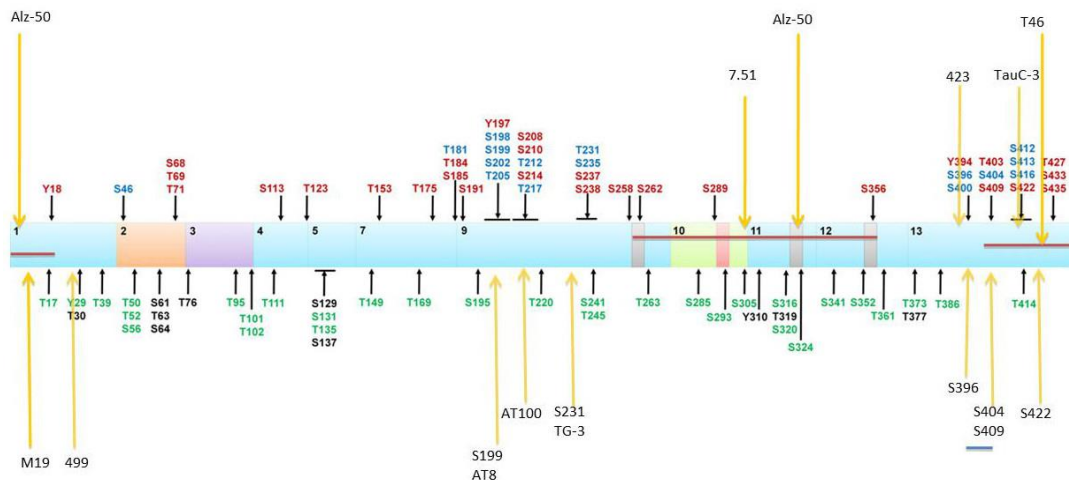


**Figure 7-9:** Phosphorylation sites on tau for normal brain activity (green), Alzheimer's activity (red) and both (blue)[91]

However, 20 years ago, this was not the case. Previously, the focus of source of Alzheimer's has been the fact that aβ plaques could be found on patients. This

created the assumption that aβ was the main suspect in causing Alzheimer's[121,122].

This shift in the focus from aβ to tau comes in large part due to how Alzheimer's is first detected and study as the disease progresses. In most cases, it is easier to see that aβ can be found in increasing numbers as the person develops Alzheimer's. However, that is not the case with Tau, in that Tau is the driving force for the cognitive decline, which will result in memory loss[121,122].

According to Dr. Melissa Murray of the Mayo Clinic in Jacksonville, FL, "Tau can be compared to a train track that brain cells use to transport food, messages and other vital cargo throughout neurons...In Alzheimer's, changes in the tau protein can cause the tracks to become unstable in neurons of the hippocampus, the center of memory. The abnormal tau builds up in neurons, which eventually leads to the death of these neurons[122]". This means that because of the affected tau not being the stabilizers that they are, this is the cause of the loss of cognition and memory.

This was performed by studying at least 3,500 brains, in which 1 out of 3 were Alzheimer's confirmed. Each brain varied as to what stage of dementia it was in when the patient died, so that it could give a time-line of brain degeneration. Each brain was not only looked at for tau severity, but also for amyloid severity. The results showed that it was tau, not amyloid, that predicted the onset of cognitive decline, duration of the disease and the deterioration of mental health. Also, when the research looked at amyloid, it was discovered that the brain-specific amyloid observed a signal from the brain scans, and not the amyloid in vessels. Furthermore, this did not correspond to tau pathology, as similar brains with similar tau effects, which led to Alzheimer's, did not lead to similar amyloid effects. It was found that some brains with a large amount

of tau influence had only a small amount of amyloid influence, and vice versa. This can show that amyloid can be found in individuals even if they have not experienced cognitive decline. Based on this reasoning, it should be pointed out that tau is now a major influence in Alzheimer's[122].

However, one cannot overlook amyloid as a cause as well. There has been research to suggest that beta amyloid can be a factor in hyperphosphorylation of tau[121,123]. Research has suggested that amyloid can cause an excessive flow of ions into neurons. This in turn can activate kinases that would undergo phosphorylation on tau[121]. One such kinase is known as AMPK (5' adenosine monophosphate-activated protein kinase). AMPK normally responds to stresses that deplete essential supplies, including low glucose and heat shock by direct phosphorylation of a number of enzymes[124]. Because of this, it can be said that beta amyloid can play a large role in the development of Alzheimer's. But in terms of finding a source and early detection, it is more reasonable to take a look into the hyperphosphorylation of tau protein.

### 7.7. Antibodies for Detection against Tau

Now that it is clear that tau is the main factor in cognitive degeneration, it is important to find a method of early detection of rogue tau. As explained in the previous part, there exists assays that can detect proteins by use of specific antibodies[19,20]. Because of this, antibodies can be created that will help in capturing tau. There have previous antibodies that have been created that have been used to detect certain areas of tau protein[90,91]. This is shown in figure 7-9 with yellow arrows. These arrows represent the different antibodies that already have been created in order to attach to tau protein[91]. These antibodies can be helpful in the identification

of tau protein in terms of both its phosphorylated and unphosphorylated forms. However, there have not been advances in terms of the number of antibodies created. Tau is a long protein and there are certain sections of it that are not associated with an antibody[91]. Because of this, there has to be a method of creating antibodies for all parts of tau.

There are numerous methods to create and extract different forms of antibodies from organisms. Some of the most popular organisms to use are mice, goats and monkeys[92]. However, this project is more concerned about seeing how antibodies can attach to tau protein. Because of this, and the severe liability risks of using animals as test subjects, it is not recommended that live animals would be used. Instead, another method has to be implemented. This method uses bacteria/E. coli in order to create antibodies. This was because of the availability of bacteria, the ease of working with it and the relatively cheap cost of using it. However, because such a simple one-celled organism is used in the creation of antibodies, these antibodies will be known as antibody fragments and would be the simplest form in which it still can detect tau[92]. Even though they cannot be properly be used in normal immunoassays, they are nevertheless important because they create the foundation for an antibody library and are the basic entities that attach to the desired antigen[92]. This will be further explained in section 7-9.

### 7.8.    Reasoning for an Antibody against Tau Protein.

Since Tau is one of the proteins that were linked to Alzheimer's disease, it would be good to create antibodies in order for it to be used in immunoassays as a biomarker. This is done by connecting the antibodies to the surface of the protein via

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the amino acids on both the protein and the antibody[92].  The diagram on the next page

shows the model of the longest Tau isoform (Tau 4R 2I) at 440 amino acids long.



**Figure 7-10:**  Diagram of tau protein where the specific antibodies will be created[91]

The two inserts are at sections 2 and 3 and the domains are located at sections

10, 11 and 12, were the second domain is the difference between 3R and 4R.  The

numbers at each amino acid spot indicates where phosphorylation can occur.  Green is

during normal brain activity, while red is observed with patients with Alzheimer's.

Blue indicates both and black is unknown.  Based on this diagram, there have been 14

known antibodies that have been discovered, based on the yellow lines.  The majority

of these occur at the front and the back of the protein.  For example, antibody T46

corresponds with the amino acids from the 420 to the 441 area (S422, T427, S443 &

S436)[91].  While there have been some antibodies that have been discovered, the

majority of the sites do not have an antibody associated with it.  For this reason, this

project will focus on finding antibodies for the first binding domain (1R).  This

domain is common in all tau isomers and is located on 256-262 range.  The peptide, or

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protein section, that this project will focus on will be the area from 251 to 270, where known phosphoylation occurs at S258 and S262.

### 7.9.    Types of Antibodies

For the basis of this project, the antibodies that are the main end product are known as single-chain variable fragments (scFvs). This form of antibody fragment is the smallest form of immunoglobulin that still retains its function in antigen-binding[125]. These small antibody fragments consist of a heavy chain ($V_H$) and a light chain ($V_L$), which are connected together by a peptide linker [92,125]. Compared to immunoglobulin (Ig) antibodies, they are about 1/6 in size (scFv ~ 25 kDa, IgG ~ 150 kDa)[92,125,126]. However, they are still able to attach to antigens as well. If one takes a look at the structure of an IgG antibody, one would notice the tips of the branches are where the antibody would bind to the antigen. These branches are known as the variable fragment region (Fv). This is similar to the scFv in that the same two chains are used in order to attach the antibody to the antigen. The only difference between the two in structure is that the scFv includes a linker, which allows the two chains to act as a single chain in name, whereas the Fvs are two separate chains that are located on the tips of the IgG. Nevertheless, both the scFv and the Fv regions of the IgG contain the same genetic sequence that allows them to bind to the same antigen. This is shown below in figure 7-11.
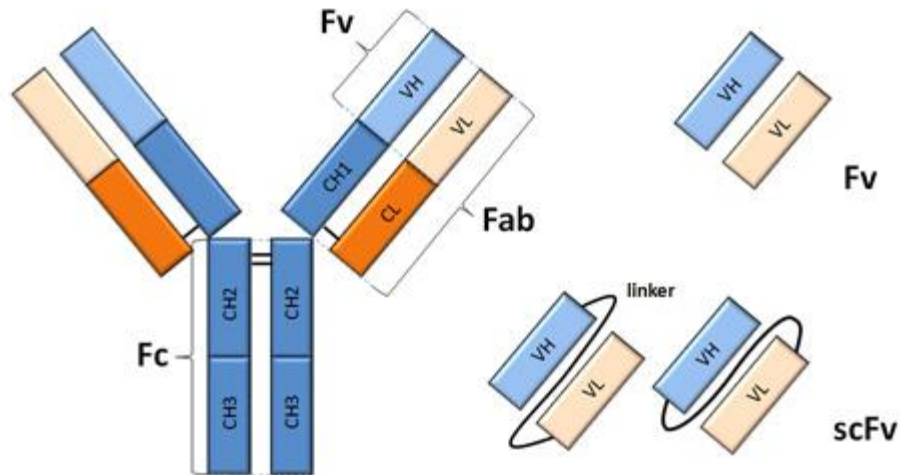
**Figure 7-11:** Diagran showing the difference between the IgG (left), Fv regions (top right) and scFv (bottom right) antibodies/antibody fragments[126]

Other types of antibody fragments include the antigen-binding fragments (Fabs)[93,127], dimetric single chain variable fragments (di-scFvs)[128] and single domain antibodies (sdAbs or VHH)[127-129]. Even though these examples are important in antibody construction, they do not hold relevance in this report because they were not fully used this study. Instead, scFvs were produced in order to find antibody fragments that were specifically designed to attach to tau protein. The upcoming sections will explain the use of recombinant antibodies and the method used to create them: phage display.

### 7.10. Recombinant Antibodies and Phage Display

Because of the previously described methods used in order to create antibodies, a new method of antibody creation has to be performed. In the last 20+ years, a new method of creating recombinant antibodies has become popular. Some of the benefits of creating recombinant antibodies are that these experiments are performed with the use of animals, which means a reduced risk of contamination due to the disease that the antibodies are being built for, they can be modified to be created for a specific

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organism in mind and they can be designed to be modified for uses that would normally not occur naturally, for example, targeting cancer cells[92]. One of the most common methods is the use of phage display. In phage display, phages, a virus, help to select and replicate antibodies for a specific protein[92,94,95,130]. An example of a phage and its structure is shown below.
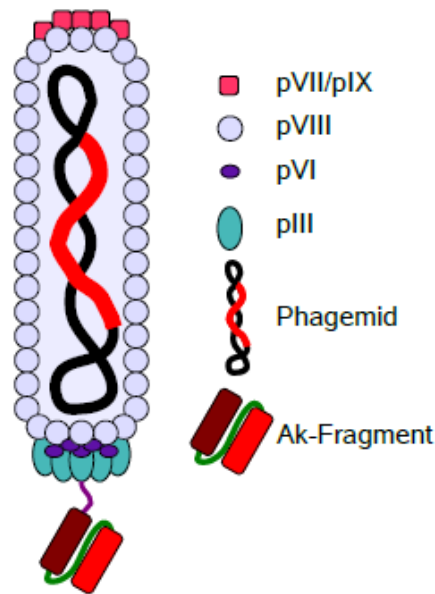


**Figure 7-12:** Diagram of phage with scFv fragment[131]

A single scFv fragment is displayed on the surface of the phage and the corresponding genetic information is encoded on a phagemid and packed inside of the phage particle. In the phagemid, the scFv fragment is fused to the phage gene III (gIII) which encodes phage protein III (pIII). This then allows the expression of a fusion protein: scFv:pIII. The fusion protein will be incorporated in the phage particle and the phagemid will be packaged in the phage during phage assembly. This results in an antibody phage[94,95,130-132]. The method of phage display is explained below on the next page.

The first step of phage display is the in vitro selection of antibody phage

particles. This is what is known as panning[94,95]. In this step, antigens in which are of importance, in the case of this thesis, tau peptides are attached to a well of a micro titer plate. An antibody phage library, in this case, HAL7/8, is incubated with these desired peptides. The antibody phage of importance binds to the peptide, while the unbound phage is washed away. The bound phage then goes through a process, known as elution, in which the phage is collected. This phage then infects an E. coli culture. Here, the phage is amplified in the cell and new antibody phage is produced with the help of helper phage. This new antibody phage will then be used in a new panning[94,95]. The reason for this is that each antigen is likely to be attached to the micro titer plate using a biotinylated linker, which is attached to streptavidin, which is in turn coated on the plate. This allows for better binding between the antigen and phage than having the antigen bind to the well due to the fact that the antigen is more intact. Because of this, there might be some phage that will actually bind with the streptavidin and the linker. By performing multiple sets of panning rounds, an optimal amount of phage and antibody that binds directly to the desired antigen will be found and a high concentration of it will be produced[92,94,95,130-132]. Each run through of this process is known as a panning round. A diagram highlighting a panning round is found in figure 7-13 on the next page.
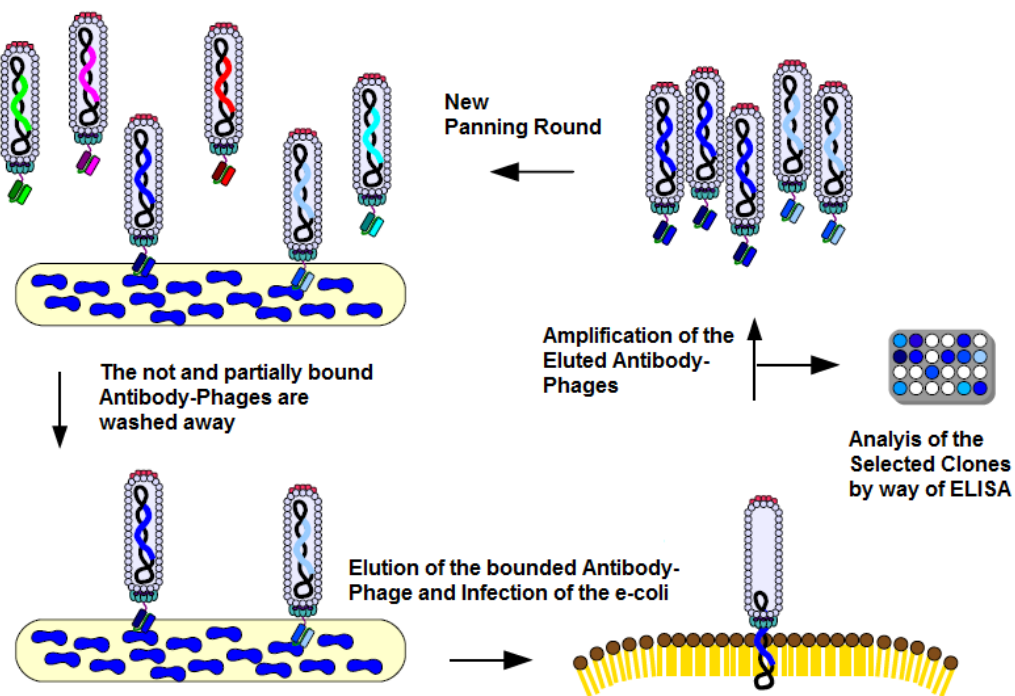
**Figure 7-13:** Diagram describing an example panning round[131]

In most cases, 3-4 panning rounds would have to be performed in order to obtain a satisfactory amount of antibody phage. During the final round (either the 3$^{rd}$ or 4$^{th}$), soluble monoclonal antibodies will be produced. These antibodies will be tested via ELISA (enzyme-linked immunosorbent assay) in order to identify positive monoclonal binders. Furthermore, the scFv encoding gene fragments can be analyzed via DNA sequencing in order to determine unique binders and the antibody fragments can be recloned into any antibody format (IgG) for further production and biochemical analysis[92,94,95,130].

### 7.11. ELISA and DNA Sequencing

Once an appropriate number of clones have been created, the next step is to make sure that there are active binders for each antigen. To test this out, an ELISA (enzyme-linked immunosorbent assay) is performed. Back in chapter 2, the different

types of ELISAs were pointed out.  These were a direct ELISA (where the antibody is attached to an unknown antigen on the bottom of a well), an indirect ELISA (where an unknown antibody is attached to the antigen), a sandwich ELISA (where the antigen is attached to a capture antibody before being detected) and a competition ELISA (where the ELISA is in direct comparison to a competing ELISA)[59].  These ELISAs share a basic construct: an antibody is used to detect an antigen and that antibody is detected via a substrate[12,133].  Because of the amount of clones that will be made based on the phage display, it would be a good idea to find scFv fragment that can attach to tau by using an ELISA.  This can be performed in order to narrow down the amount of clones that have been made, in order to find an antibody fragment that will properly attach to tau.  This is what is known as a screening ELISA.  In this screening ELISA, clones are picked out and the scFvs are extracted from the E. coli culture.  These fragments are then placed in a micro titer plate with the antigen already in place.  After the fragments have incubated and attached to the protein, detection antibodies are applied and incubated for some time.  The substrate is then applied and clones are then taken into account based on the signal given off.  This is due to the color change in substrate. The darker the color, in most cases, the higher the signal[12,59,133,134].  From there, clones that have shown to emit a high signal can be removed.  An example of a screening ELISA is found on the next page.
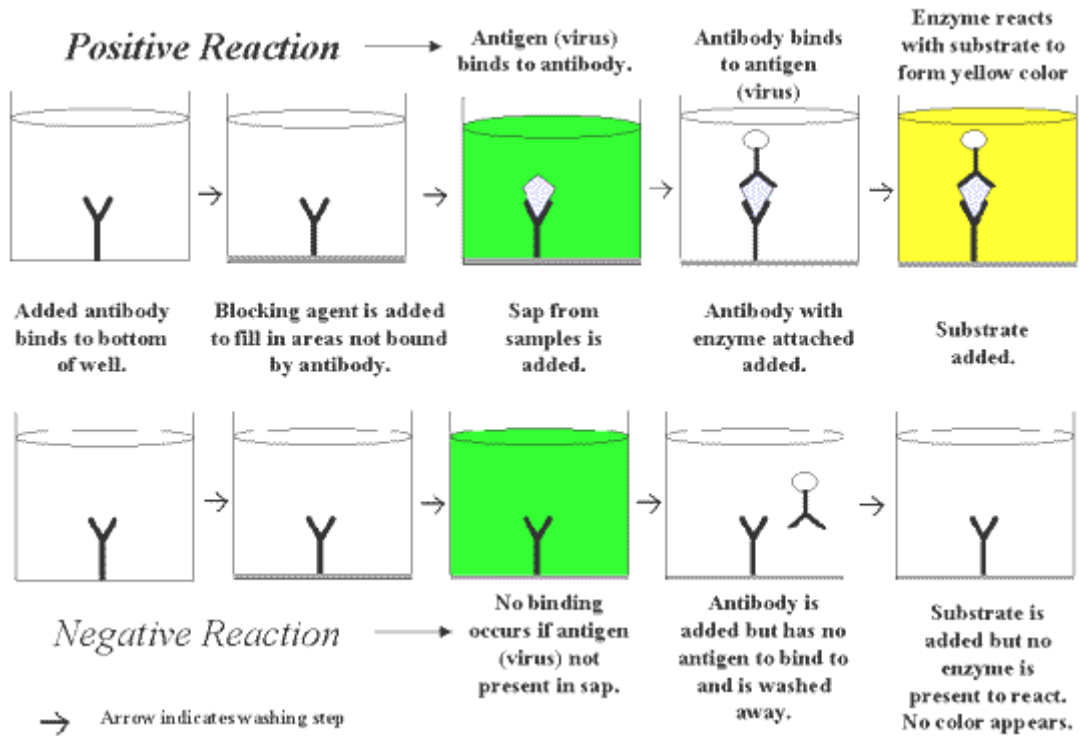
**Figure 7-14:** Diagram of the steps for a screening ELISA. Top represents a well with a desired antigen signal, bottom represent no antibody signal[134]
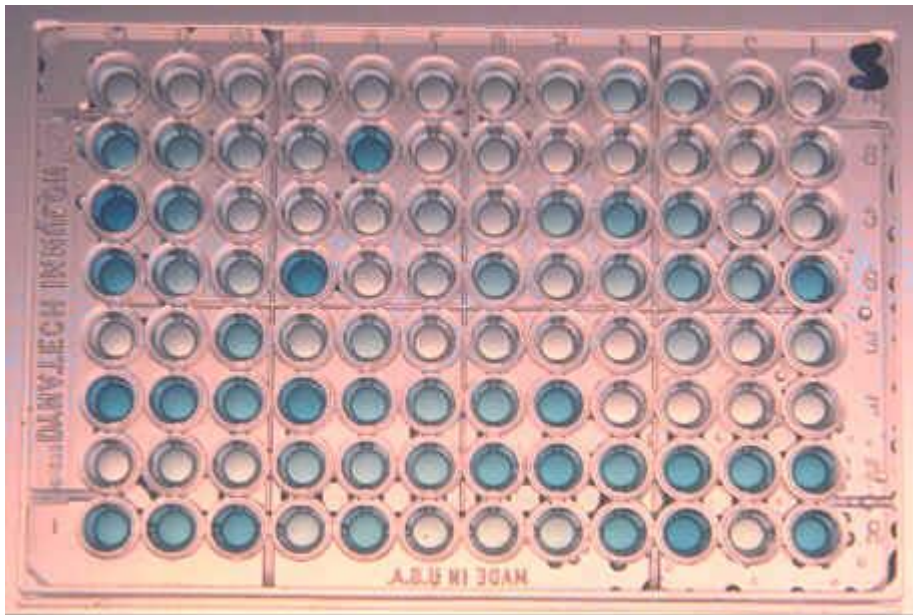


**Figure 7-15:** Example of a completed screening ELISA. Note that the dark blue well represents a strong signal[135]

Sometimes even the screening ELISA can produce more clones than needed as well.  This means that there might be multiple clones with the same structure.  If this is the case, it would be necessary to perform a BStN digest.  This digest is a type of electrophoresis by using an agarose gel.  In this digest, fragments are separated and sorted by size through a gel by the use of an electric field[136-138].  Because each fragment has a different make up and a different size, they can flow through the gel at different speeds.  The larger the particle, the slower it will move through the gel[136-138].  This will create bands in the gel when exposed to UV light.  Based on these bands, it can be determined what would be the makeup of these fragments.  Similar fragments should look the same under the light, while different fragments should have bands[136-138].  From there, the number of possible clones can be further lowered.  An example of this digest is shown below.
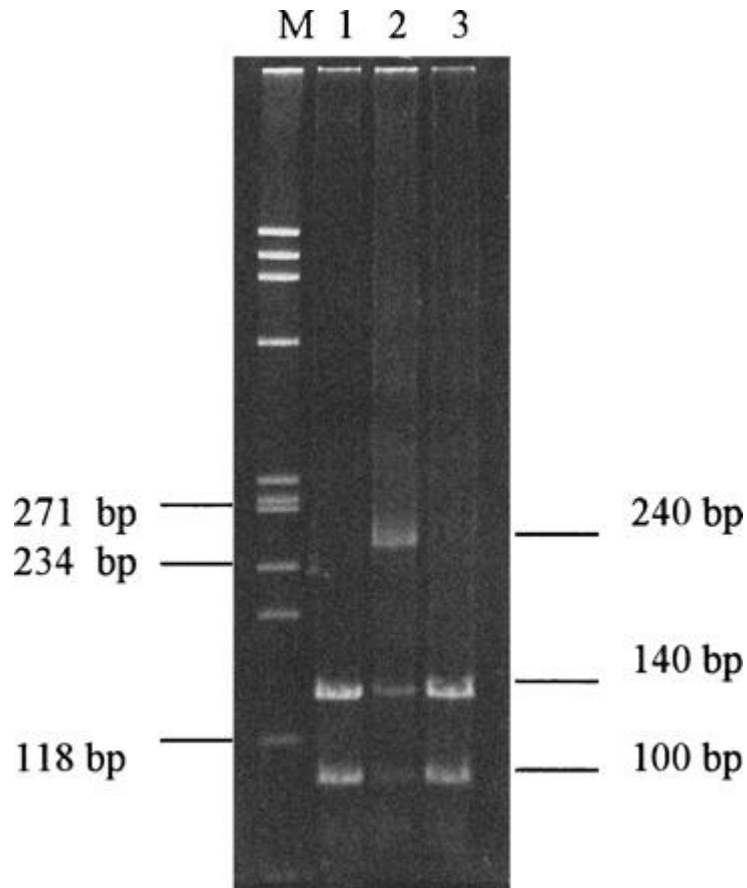
**Figure 7-16:** Example of a BStN1 digest[139]

Once a suitable number of clones have been found, the next step would be to

create titration curves for these fragments. This is in order to make sure that not only

these fragments are able to attach to their specific antigens, but also to find out the

strength of each antibody fragment. This is performed by using a titration ELISA[140].

In this ELISA, the concentration of antigen or antibody fragment is decreased along

the length of the micro titer plate. When substrate is then added to the wells, the color

should gradually become lighter as the concentration of antigen or antibody fragment

decreases[140,141]. An example of this can be found in figure 7-17. From this, the

overall strength of the antibody or antigen can be determined and how much of each

would be needed in order to produce a successful signal.

**Figure 7-17:** Example of a titration ELISA[141]

Once both ELISAs are performed and there are clones that have been chosen

based on these two ELISAs, these fragments are then broken up through a process

known as DNA sequencing. This allows the DNA strand on the fragment which

attaches to the antigen to be read in order to figure out its binding structure. One of

the most common methods is known as the Sanger Method[142]. In this method, the

fragments are broken into plasmids. These plasmids are then denatured by using heat.

This turns the plasmids into single strand structures of DNA. Next, a primer, or a

chain of 3 nucleotides is added to the ends of each strand. The strands of DNA are

then separated into one of four tubes containing dideoxynucleotides, one for each of

the four bases: adenine, thymine, cytosine and guanine. Each tube also contains DNA

polymerase, which attaches the nucleotides to the DNA. In each tube, the nucleotides

will attach to the strands until the specific nucleotide for that tube attaches to it. From there, the four tubes will go through gel electrophoresis, in which each strand is separated and the sequence can be read from top to bottom[142]. An example is shown below in figure 7-18
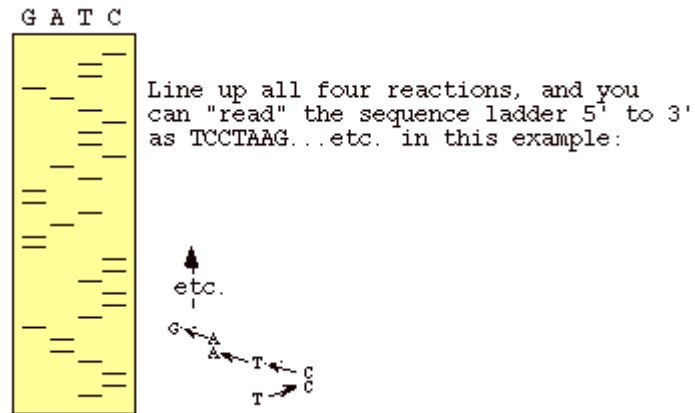


**Figure 7-18:** Example of DNA Sequencing using the Sanger Method[142]

Since this process is more involved, for the purposes of this project, the DNA sequencing will be performed offsite. The main goal for this however is to see how the antibody fragments attach to the tau and if there are multiple fragment clones that attach, either more than one clone with the same structure, or more than one clone with different structures. From DNA sequencing, it will be determined how these fragments can attach to the desired proteins. In the upcoming sections, the procedure for these experiments will be presented.

# CHAPTER 8

## METHODOLOGY

### 8.1 Phage Display

#### 8.1.1. Panning using Agar Plates

To begin with the experiments, it is important to mention the tau that was used.

The tau peptides that were used are located in the area shown in orange in figure 8-1.
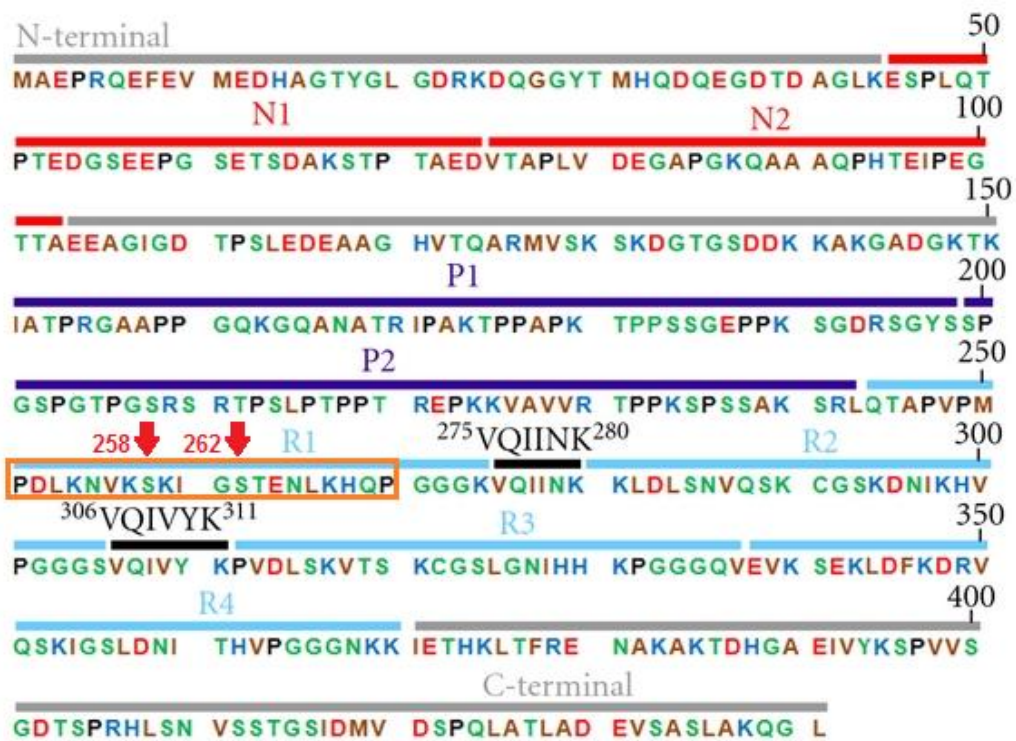


**Figure 8-1:** Tau protein with DNA sequence with peptide area highlighted in orange and phosphorylation sites in red[98].

Above is the sequence for tau protein, length 441. The box highlighted in

orange shows where on the tau the peptides used for this project come from. This

peptide is located between amino acids 251 and 260. It is 20 amino acids in length

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and has phosphorylation areas at site 258 (S) and 262 (S).  These are of importance

because they are phosphorylation areas in which Alzheimer's is prevalent.  In this

project, two types of peptides will be used: a peptide without phosphate groups at

those sites (Tau) and a peptide with phosphate groups at both sites (pTau).  Also, two

forms of each peptide will be used in these experiments.  The reason for this being that

micro titer plates coated with streptavidin will be used to carry out the phage display

and the ELISA for the peptides to attach to the well rather than capture the fragments.

For this reason, each peptide will be attached to one of two biotinylated linkers.  The

reason that two linkers will be used is that each linker has its own set of amino acids

connecting between the biotin and the peptide.  Because of this, it is possible for the

phage/scFv to attach to the linker.  Because of this, each peptide will either have what

will be known as linker 1 (L1) or linker 2 (L2).  With multiple panning rounds, each

round will alternate between the two linkers so that no phage/scFv that attaches to the

linker will be detected.  The following table lists the four different peptides that will be

used.

| Peptide Number | Name | Linker | Sequence |
|---|---|---|---|
| 1 | Tau L1 | eBio-*bAbAbAbAbA*- | PDLKNVKSKIGSTENLKHQP |
| 2 | Tau L2 | eBio-*GGSGGS*- | PDLKNVKSKIGSTENLKHQP |
| 3 | pTau L1 | eBio-*bAbAbAbAbA*- | PDLKNVK**p**SKIG**p**STENLKHQP |
| 4 | pTau L2 | eBio-*GGSGGS*- | PDLKNVK**p**SKIG**p**STENLKHQP |

**Table 8-1:** Peptides used in project with sequence (**p** – phosphate group) and linkers

There are four total experiments for this project, each with the same

procedures.  The only difference between them is the types of peptides being used.

The first two experiments focus on obtaining an scFv fragment by alternation.  This

will be performed with Tau only for experiment 1 and pTau only for experiment 2.  In

these experiments, peptides 1 & 2 (Tau L1 & Tau L2) and peptides 3 & 4 (pTau L1 & pTau L2) will alternate for each panning round. In experiment 1, peptide 1 will be used in rounds 1 & 3 while peptide 2 will be used in round 2. The same can be said for peptides 3 & 4 in experiment 2 (peptide 3 for rounds 1 & 3, peptide 4 for round 2). This method will not only make sure that only the selected phage at the end of the experiments will only bind to the peptide, but also with the additional third round, be in greater numbers. With this method, a scFv clone can be found that only attaches to the peptide.

The other two experiments focus on competition. This will be performed with the L1 peptides for experiment 3 and the L2 peptides for experiment 4. In these experiments, pTau peptides are in competition with the Tau peptides with the same linker. This means that before the phage comes in and attaches with pTau, some of the phage that either attaches to Tau or the linker will be removed. This is done in order to find phage that will attach to pTau at a simpler rate due to the fact that less phage is located in the solution. This can be reversed in order to locate phage for Tau with pTau in competition, but for these experiments, only phage against pTau will be considered.

### 8.1.2. Panning using Agar Plates

The first part of this project is to perform the panning rounds in order to retrieve the scFvs used for detection. There were two different methods used to perform the panning rounds. The first and easier method is to use agar plates to cultivate E. coli cultures to extract scFvs. To perform this experiment, a total of three panning rounds were performed to maximize the amount of cultures created. In this

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experiment, this procedure was taken from the protocol "Panning Ab-libraries:

Panning mit biotinylierten Oligopeptiden (Panning with biotinylated oligopeptides)"

developed by Michael Hust of the Institute of Biotechnology, TU Braunschweig,

Braunschweig, Germany[143] (see appendix B.1 for a English translation). To begin the

first round of panning, 1 well of a NUNC Streptavidin plate (Item no: 436014, Lot no:

123305, Roskilde, Denmark) was coated with 1 µg (1µL) of tau peptide diluted in 150

µL of PBS (Phosphate Buffer Saline). This was used as the coating well, which was

used as the main site for the phage to antigen binding. A second well was coated with

350 µL 2% mPBS (PBS + milk powder). This was the pre-adsorption well, which was

used to remove phage that bound either to the streptavidin or to other antigens. This

plate with these wells was stored overnight at 4°C without shaking. Diagrams

showing the steps are found below in figure 8-2.
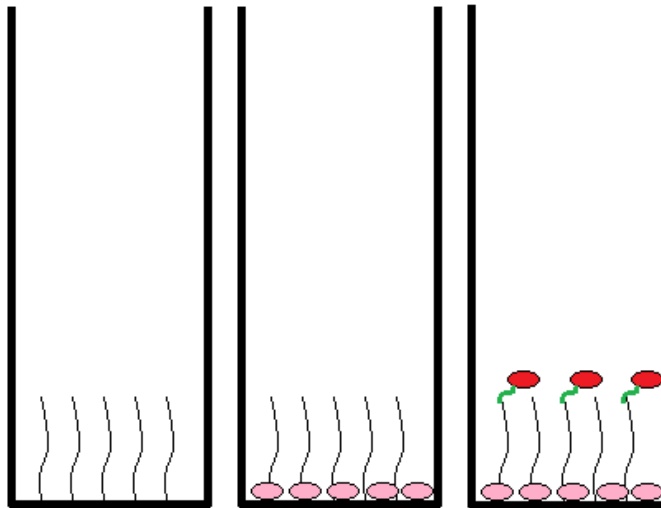


**Figure 8-2:** Diagrams showing the panning process with the Streptavidin on the well (left), addition of 2% mPBS for blocking (center) and the addition of biotinylated tau peptide (right)

In conjunction with this, a 50 mL 2×YT-T (Yeast Extract Tryptone Medium

with 20µg/mL tetracycline) overnight culture inoculated with E. coli XL1blueMRF'

(LOT#00016047973 (Patrick Droste), Institute of Biotechnology, TU Braunschweig, Braunschweig, Germany) was also prepared and incubated overnight at 37°C at 250 RPM (INFORD Thermotron High Temperature Shaking Incubator, Rose Scientific LTD, Edmonton, AB, Canada).  This was the first bacterial culture that was used to let the phage attach itself to the E. coli.  After incubation of the bacteria culture, 40 ml of 2xYT (Yeast Extract Tryptone Medium without tetracycline) was inoculated with 37°C overnight culture.  From there, the optical density (OD) was measured.  To ensure that optimal growth occurred, the solution was inoculated until the OD reached 0.1.  It was then incubated at 37°C and at 250 RPM and measured for OD until the OD reaches 0.5.  This process took about 1.5 hours.

While this process was going on, the incubation and phage sorting on the streptavidin plate was being performed.  These incubation steps were performed on a shaker at room temperature.  The first step was to wash the wells three times with 350 mL with 1X PBS with 0.05% Tween (PBST) using a microplate washer.  The washer that was used was a TECAN Columbus microplate washer (Männedorf, Switzerland).  Afterward, the pre-absorption wells were coated with 350 µL Panning block (2% mPBS + 2% BSA), while the coating wells were coated with 350 µL 2% mPBS.  It was then incubated for one hour.  The next step was to remove the panning block from the pre-absorption wells and add 150 µL Panning block with $5 \times 10^{11}$ clones of the antibody phage library to it.  This was performed with the HAL 7/8 library and the total volume of the library added was between 4 and 6 µL.  An additional 5 µL phage was added to the well to eliminate further binding to the streptavidin.  This was then incubated for one hour.  Afterwards, the panning block and phage were removed from

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the pre-absorption wells and placed in separate vials to be used in the next part. The

wells were then washed three times with 350 mL with 0.05% PBST. After this, the

phage was then added to the coating wells incubated for two hours. The wells were

then washed ten times with 350 mL with 0.05% PBST. This was to remove all signs

of possible excess phage and leave only phage that stuck to the antigen. After

washing, 200 µL Trypsin was added to re-suspend the phage in solution. This was

incubated at 37°C for 30 minutes (Memmert Incubator, Memmert, Schwabach,

Germany). The phage was then collected to be eluted and coated on agar plates.

Figure 8-3 highlights the addition of phage into the wells.
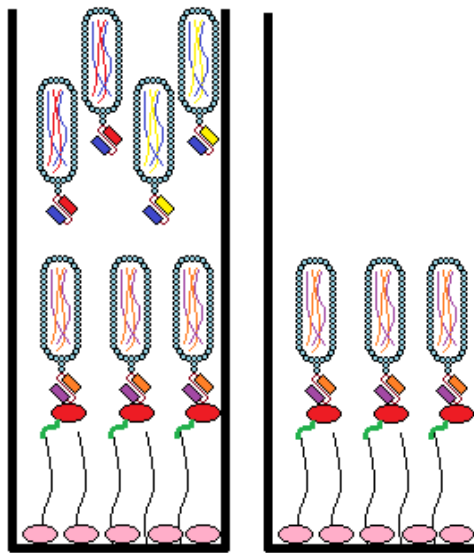


**Figure 8-3:** Diagrams of wells with phage entering and binding to the tau peptides
(left) and after removal of unbound phage (right)

The next part is to create and grow the bacteria cultures that will create the

scFvs. For this part, two sections will be performed. The first is to create the main

culture. For this step, 100 µL Phage was added to 20 mL 2YT Medium and incubated

at 37°C for 30 min. After incubation, this solution was centrifuged (Eppendorf

Cenrtrifuge 5810 R, Eppendorf, Hamburg, Germany) at 4000 RPM for 10 minutes.

The supernatant was then decanted and the remaining pellet was re-suspended with 250 µL 2YT medium. This solution was spread on a 15 cm 2YT-AG agar plate and incubated overnight at 37°C.

The second section was the elution of the phage. For this section, 10 vials were set up it create the dilutions. In five of these vials, PBS was added: two with 900 µL, one with 990 µL, one with 60 µL and one with 10 µL. After this was performed, 10 µL of a positive control (pHAL14-HT186-D11) was added to a sixth vial. Once this was completed, 10 µL Trypsin + Phage was added to the vial with 990 µL PBS. This dilution was 1/100 of the original. From there, two more dilutions were made. A second dilution was created when 100 µL of the first dilution was added to 900 µL PBS, which created a 1/1000 dilution. A third dilution was also created when 100 µL of the second dilution was added to 900 µL PBS, which resulted in a $1/10^4$ dilution. From there, 10 µL of each dilution was added to three more reaction caps, creating a further drop in dilutions. To perform this, 10 µL was taken from the $1/10^4$ dilution to create a $1/10^6$ dilution, 10 µL was taken from the 1/1000 dilution to create a $1/10^5$ dilution and 10 µL was taken from the 1/100 dilution to create a $1/10^4$ dilution. From there, the incubated XL1 culture is then added to these vials. The following vial had 50 µL added to it: the $1/10^4$ dilution, the $1/10^5$ dilution, the $1/10^6$ dilution, the positive control and the vial with 10 µL PBS. An additional 60 µL was added to an empty vial. After this, the following vials were incubated for 30 minutes at 37°C: the $1/10^4$ dilution, the $1/10^5$ dilution, the $1/10^6$ dilution, the positive control, the 60 µL XL1 culture, the 60 µL PBS and the 50 + 10 µL XL1 + PBS. After incubation, the three elutions were then spread on three separate 10 cm 2YT-AG agar plates, while the

remaining four were added drop wise onto a fourth plate.  These plates were then

incubated overnight at 37°C.

After incubation, each plate was checked for bacterial growth and all colonies

on the small plates were counted (see appendix B.3 for total counts).  After this step,

the amplification of the phage began.  In this stage, the scFvs are extracted from the

bacteria and are placed inside phage again for another panning round.  To begin with,

the 15 cm plate was washed with 5 mL 2YT-AG (2YT Medium + 1:1000 ampicillin +

1:20 glucose) medium.  From there, the suspension was mixed with a 40 mL solution

of 2YT-AG Medium until the OD of the solution was at 0.1.  The remaining

suspension was stored at 4°C.  This solution was then incubated at 37°C at 250 RPM

until the final OD was around 0.5.  Depending on the solution, this could take between

2 and 5 hours.  This is because during incubation, there is some bacterial growth, then

decay and then growth again.  Once the solution reaches an OD of 0.5, 5 mL (~ 2.5 X

$10^9$ cells) was extracted and  infected with a 1:20 ratio of M13K07 (5 X $10^{10}$ pfu)

helper phage.  This was then incubated twice: once for 30 min at 37°C without

shaking and again for 30 min at 37°C at 250 RPM.  Once incubation was complete,

the culture was then centrifuged at 4000 RPM for 10 minutes.  After, the supernatant

was decanted, the pellet was re-suspended with 1 mL 2YT medium and the total

solution is added to 30 mL to 2YT-AK (YT Medium + 1:1000 ampicillin + 1:1000

kanamycin) medium.  This was incubated overnight at 30°C at 250 RPM.  After

incubation, the solution was filtered and stored in separate vials.  This ended the first

panning round[143].

The remaining panning rounds (second and third) were performed in a similar

fashion as the first with some notable exceptions. First, different antigens were used during each panning round. This will be explained later on in the procedure. Second, the antibody library that was placed in the pre-adsorption well was from the previous panning round. This amount was 150 µL that was added without panning block. This was for both the second and third rounds. Third, the final was increased after complete incubation. The number of total washed in the final wash was the round multiplied by 10 (first round: 10 washes per well, second round: 20 washes per well, third round: 30 washes per well). The final variation was during the elution step. This step occurs normally as before, except with one additional part: amplification. In this part, 9 vials were used to create amplification. In four vials, 990 µL PBS were added and in two additional vials, 900 µL PBS were added. In the first 990 µL vial, 10 µL Trypsin + Phage was added to create a dilution that was 1/100 of the original. From there, five more dilutions were made. A second dilution was created when 10 µL form the 1/100 dilution was added 990 µL PBS, which resulted in a $1/10^4$ dilution. This was repeated with the remaining 990 µL PBS vials resulting in a $1/10^6$ dilution and a $1/10^8$ dilution. Then 100 µL was added from the $1/10^8$ dilution to a 900 µL PBS vial, creating a $1/10^9$ dilution and that was repeated with the last 900 µL vial to create a $1/10^{10}$ dilution From there, 10 µL of each dilution was added to three more reaction caps, creating a further drop in dilutions. To perform this, 10 µL was taken from the $1/10^{10}$ dilution to create a $1/10^{12}$ dilution, 10 µL was taken from the $1/10^9$ dilution to create a $1/10^{11}$ dilution and 10 µL was taken from the $1/10^8$ dilution to create a $1/10^{10}$ dilution. From there, 50 µL incubated XL1 culture is then added to these vials. Then three 10 µL samples of each were dropped on a 10 cm agar plate and incubated

overnight at 37°C.  The following day, each sample was counted for bacteria cultures[143].

### 8.1.3. Panning using Micro Titer Plates

Using micro titer plates during the panning rounds is seen as a faster method than the straightforward method of using agar plates for panning.  During the first panning round, the same methods used to find phage for scFvs up to when the bacteria are added to the phage.  Instead of using different sized agar plates, the bacteria cultures were placed in small amounts in micro titer plates for growth and cultivation.  In this experiment, this procedure was taken from the protocol "Panning Ab-Libraries: Panning in Immunotubes (MTPs)" developed by Michael Hust of the Institute of Biotechnology, TU Braunschweig, Braunschweig, Germany[144] (see appendix B.2 for an English translation).  To perform this, the exact same procedure for the first panning round was used (see 8.1.2. Panning in Agar Plates) until it was time to perform the elution.  To perform the elution, 50 µL of Phage was added in one well of a micro titer plate, along with 130 µL of 2YT-XL1 Culture.  This was then incubated at 37°C for 30 min.  Added to the well after incubation was 20 µL of 10XGA (2YT + 1:20 Glucose + 1:1000 Ampicillin) medium to the culture.  This was then incubated overnight at 37°C at 850 RPM (LabNet VorTemp 56 Shaking Incubator, LabNet, Edison, NJ).

Amplification of the phage was also different than in the previous experiments.  To begin amplification, 5 µL overnight culture was added to 180 µL 2YT-AG medium in one well in a new micro titer plate and 10 µL was added to 180 µL 2YT-AG medium in a second well in that same plate.  This was then incubated at 37°C and 850

RPM.  The remaining culture was stored at 4°C.  The OD was then found and recorded.  This was found by using a TECAN Sunrise microplate reader (Männedorf, Switzerland).  However, in previous experiments, the OD was measured at a wavelength of 600 nm.  In this experiment, the wavelength was measured at 620 nm.  To find the optimal OD, the following measurement had to be used: an $OD_{620}$ between 0.3 and 0.4 is around an $OD_{600}$ between 0.4 and 0.5.  The incubation time varied between 1.5 and 2.5 hours.  After incubation, 10 µL M13K07 helper phage was added to each well and the cultures were incubate at 37°C without shaking for 30 min and at 37°C and 850 RPM for 30 min.  Each culture was centrifuged at 4000 RPM for 10 min and the supernatant was decanted.  180 µL 2YT-AK medium was added per well and the cultures were incubated overnight at 30°C and 850 RPM.  The next day, the cultures were centrifuged for 10 min at 4000 RPM.  100 µL of supernatant were collected in vials and were used in the next round of panning, where the 100 µL of supernatant was added to the pre-adsorption wells as in the previous experiment.  This was repeated during the second round of panning.  However, the third round of panning, agar plates were used again during the colony growth for use in the upcoming screening ELISA.  This procedure was shown in the previous section[144].

## 8.2.  ELISA and scFv Detection

### 8.2.1.  Screening ELISA

During the Screening ELISA, the scFv clones go through a process where they are attached to a piece of antigen and and then detected with detection antibodies to see if they attached to the antigen and how strong the signal is.  In all ELISA experiments, the procedure was taken from the protocol "ELISA" developed by

Michael Hust of the Institute of Biotechnology, TU Braunschweig, Braunschweig, Germany[145]. To begin this section, cultures were picked from the cultivation plates from the panning round and placed in a well of one of two 96 well Costar micro titer plates (Corning Inc., Corning, NY). Each well was filled with 180 μL 2xYT-AG Medium. In total, 92 wells had a clone culture, 2 wells (H3 & H6) were left blank for the negative control and 2 wells (H9 & H12) had a positive control (pHAL14-HT186-D11) inside. Each plate was incubated overnight at 37°C at 800 RPM. There were two plates that were used because of the amount of colonies that were found after the third panning round. After incubation of the micro titer plates, a second set of plates were used for bacterial growth of the overnight cultures. In these plates, 170 μL of 2YT-AG Medium along with 10 μL of the overnight culture was added per well. The remaining clones were stored at 4°C. These new plates were incubated at 37°C at 800 RPM for 2 hours. After incubation, the solutions were centrifuged at 4000 RPM for 10 minutes. The supernatant was then decanted and a solution of 180 μL 2YT-AI (2xYT Medium + 1:1000 ampicillin + 1:20000 IPTG (Isopropyl β-D-1-Thiogalactopyranoside)) medium per well. These plates were incubated overnight at 30°C and 800 RPM.

At the same time, the ELISA plates were set up. For one micro titer plate that had picked colonies inside the wells, 2 ELISA plates were used during the run. One plate was used as the positive control, where there the antigen used in the phage display will be used in the experiment to see if the scFv bound to the antigen. The other plate contained no antigen and was used to see if that scFv bound to the streptavidin. Both ELISA plates were coated with streptavidin, (100 μL at a

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concentration of 2 µg/mL), except for the positive controls, wells H9 & H12, which were coated with 100 µL of 1 µg/ml MucI. These plates were then stored overnight at 4°C to allow for coating of the wells.

After the overnight incubation for the ELISA plates, the streptavidin was discarded and both plates were blocked with 350 µL of 2% BSA in 0.1% PBST. This was incubated for one hour. Then the plates were washed three times with 0.05% PBST. The next step was to add 100 ng of the tau antigen in 100 µL PBS to each well of the one of the plates. This was added for all wells, except for the positive controls, where 100 µL hMucI was added to wells H9 & H12. This plate was used as the positive control during the ELISA. In the other plate, only 100 µL of PBS was added to each well, except again for the two wells used as the positive control. This plate represented the negative control during the ELISA. Both plates were incubated for an hour and washed three times with 0.05% PBST.

During this part of the experiment, the overnight cultures were centrifuged at 4000 RPM for 10 minutes. After centrifugation and the ELISA plate washing, the scFv clones were transferred to the ELISA plates. This was performed by adding 50 µL of 2% BSA in PBS and transferring 50 µL of the scFvs into those ELISA plates, making sure that the clone in a given well went to the same well in the ELISA plates. Both plates were incubated for an hour and washed three times with 0.05% PBST. After washing, 150 µL of 9E10 primary antibody (goat anti myc antibody, 1:1000) in 2% BSA in PBS were added to each well in both plates. These plates were then incubated for an hour and washed three times with 0.05% PBST. Afterwards, 150 µL of a secondary antibody (goat anti mouse with a tag, 1:1000) in 2% BSA in PBS was

added to each well.  These plates were incubated one last time for one hour and washed three times with 0.05% PBST.

The last step was to perform the detection of the ELISA.  For this, a TMB (3,3',5,5'-Tetramethylbennzidine) mixture was used to detect the antibodies.  To perform this detection, 100 µL of a 20:1 ratio of TMB-A:TMB-B was added to each well.  From there, the detection began when the colors of the wells changed from clear to blue.  The darker the blue, the more concentrated the scFvs are in the well.  In order to make sure that the experiment worked, all the positive control wells had to be blue, all the negative control wells had to be clear and the wells on the positive control plate had to be blue, while the wells on the negative control plate had to be clear.  The first signs of detection in the plates were found within the first five minutes.  After 20 – 30 minutes, the reaction slowed down and the background started to come into play.  At this point, the experiment detection was stopped by adding 100 µL of Sulfuric Acid in each well.  This turned the solution from blue to yellow and allowed the experiment to stop so that the OD of each well could be measured.  To measure the OD, the plate was placed in an ELISA reader (TECAN Sunrise) and the reader was set to measure the OD at a wavelength of 450 nm, but have a reference wavelength at 620 nm.  After, the plates were read, the results were graphed and clones that worked well in the experiment were chosen based on two factors: intensity and high positive:negative ratio[145].  Once the clones are chosen, they are grown overnight in 5 mL 2YT medium.  After incubation, 80 µL of culture is added to a sterile vial, along with 20 µL of glycerol, mixed together and stored at -80°C for further use in experiments.  Diagrams explaining the process are shown in figure 8-4.

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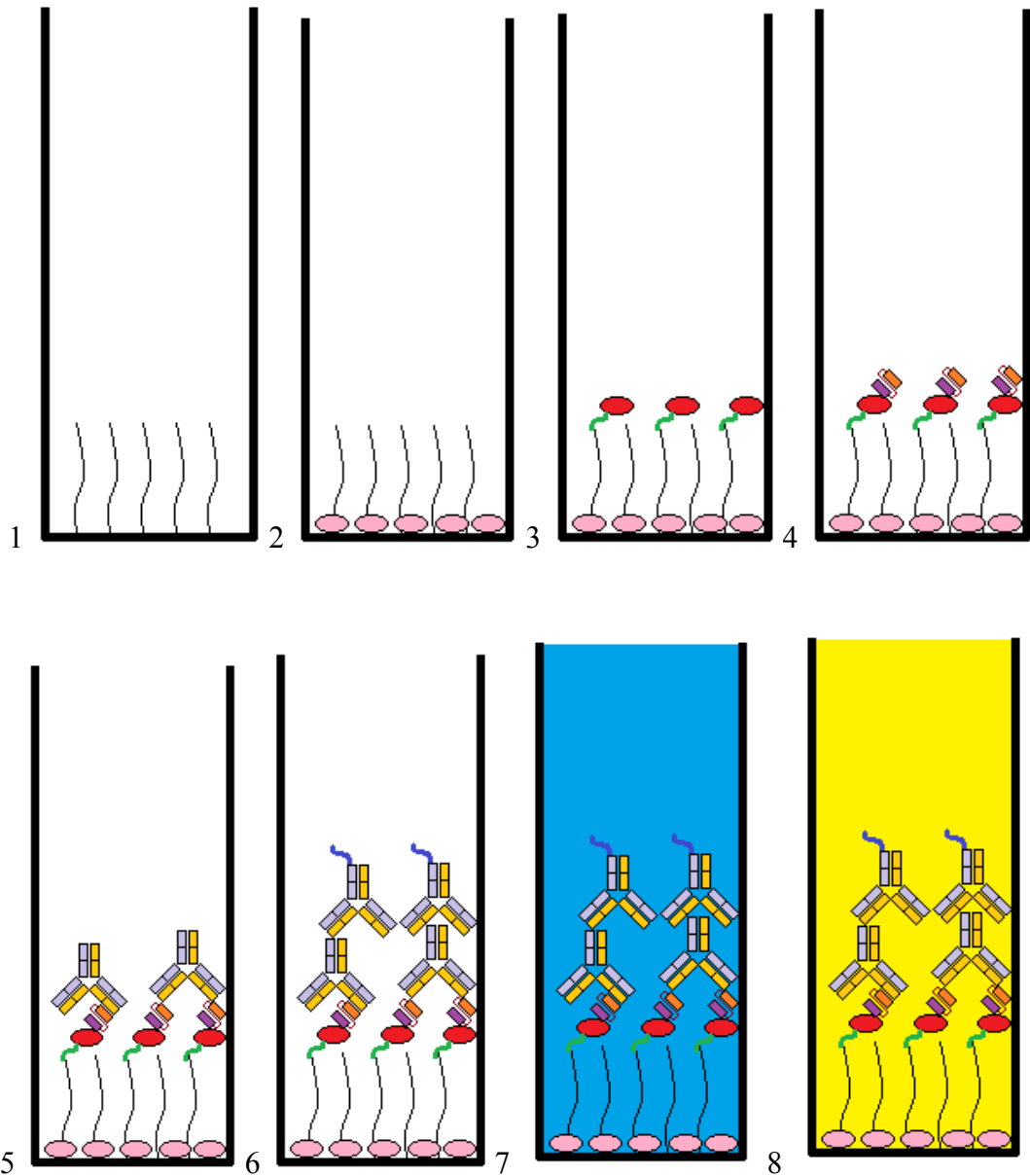
**Figure 8-4:** Diagram showing the ELISA steps (1: coating of streptavidin, 2: blocking with 2% BSA in PBS, 3: addition of tau, 4: addition of scFv clones, 5: primary antibody, 6: secondary antibody, 7: TMB, 8: sulfuric acid)

### 8.2.2. BStN Digest.

After the screening ELISA, the clones that gave off a high signal and aspect ratio were chosen to be sequenced for possible antibodies. However, some screening ELISAs can display multiple positive clones. These clones can be reduced by selecting a minimum OD level in the ELISA. However, at times, there can be

upwards to 20 clones within a given OD range. When this happens, it is necessary to perform a BStN Digest. This digest breaks down the scFvs into smaller components and sorts the components based on mass. It is used to find out if there are any identical clones in the system. Once the digest is performed, the number of possible binders is greatly reduced because it can be shown that for multiple picked clones, they have the same structure and DNA makeup.

To perform the digest, the procedure is broken up into four parts: breakdown of scFvs, building of the separation gel, separation of the scFvs and optical detection. To begin the experiment, the scFvs have to be broken down. This starts with a master mix to use in the Thermal Cycler (MJ Research PTC 200, MJ Research, St. Bruno, QC, Canada) in order to perform a polymerase chain reaction (PCR). Each clone needs the following amount of these ingredients to be in the vial for breakdown:

- 2 μL of 5X GoTaq Flexi Buffer

- 0.4 μL of Magnesium Chloride

- 0.2 μL of Deoxynucleotide Triphosphates (dNTPs)

- 0.5 μL of MHgIII_r Primer

- 0.5 μL of LacZpro_f Primer

- 0.05 μL of GoTaq Polymerase

- 6.35 μL of distilled water

A small amount of scFv is scraped and placed inside a vial with the master mix. From there, the PCR is activated and the following program is used during the process:

1. 95°C for 2 minutes

2. 94°C for 20 seconds

3. 56°C for 15 seconds

4. 72°C for 90 seconds

5. Repeat steps 2 – 4 a total of 29 more times

6. 72°C for 3 minutes

7. End program at 16°C

From there, 3 µL of solution is transferred to a second vial that contains the following:

- 0.5 µL of 10X Nuclear Extraction Buffer 2 (NEB2)

- 0.5 µL of 10X Bovine Serum Albumin (BSA)

- 0.2 µL of BstNI (10 U/µL)

- 0.8 µL of distilled water

From there, the PCR is activated and the following program is used during the process:

1. 60°C for 1 hour

2. 65°C for 10 minutes

3. End program at 16°C

At the same time the agarose gel has to be assembled.  To assemble the gel, the following procedure is used.  In a clean glass molding, the following mixture is added in two parts.  It is added until about halfway, hardened, and completely filled:

- 1.55 mL of distilled water

- 3.2 mL of 30% Acrylamid mix

- 1.2 mL of 5X Tris/Borate/EDTA (TBE) buffer

- 60 µL of 10% Ammonium Persulfate (APS)

- 2.4 µL of Tetramethylethylenediamine (TEMED)

Both the chemicals used for the PCR and agarose gel came from New England Bio Labs in Ipswich, MA.  Slots are added during the making of the top layer, so that the clones are easily inserted inside the gel.  Once the samples have been prepared and the gel has been hardened, the digest is then able to begin.  1 – 2 µL of dye is added to each sample to where each line in the digest is and to give an estimate as to where the samples have traveled down the gel.  In total: 5 – 7 µL of sample was added to each well as well as a 4 µL sample of marker it show where the size of each particles is separated along the gel.  To run the experiment after all the samples have been loaded, the gel and casing is attached to a container, which is filled with 1% TBE Buffer solution.  An electrical charge runs through the gel at 300V and 50mA for 45 minutes in order to pass the samples through the gel.  After 45 minutes, the gel is removed, and added to a petri dish with 40 µL of Ethidium Bromide (c = 10 µg/mL) 100 mL TAE (Tris/Acetate/EDTA) Buffer and incubated at room temperature for 15 minutes.  This constitutes the developing stage so that the lines can be easily seen.  After development, the gel is placed in a UV scanner, where ultraviolet rays allow the bands to be seen inside the gel.  From there, one would be able to decipher which clones are identical and which ones had some abnormalities in the DNA structure.  From there, the total number of possible clones can be decreased based on similar structures, as

one will be able to see by way of DNA sequencing.

### 8.2.3.  Titration ELISA

During the titration ELISA, each clone is placed into two different tests on the same ELISA: to make sure that the scFv only binds to its proper antigen and that the limit of detection for each scFv clone is found.  The procedure for this ELISA is similar to that of the screening ELISA, except for some differences during the addition of the scFv clones and the antigens used to attach to the scFvs[145].  The experiment starts with a 96 well micro titer plate, where 4 wells per clone is filled with 180 µL of 2xYT-AG medium**.**  Each culture is reused from cryotubes that were prepared earlier after the screening ELISA for each specific binder and each culture was placed in the filled wells.  One of the wells had a positive control inside so that it was to be shown that the experiment functioned properly.  This plate was incubated overnight at 37°C at 800 RPM.

After overnight incubation overnight a second 96 well micro titer plate is used for the growth of the clones. 170 µL of 2YT-AG medium is added to the same locations as the first plate.  10 µL of the overnight culture is then added per well.  This plate is then incubated at 37°C at 800 RPM for 2 hours.  Once incubation is completed, the plates are spun down and the supernatant is decanted.   180 µL of 2YT-ASI medium is added per well and the plates are incubated overnight at 30°C and 800 RPM.  At the same time, the ELISA plates are being prepared.  The Costar ELISA plates are coated with 100 µL Streptavidin (100ng/well), except for one well, which was coated with 100 µL of MucI (1µg/well) to be used as a positive control.  The plates are incubated for 1 hour at room temperature.  From there, the Streptavidin is

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discarded and each well was blocked with 2% BSA in 0.1% PBST. These plates are then stored overnight at 4°C.

The following day, the plates are washed three times with 0.05% PBST. At this point, the antigens added to the plate. Each antigen was added so that there was one antigen per row. For each clone, two rows are used for the antigen the scFv should bind to, one row is used for the antigen that the scFv should not bind to and one row does not have antigen, in order to see if there was binding to the streptavidin. In each well with antigen, add 100 ng of antigen is added to 100 µL of PBS. In the rows where no antigen is added and the well with the positive control, only 100 µL of PBS is added. The plates are then incubated for 1 hour and washed three times with 0.05% PBST. In the meantime, a 96 well micro titer plate is used per ELISA plate for the transfer of scFvs. In each well, 100 µL of 2% BSA in PBS is added. The overnight culture is spun down and the supernatant is transferred in the following way: 100 µL of supernatant for a specific clone is added to each well in the first column of that 96 well plate. After mixing, 100 µL of mixture in the first column is transferred to the second column. After mixing, 100 µL of mixture in the second column is transferred to the third column. This process is repeated for each column until the last column is reached. Then, starting with the second to last column, 100 µL of mixture is transferred from the micro titer plate to its exact column on the ELISA plate. This is done by transferring the second to last column, then the third to last column, and so on until the first column is transferred. This is done so that the results can be as accurate as possible. In the last column of the ELISA plate, 50 µL of 2% BSA in PBS is added, as well as 50 µL of the overnight culture for the positive control. These plates are then

incubated at room temperature for 1.5 hours and washed three times with 0.05% PBST.

The remaining steps are the same as for the screening ELISA. After washing, 150 µL of 9E10 primary antibody (goat anti myc antibody, 1:1000) in 2% BSA in PBS were added to each well in both plates. These plates were then incubated for an hour and washed three times with 0.05% PBST. Afterwards, 150 µL of a secondary antibody (goat anti mouse with a tag, 1:1000) in 2% BSA in PBS was added to each well. These plates were incubated one last time for one hour and washed washed three times with 0.05% PBS.

The last step was to perform the detection of the ELISA. For this, a TMB mixture was used to detect the antibodies. To perform this detection, 100 µL of a 20:1 ratio of TMB-A : TMB-B was added to each well. From there, the detection began when the colors of the wells changed from clear to blue. The darker the blue, the more concentrated the scFvs are in the well. In order to make sure that the experiment work, all the positive control wells had to be blue, all the negative control wells had to be clear and the wells on the positive control plate had to be blue, while the wells on the negative control plate had to be clear. The first signs of detection in the plates were found within the first five minutes. After 20 – 30 minutes, the reaction slowed down and the background started to come into play. At this point, the experiment detection was stopped by adding 100 µL of sulfuric acid in each well. This turned the solution from blue to yellow and allowed the experiment to stop so that the OD of each well could be measured. To measure the OD, the plate was placed in a machine and the reader was set to measure the OD at a wavelength of 450 nm, but have a

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reference wavelength at 620 nm[145].  If the experiment worked, the intensity would fade from left to right in the two rows where the antigen that is supposed to attach to the scFv, while the other rows would give off no signal.  From there, the best shown clones would be then chosen to be sequenced to show the DNA structure and to see any common areas in the structure or if they were identical.

### 8.2.4.  DNA Sequencing

The final step for the whole experiment is the DNA sequencing.  In this part, the structure of each scFv is found and one can see if there is more than one clone that can be used to detect antigens.  To begin with the sequencing, an overnight culture is first made by inserting a small amount of culture for a specific clone in a test tube filled with 5 mL of 2xYT-AG medium.  This test tube is incubated overnight at 37°C at 250 RPM.  The following day, the overnight culture is poured into a 1.5 mL reaction cap, one milliliter at a time and centrifuged for 1 minute at 10X g (Eppendorf Centrifuge 5415 D, Eppendorf, Hamburg, Germany).  The supernatant is discarded and the process is repeated until all the culture is used up and the pellet remained in the reaction cap.  The next part of the experiment used the peqGOLD Plasmid Mini Kit (PeqLab, Erlangen, Germany) in order to extract the DNA.  The first step is to suspend the pellet completely in 250 µL of Solution I, which is mixed with Rnase.  Then 250 µL of Solution II is added and the cap is gently inverted until a clear lysate formed.  After that, 350 µL of Solution III is added and the cap is again gently inverted until a white precipitate formed.  The cap is then centrifuged for 10 min at 10X g.  The resulting supernatant is then transferred to a peqGold membrane (~750 µL) and centrifuged for 1 minute at 10 X g.  After centrifugation, 750 µL DNA Wash Buffer,

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which was mixed with ethanol, is added to the membrane and centrifuged for 1 minute at 10 X g.  This last step is repeated one more time.  Then the membrane was dried by centrifugation for 2 minutes at 10 X g.  Following drying, 100 mL of elution buffer is added to the membrane, centrifuged for 1 minute at 5X g and collected in a clean reaction cap.  This elution is stored at 4°C.

To prepare the elution for sequencing, new reaction caps containing the plasmids must be sent to a GATC Biotech AG (Konstanz, Germany) for sequencing. These reaction caps had to contain a plasmid concentration between 30 and 100 ng/µL, while the total volume had to be equal to 30 µL.  To obtain this precise concentration and volume, elution buffer was added to the specific volume of plasmid to obtain the desired concentration at 30 µL of solution.  The caps were shipped and in a few days, the sequencing came back as a downloadable note file, which could be run on VBASE2 (www.vbase2.org) to give the proper sequence for each scFv clone Multalin (multalin.toulouse.inra.fr/multalin/) in order to see if multiple clones were similar to each other, but how they lined up against each other.

FINDINGS

## 9.1. Non-phosphorylated-Tau Protein

### 9.1.1. Screening ELISA
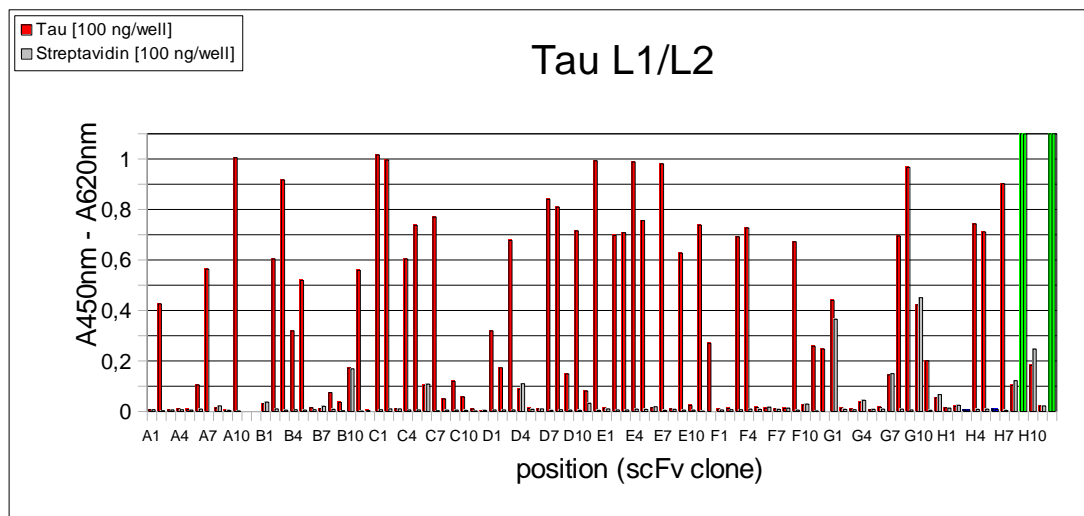


**Figure 9-1:** Screening ELISA for Tau

The main objective for obtaining results with non-phosphorylated Tau is to show what type of scFvs can be created with a version of Tau that was not only healthy and did not immediately show the possible effects of phosphorylation, but also that there was a baseline for detection, that strands of Tau could be found and not render the experiment obsolete. After three panning rounds, 92 E. coli samples from were collected, cultivated, had all possible amounts of scFvs extracted, and underwent a screening ELISA. The results are as follows: Out of a possible 92 potential clones, 37 clones had an OD greater than or equal to 0.3. However, two of these clones also had a positive signal:negative signal under 5. The reason that there needs to be a

positive:negative ratio over 5 is that there is only streptavidin in the wells of the negative control. This is to make sure that the scFvs will ONLY attach directly to the antigen and not anywhere else, including the streptavidin. Out of the possible 35 clones, that could be of use, 31 had an OD greater than 0.5. However, to further show that the amount of scFvs can be maximized, 11 had an OD greater than 0.8 and only 4 had an OD greater than 1.0. To show which clones were the best during this ELISA, all clones that exhibited an OD greater than 0.8 were used in the following BStNI digest.

| A10 | B3 | C1 | C2 | D7 | D8 |
|-----|-----|-----|-----|-----|-----|
| D12 | E4 | E7 | G9 | H7 | |

**Table 9-1:** Chosen clones for BStN digest for Tau

### 9.1.2. BStN Digest
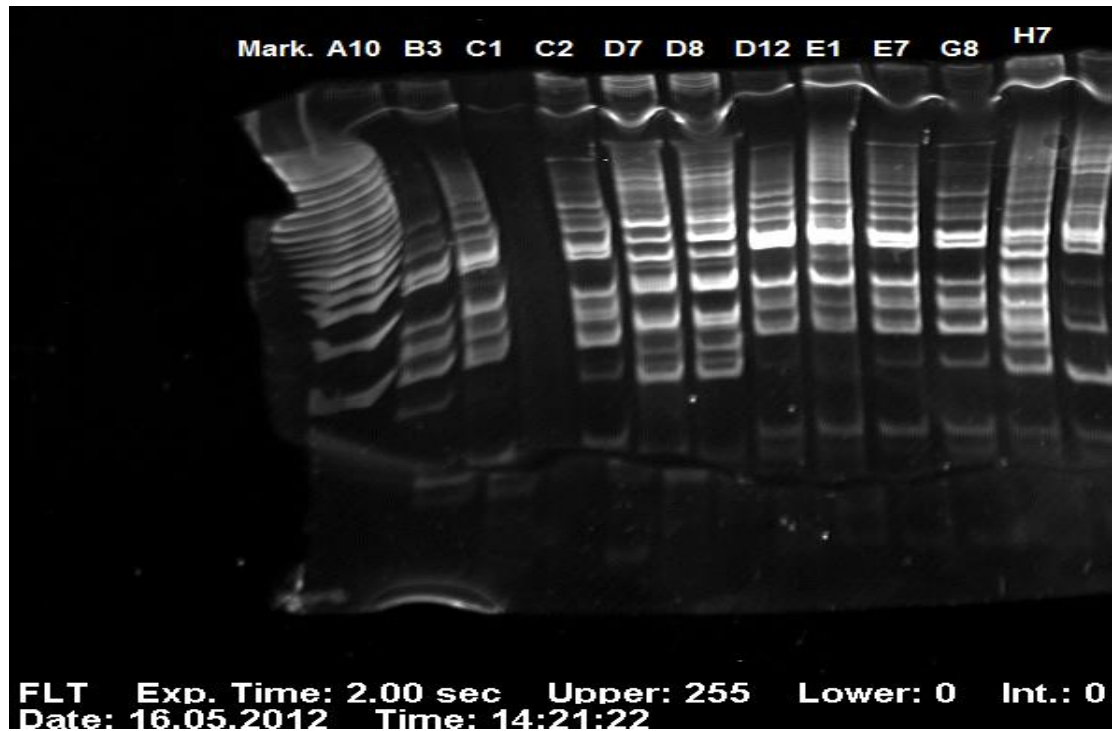


**Figure 9-2:** BStN Digest of Tau clones from A10 through H7

The use of the BStNI digest is to determine if there are any differences in the makeup of each clone.  Each scFv component is broken up into smaller fragments and separated an agarose gel using electrophoresis.  During the experiment, each component is separated based on mass (the greater the mass, the higher up in the column it would be), with a band showing the location of each particle.  Although the bands cannot be seen in the gel directly, they can be detected via UV light.  For this experiment, 11 clones were prepared and separated in the aforementioned gel.  As a result of this separation, there were 5 different sets of bands that could be used to differentiate each scFv (A10-C2-D12-G8, B3-E1, D7-D8, D12-E7, H7, with C1 not displaying any signal).  However, in some cases, there seemed to be different variations of intensities that corresponded with each clone, as shown with clones E7 & G8.  Also, on some of the clones, there appears to be an extra band near a specific group of bands, which could mean an error in the process of splicing or a totally different clone altogether.  This was shown for clones D12 and E1, for example.  Because of these bands, 6 clones will go through DNA sequencing and a titration ELISA.

| A10 | C1 | D7 | E4 | E7 | H7 |
|-----|-----|-----|-----|-----|-----|

**Table 9-2:** Chosen clones for Titration ELISA and DNA sequencing for Tau

### 9.1.3. Titration ELISA

## Tau L1/L2 Titration ELISA
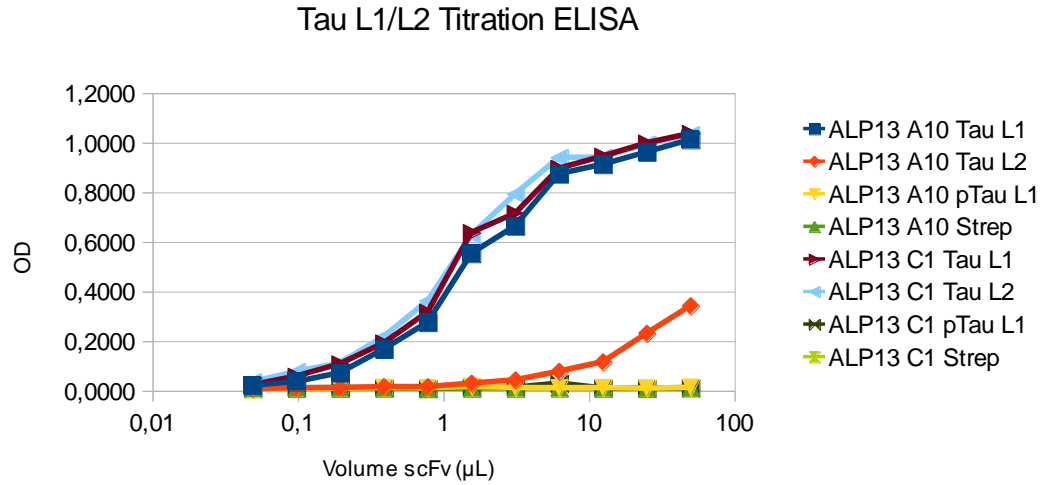


**Figure 9-3:** Titration ELISA of Tau against Tau (linkers 1&2), pTau (linker 1) and Streptavidin for clones A10 and C1

## Tau L1/L2 Titration ELISA
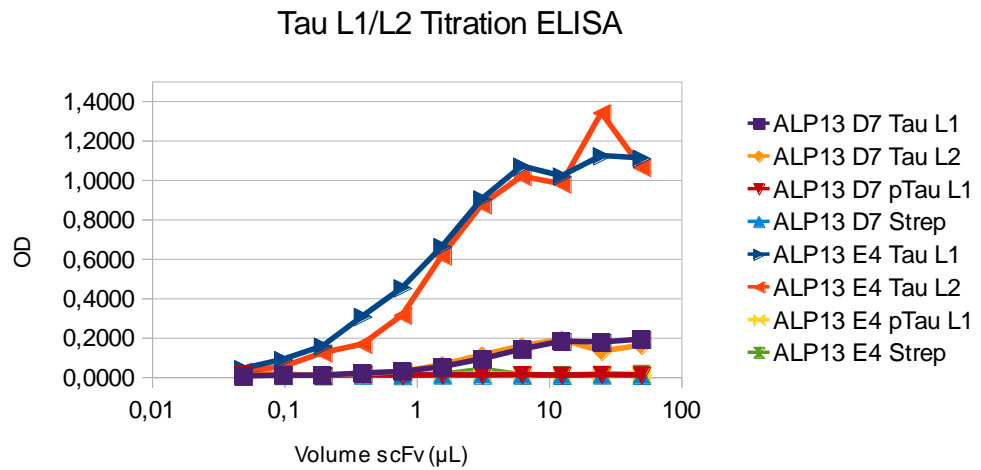


**Figure 9-4:** Titration ELISA of Tau against Tau (linkers 1&2), pTau (linker 1) and Streptavidin for clones D7 and E4
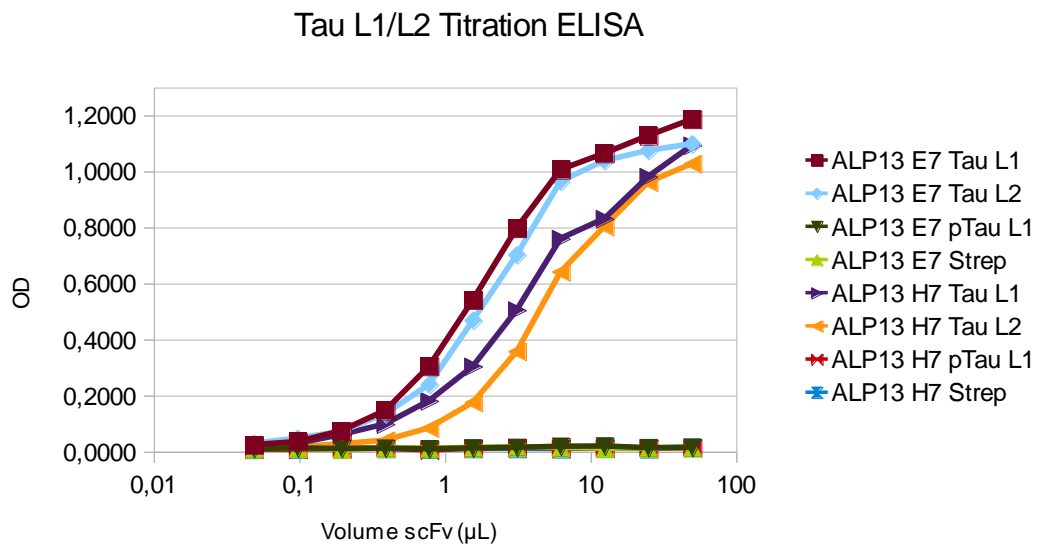
**Figure 9-5:** Titration ELISA of Tau against Tau (linkers 1&2), pTau (linker 1) and Streptavidin for clones E7 and H7

The purpose of the titration ELISA is to determine the intensity of specific concentrations of each scFv clone, or the limit as to how intense the detection is. In this experiment, with the binders for non-phosphorylated tau, we see that the most concentrated samples for 4 clones show a standard OD of between 1.0 and 1.4. Only one clone had one peptide at an OD of 0.4 and one clone had both peptides at around 0.2 at the maximum concentration. Because of this, these clones are not well fit to be used for further experiments involving Tau. What is also worth noting is that the detection signal is still strong (similar OD), even at 1/8 the concentration of scFvs. After this is when the signal becomes weaker with decreasing concentration. However, at an OD at 0.2, the intensity of this decreasing OD lessens and becomes more gradual towards an OD between 0.03 and 0.1. This occurs at an scFv concentration 1/64 – 1/128 the size of the original concentration. Therefore, we can see that based on this observation, these scFv clones have a limit of detection at 1/128 the original concentration, with a high amount of detection starting at 1/8 the original

concentration.  Because of this, all six clones will be sequenced: A10, C1,D4, E4, E7 & H7.

### 9.1.4.  DNA Sequencing:

With the DNA sequencing, the pattern of each attaching points on the scFvs for both the heavy chain and the light chain are found and the antibody library can be made for that specific scFv that attaches to the non-phosphorylated Tau.  For this, it was ideal to base the clone selection from a CDR comparison (due to an IP relevance, the CDR comparisons cannot be shown, but the structure of the scFvs for both Tau and pTau will be presented in section 9.5).  Based on the results of the sequencing, only three clones had full heavy and light chains that were similar: A10, C1& E4.  Clone E7 had the same heavy chain, but no light chain, which made it unsuitable for further study.  The other two clones, D7 & H7, were sequenced, but also had unfavorable results.  Clone D7 had a sequence that was completely different than the other three clones, while clone H7 was missing completely.  It was later re-sequenced, but the light chain sequence was also missing.  Also, it is important to note that even though the light chain was missing for two of the clones, the heavy chain was still identical in sequence as with the three best clones.  In terms of choosing a clone, it was determined that based on the ELISAs and sequence, that clone E4 will be used as the biomarker against Tau..  However, even though clone D7 did not have a high signal in the titration ELISA, because of the difference in the CDR comparison, this could be a second possible candidate for the scFv against Tau.

## 9.2. Phosphorylated-Tau Protein

### 9.2.1. Screening ELISA



**Figure 9-6:** Screening ELISA for pTau

The main objective for obtaining results with phosphorylated Tau is to show what type of scFvs can be created with a version of tau that was phosphorylated. This is because unlike non-phosphorylated tau, this type of tau is known as a signaler for Alzheimer's disease. After three panning rounds, 92 E. coli samples from were collected, cultivated, had all possible amounts of scFvs extracted, and underwent a screening ELISA. The results are as follows: Out of a possible 92 potential clones, 29 clones had an OD greater than or equal to 0.6. None of these clones had low positive to negative control ratio, meaning that it is with the upmost confidence that these clones will only attach to the antigen and not the streptavidin. Out of these possible 29 clones, that could be of use, 16 had an OD greater than 0.8, with the majority of these clones within the same OD range. These clones were used in the following BStNI digest.

| | | | |
|---|---|---|---|
| A2 | A5 | B7 | B8 |
| B9 | B10 | C3 | C6 |
| C12 | E3 | E11 | F3 |
| F5 | G7 | H4 | H5 |

**Table 9-3:** Chosen Clones for BStN Digest for pTau

### 9.2.2. BStN Digest
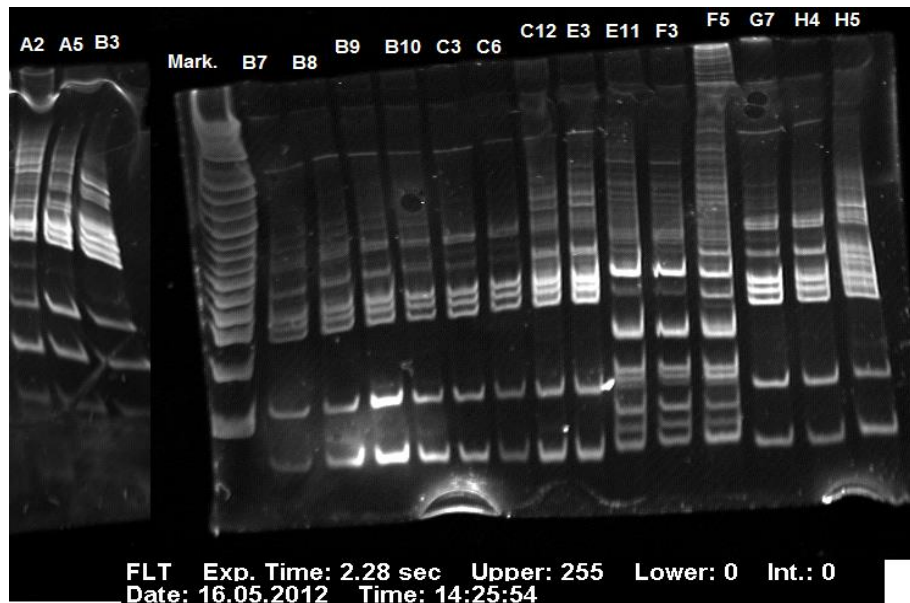


**Figure 9-7:** BStN Digest of pTau clones B7 through H5

For the BStNI digest, 16 clones were prepared and separated in an agarose gel.
As a result of this separation, there were 5 different sets of bands that could be used to
differentiate each scFv (B7-B8-B9-B10-C3-C6-G7-H4, C12-E3, E11-F3, F5 & H5).
However, like in section 9.1.2, in some cases, there seemed to be different variations
of intensities that corresponded with each clone (C6 and G7 for example). Because of
these bands, 10 clones will go through DNA sequencing and a titration ELISA.

| | | | | |
|---|---|---|---|---|
| A2 | A5 | B3 | B10 | C3 |
| E3 | E11 | F5 | H4 | H5 |

**Table 9-4:** Chosen clones for Titration ELISA and DNA Sequencing for pTau

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### 9.2.3. Titration ELISA

In terms of this round of the titration ELISA, this experiment had to be performed twice. The first time, there was an issue with pTau L1. The scFvs were binding properly to pTau L2 but not to pTau L1. It was then determined that the peptides were old and contaminated during the course of the screening ELISA, with a new set of peptides, the screening ELISA could resume and the results shown below and on the next page display the actual final product.
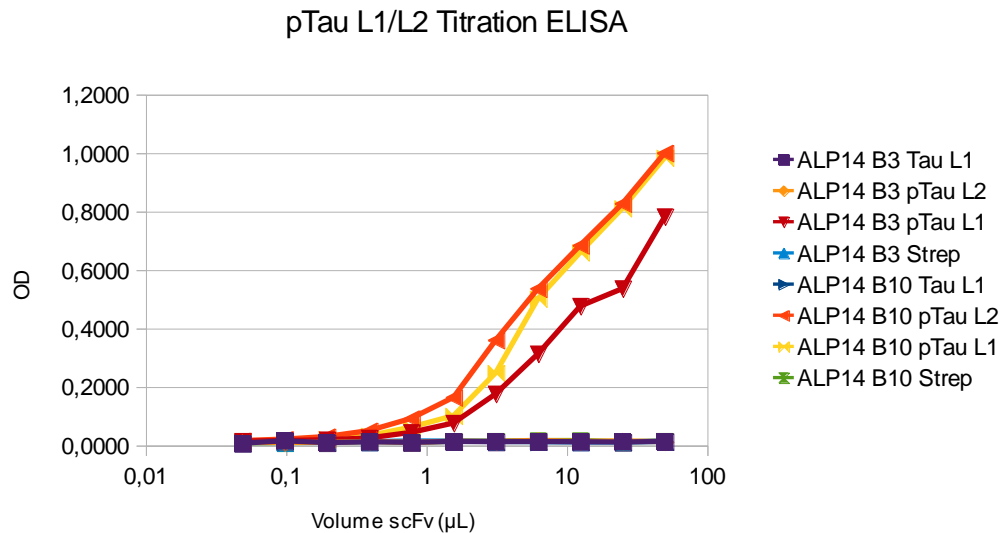


**Figure 9-8:** Titration ELISA of pTau against Tau (linker 1), pTau (linkers 1&2) and Streptavidin for clones A2 and A5

**pTau L1/L2 Titration ELISA**

- ALP14 A2 Tau L1
- ALP14 A2 pTau L2
- ALP14 A2 pTau L1
- ALP14 A2 Strep
- ALP14 A5 Tau L1
- ALP14 A5 pTau L2
- ALP14 A5 pTau L1
- ALP14 A5 Strep
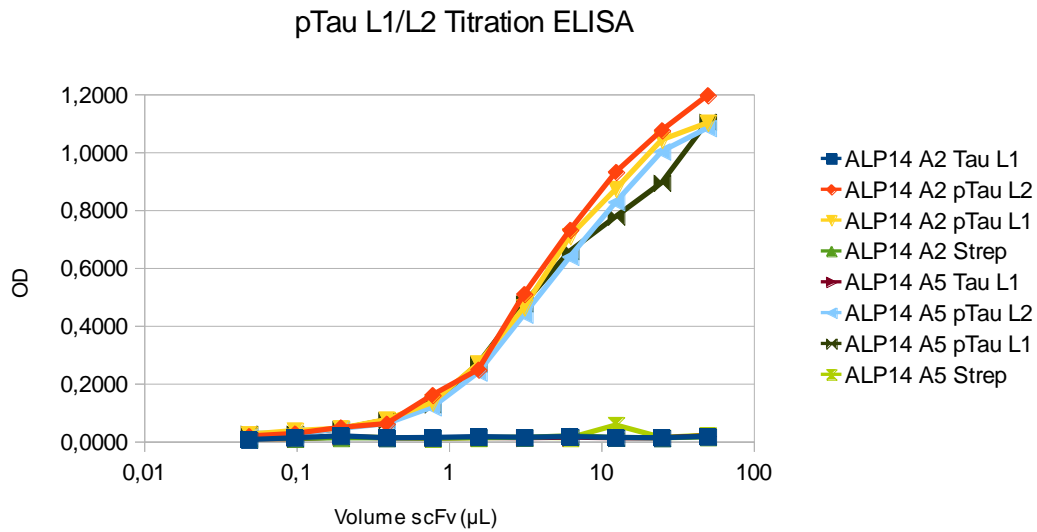
**Figure 9-9:** Titration ELISA of pTau against Tau (linker 1), pTau (linkers 1&2) and Streptavidin for clones B3 and B10



**pTau L1/L2 Titration ELISA**

- ALP14 C3 Tau L2
- ALP14 C3 pTau L1
- ALP14 C3 pTau L2
- ALP14 C3 Strep
- ALP14 H4 Tau L2
- ALP14 H4 pTau L1
- ALP14 H4 pTau L2
- ALP14 H4 Strep
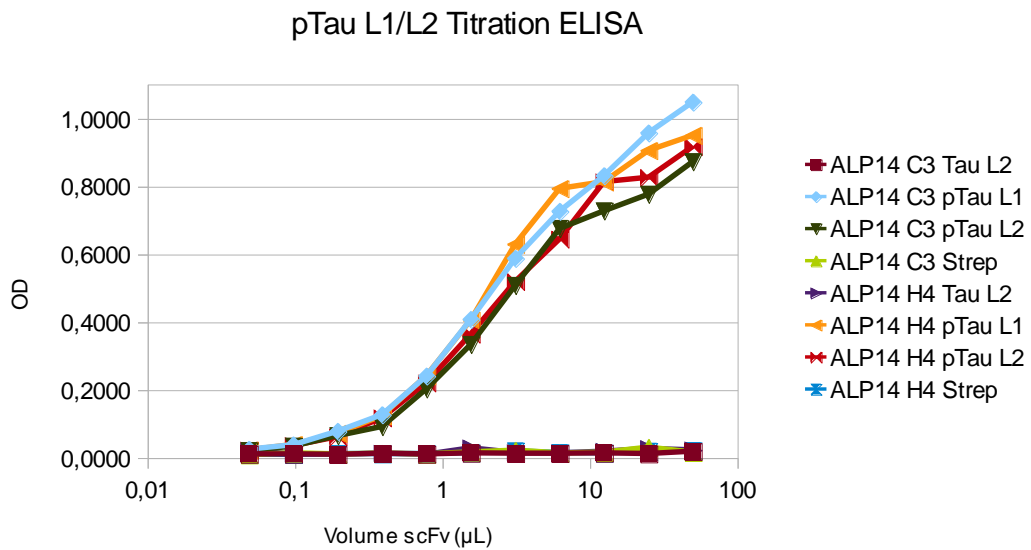
**Figure 9-10:** Titration ELISA of pTau against Tau (linker 1), pTau (linkers 1&2) and Streptavidin for clones C3 and H4

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**pTau L1/L2 Titration ELISA**

Legend:
- ALP14 E3 Tau L2
- ALP14 E3 pTau L1
- ALP14 E3 pTau L2
- ALP14 E3 Strep
- ALP14 E11 Tau L2
- ALP14 E11 pTau L1
- ALP14 E11 pTau L2
- ALP14 E11 Strep
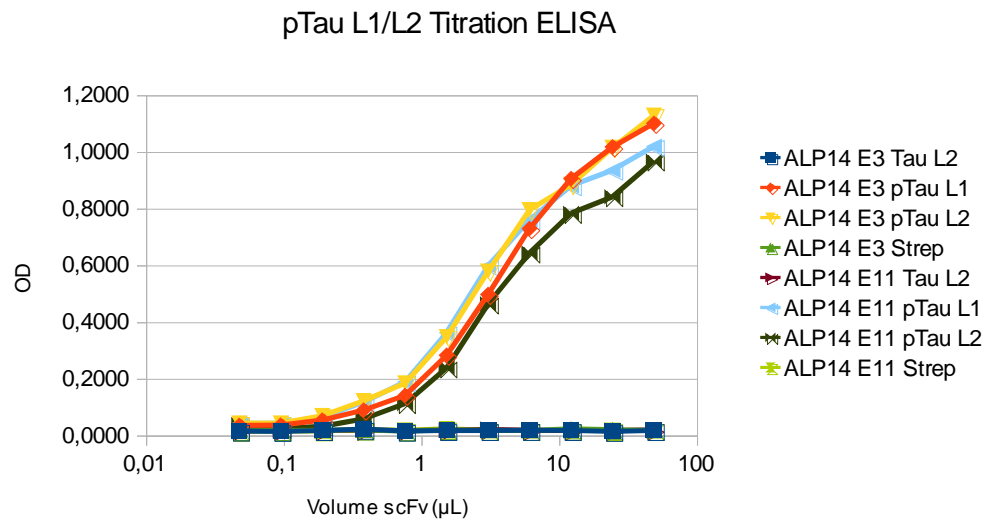
**Figure 9-11:** Titration ELISA of pTau against Tau (linker 1), pTau (linkers 1&2) and Streptavidin for clones E3 and E11

With a new set of antigens, the experiment was a success as the scFv clones attached to both antigens (the phosphorylated ones), with the exception of clone B3 which did not bind to pTau L2. At the highest scFv concentration possible, the OD values ranged between 0.8 and 1.2. However, there was a slight difference between the result in the last experiment and the current experiment. In the last experiment, as the scFv concentration decreased, the OD stayed relatively flat until it the concentration reached 1/8 the original. At that point, the concentration started to sharply decreased until it was about 1/64 the original, where it then leveled off. In this experiment, as the concentration started to decrease, the OD also decreased. At the original concentration, the OD was between 0.8 and 1.2. At 1/2 the original concentration, the OD was between 0.55 and 1.1. The OD at 1/4 the concentration was between 0.5 and 0.95. This steady decrease continued until the concentration was 1/128 the original concentration. Because of this observation, these scFv clones have

a limit of detection at 1/128 the original concentration, but unlike the last experiment where there is no increase in signal after 1/8 the concentration, there can be an increase in signal as the scFv increases.  Because of this, 10 clones will be sequenced: A2, A5, B3, B10, C3, E3, E11, F5, H4 & H5.

### 9.2.4. DNA Sequencing:

Based on the CDR comparison, out of the possible 10 clones, only three clones were bad.  Clone B3 was missing part of the light chain, which made it unsuitable for further study, which explains the titration curve, clone A2 was missing the light chain and clone E11 was missing completely. On the other hand, the other seven clones that were still intact had identical sequences, meaning that there was only one sequence for this antigen.  One other thing to note is that even though some scFvs were completely missing the light chain, their heavy chains were identical to the rest of the suitable clones.  Based on these results, it was decided that clone B10 would be used as a possible biomarker against pTau.

### 9.3.     Phosphorylated-Tau Protein Linker 1 with Competition

### 9.3.1.  Screening ELISA
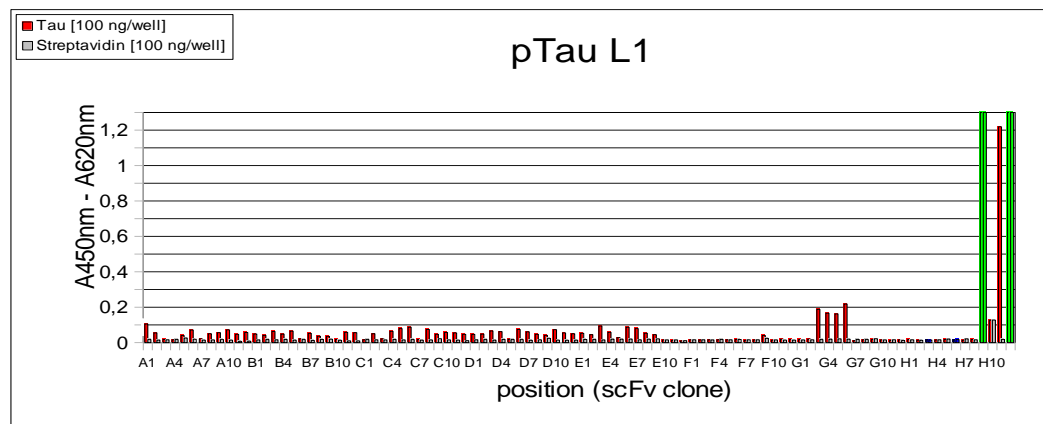


**Figure 9-12:** Screening ELISA for pTau L1 in competition with Tau L1

The main objective for obtaining results with phosphorylated Tau is to show

what type of scFvs can be created with a version of Tau that was phosphorylated.

However, what is different with this experiment is that competition is used. During

competition, a second antigen is used in the panning process. While the target scFvs

are being detected in this step, another antigen is being used to remove other scFv's

that might cause interference. For this experiment, the main antigen being used for

detection is phosphorylated Tau with the first linker, with non-phosphorylated Tau

with the first linker being used as competition. This is done in order to remove any

binding to the non-phosphorylated Tau and the linker beforehand. After three panning

rounds, 92 E. coli samples from petri dishes with agar were collected, grown and had

all possible amounts of scFvs have been extracted, a screening ELISA was performed.

The results are as follows: Out of a possible 92 potential clones, 5 clones had an OD

greater than or equal to 0.1. None of these clones had low positive to negative control

ratio, meaning that it is with the upmost confidence that these clones will only attach

to the antigen and not the streptavidin. However, it should be noted that one clone

gave off a really high signal compared to the others (> 0.7), and was next to one of the

positive controls. This could mean that there might have been a chance for a possible

contamination. These 5 clones were used in the following BStNI digest.

| G3 | G4 | G5 | G6 | H11 |
|---|---|---|---|---|

**Table 9-5:** Clones chosen for BStN digest for pTau L1

### 9.3.2. BStN Digest
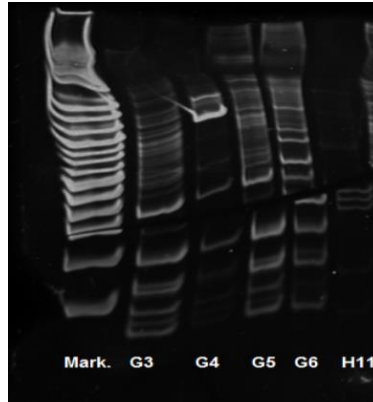


**Figure 9-13:** BStN Digest of pTau L1 with competition clones G3 to H11

For the BStNI digest, 5 clones were prepared and separated in the aforementioned gel. As a result of this separation, there were 4 different sets of bands that could be used to differentiate each scFv. There is only one pair of clones that were similar: clones G5 & G6. The rest either varied in intensity in the bands (G4) or exhibited an extra band that was not common among the other clones (G3). Also, H11, the clone that exhibited a higher peak than the rest, was completely different than the others in the digest. As a result of this, all five clones will be used in the titration ELISA and DNA sequencing.

| G3 | G4 | G5 | G6 | H11 |
|----|----|----|----|-----|

**Table 9-6:** Clones chosen for Titration ELISA and DNA Sequencing for pTau L1

### 9.3.3. Titration ELISA
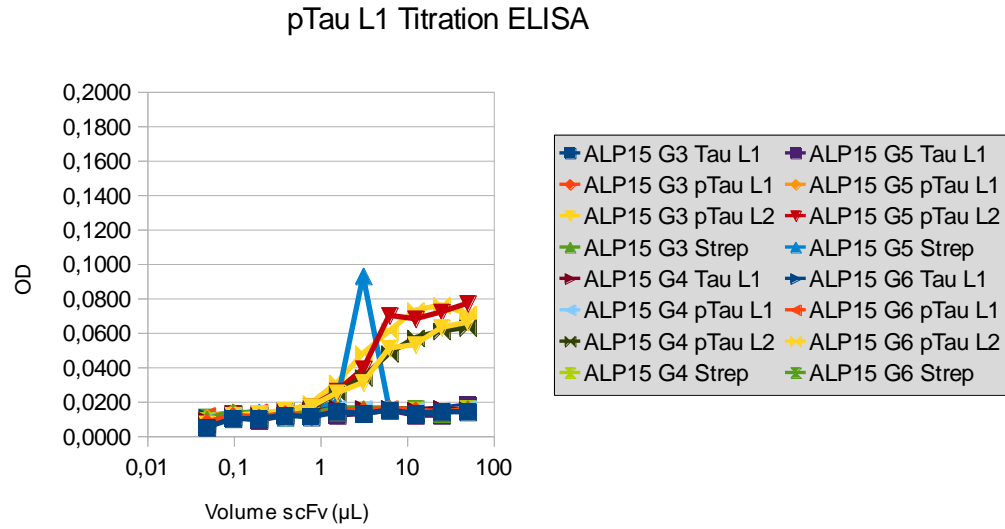


**Figure 9-14:** Titration ELISA of pTau with linker 1 with competition from Tau with linker 1 against Tau (linker 1), pTau (linkers 1&2) and Streptavidin, without clone H11
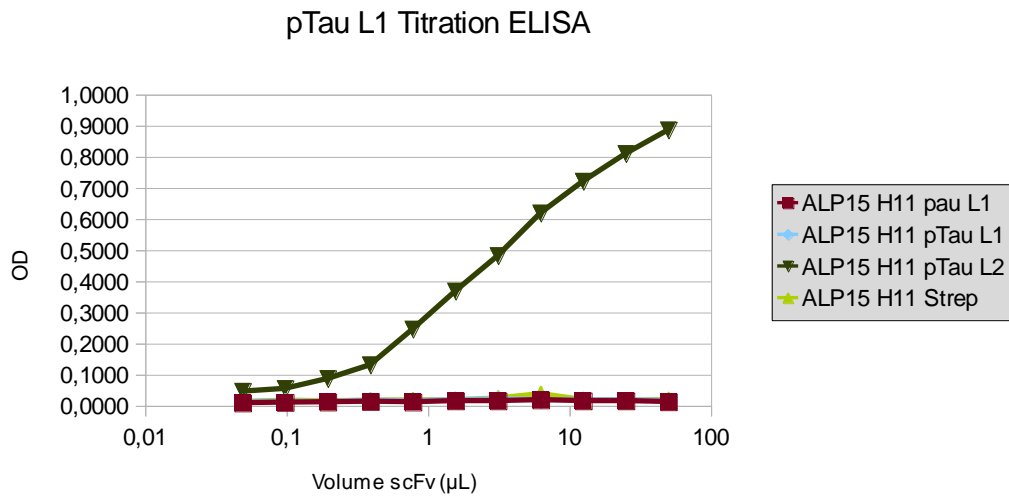


**Figure 9-15:** Titration ELISA of pTau with linker 1 with competition from Tau with linker 1 for clone H11 against Tau (linker 1), pTau (linkers 1&2) and Streptavidin

As shown by the titration graphs on the previous page, the experiment performed well pTau L2, but not with pTau. This was explained in section 9.2.3. Because of this, new peptides were prepared and the titration was performed again.
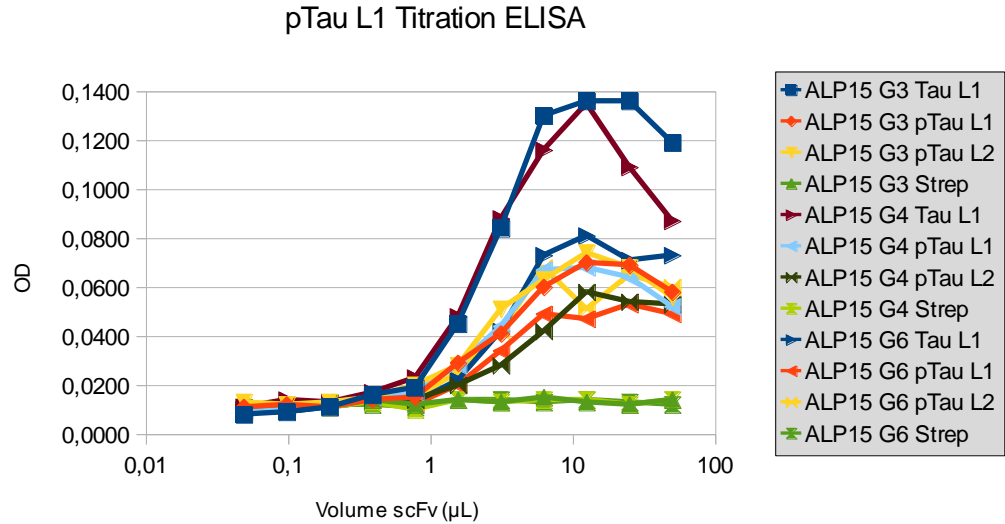


**Figure 9-16:** Redo of Titration ELISA of pTau with linker 1 with competition from Tau with linker 1 against Tau (linker 1), pTau (linkers 1&2) and Streptavidin, without clone H11
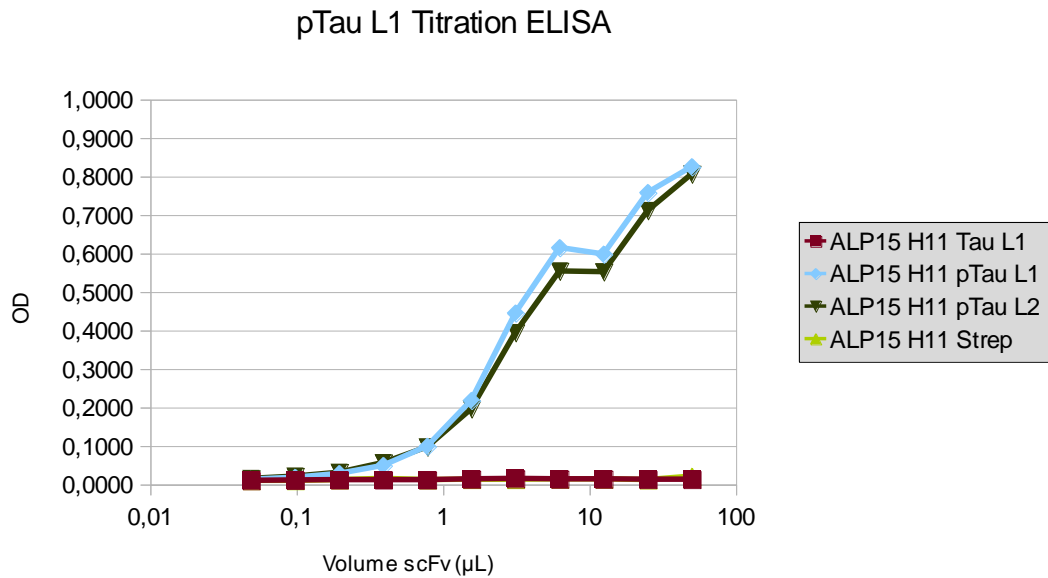


**Figure 9-17:** Titration ELISA of pTau with linker 1 with competition from Tau with linker 1 for clone H11 against Tau (linker 1), pTau (linkers 1&2) and Streptavidin

In this second run, it was a success as the scFv clones attached to both antigens (the phosphorylated ones). At the highest scFv concentration possible, the OD values for the clones, except for H11, ranged between 0.04 and 0.4. There was a decline after this, but the signal was too weak to consider any further experiments with these antibodies. However, the clone H11, the one that exhibited a high signal performed well. The OD at the maximum concentration was around 0.8, with a steady decrease as the concentration also decreased. At 1/64 the original concentration, the OD started to level off towards an OD of zero. Because of this, only clone H11 underwent DNA sequencing.

### 9.3.4. DNA Sequencing:

Based on the results of the CDR comparison, clone H11 displayed a complete sequence. Moreover, compared to the previous experiment with no competition, the DNA sequence was exactly the same as the other ones sequenced. Based on this observation, this clone can be used with the others as a possible scFv to detect phosphorylated Tau.

## 9.4. Phosphorylated-Tau Protein Linker 2 with Competition

### 9.4.1. Screening ELISA
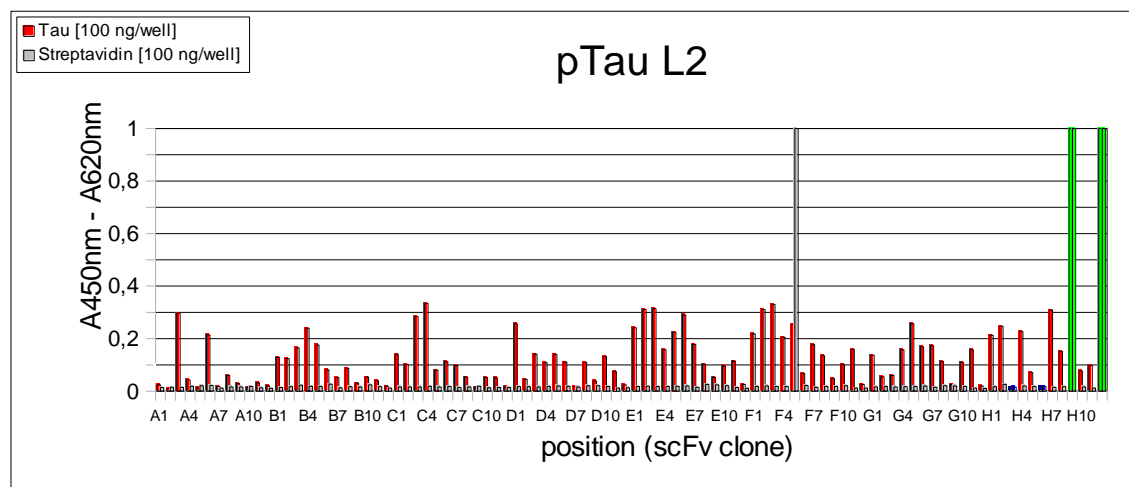


**Figure 9-18:** Screening ELISA for pTau L2 in competition with Tau L2

Like back in section 9.3, this screening ELISA encompassed the use of competition. Except this time, the peptides with linker 2 were used. After three panning rounds, 92 E. coli samples from petri dishes with agar were collected, cultivated, had all possible amounts of scFvs extracted and underwent a screening ELISA. The results are as follows: Out of a possible 92 potential clones, 19 clones exhibited an OD reading greater than 0.2. These clones were the best read clones out of all the possible clones that were produced. However, with one of the clones, clone F4, the negative control was abnormally higher than what it should have been. This could either be a case where there was contamination in the negative control or there was an unknown error during the running of the experiment. Nether the less, because of this error, this one specific clone cannot be used in any further experiments. Instead, the other 18 clones can be used for a BStNI digest.

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| A3 | A6 | B4 | C3 | C4 | D1 |
|----|----|----|-----|----|----|
| E1 | E2 | E3 | E5 | E6 | F1 |
| F2 | F3 | F4 | F5* | G5 | H1 |
| H2 | H4 | H7 |  |  |  |

**Table 9-7:** Chosen clones for BStN digest for pTau L2(*Clone F5 had a higher negative signal than positive)

### 9.4.2. BStN Digest
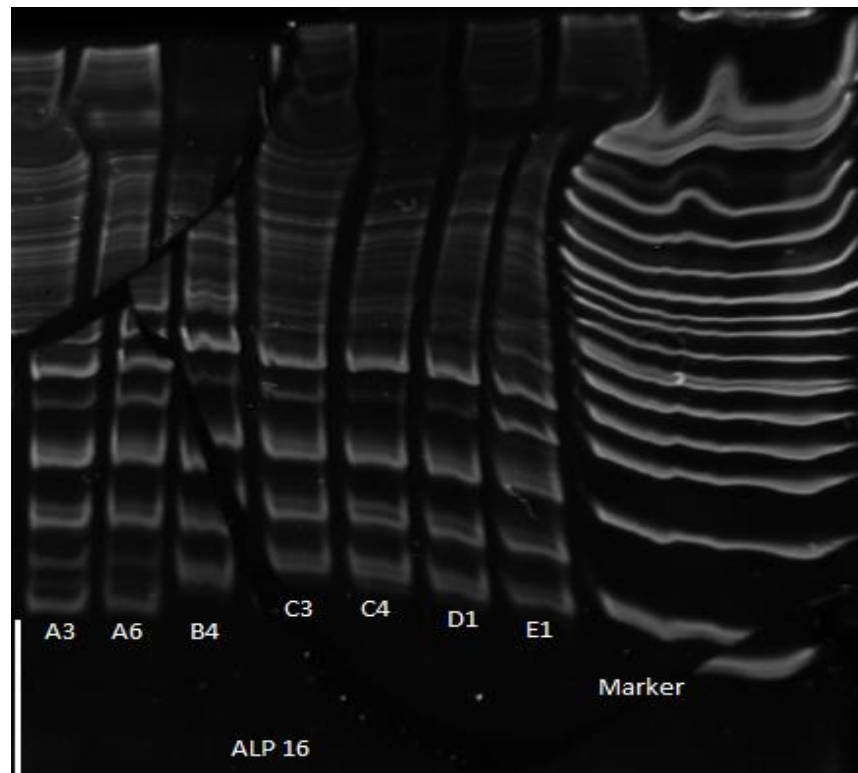


**Figure 9-19:** BStN Digest for pTau L2 with competition clones A3 through E1
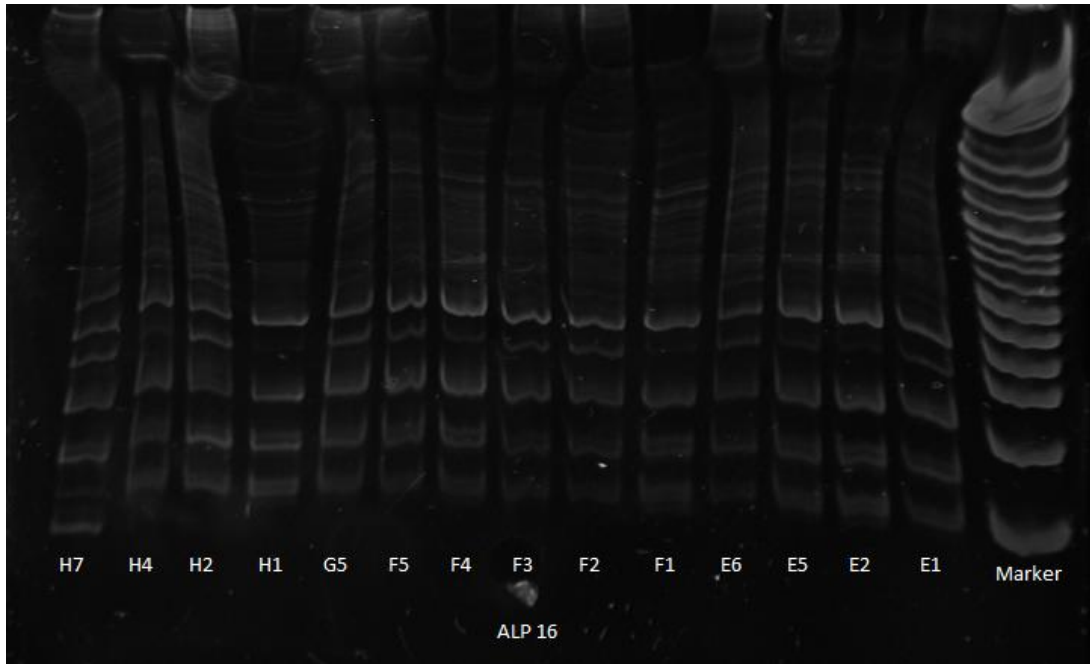
**Figure 9-20:** BStN Digest for pTau L2 with competition clones E2 through H7

In this digest, 18 clones were prepared and separated in the agarose gel. As a result of this separation, most of the clones were similar in band length and distance. However, some of the clones did experience a small brightness in the type of band (clone E1, compared to D1), while other bands were just a little bit off from what the majority of the bands (clone H7 compared to clone H4). As a result of this digest, 14 different clones will undergo a titration ELISA and DNA sequencing.

| A3 | A6 | B4 | C3 | C4 | D1 |
|----|----|----|----|----|----|
| E1 | E2 | F1 | F3 | G5 | H1 |
| H2 | H7 |    |    |    |    |

**Table 9-8:** Chosen clones for Titration ELISA and DNA sequencing for pTau L2

### 9.4.3. Titration ELISA



**pTau L2 Titration ELISA**

Legend:
- ALP16 A3 Tau L2
- ALP16 A3 pTau L1
- ALP16 A3 pTau L2
- ALP16 A3 Strep
- ALP16 A6 Tau L2
- ALP16 A6 pTau L1
- ALP16 A6 pTau L2
- ALP16 A6 Strep

X-axis: Volume scFv (µL)
Y-axis: OD
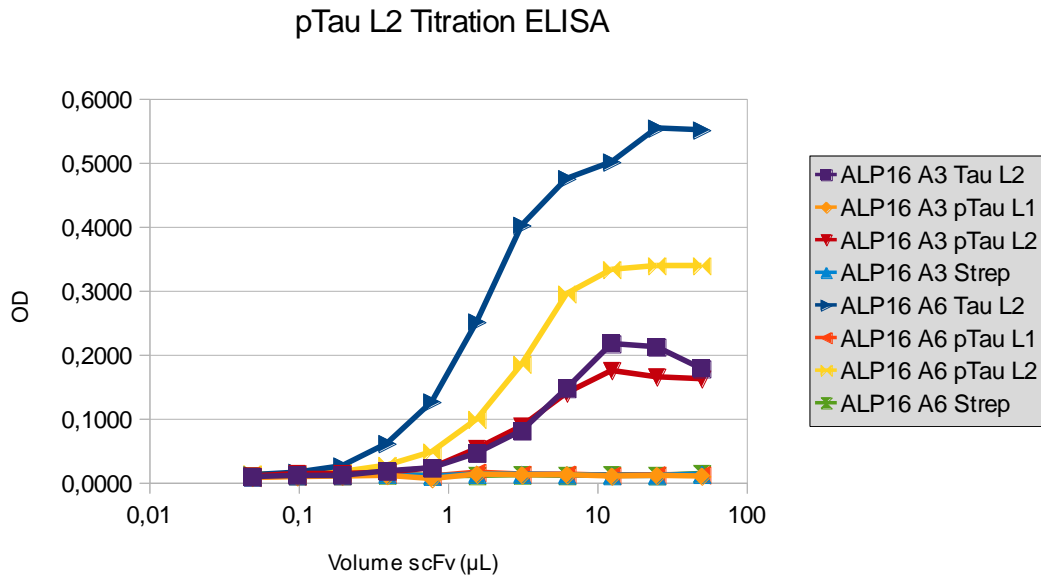
**Figure 9-21:** Titration ELISA of pTau with linker 2 with competition from Tau with linker 2 against Tau (linker 2), pTau (linkers 1&2) and Streptavidin

Like the last experiments, the scFv clones attached well to the target antigen (pTau L2), but not pTau L1.  Not only that, but that same scFv also attached to one of the negative controls, Tau L2.  This was the control where the antigen was non-phosphorylated, but it used the exact same linker as before.  Because of this, it is safe to assume that these scFvs somewhat bind to the antigen, but there is binding at the linker.  A further titration ELISA was performed but it resulted in similar results, except that the OD measurements were lower than before.  Because of these reasons, this experiment was not further investigated because the focus is only on scFvs that attach directly to the antigen and nothing else.

### 9.5. Titration ELISA Using the Best scFv Clones against Tau, pTau and Other Antigens

In these final experiments, the optimal scFv clones against Tau and pTau (Tau clone E4 and pTau clone B10) underwent a titration ELISA against not only the forms of Tau that were used, but also against other antigens. In a 96 well ELISA plate, the following order was used for each antigen (from top to bottom): Non-Phosphorylated Tau – Linker 1 (Tau L1), Non-Phosphorylated Tau – Linker 2 (Tau L2), Phosphorylated Tau – Linker 1 (pTau L1), Phosphorylated Tau – Linker 2 (pTau L2), Beta Amyloid 42 Fibrils (aß 42), Phosphorylated Fibronectin 1 – Linker 1 (pFN1 L1), Phosphorylated Fibronectin 2a – Linker 1 (pFN2a L1), Streptavidin (negative control). This is to make 100% sure that each scFv only binds to its own antigen and not any others.
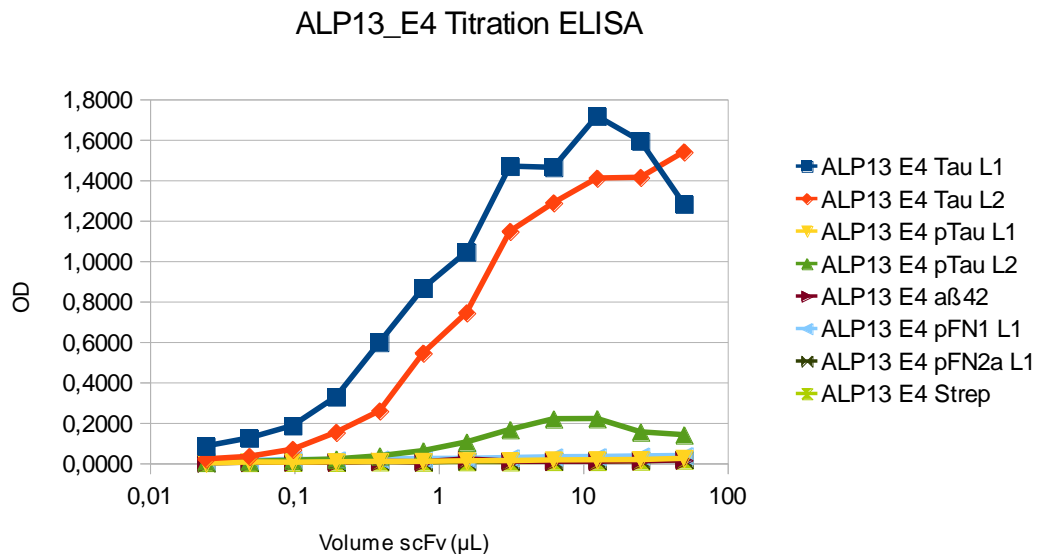


**Figure 9-22:** Titration ELISA for Tau scFv clone E4 against Tau (linkers 1&2), pTau (linkers 1&2), aß$_{42}$, pFN1 (linker 1), pFN2a (linker 1) and Streptavidin

The ELISA that used the Tau scFv only bound to the non-phosphorylated Tau. At the highest concentration possible, the OD measured between 1.3 and 1.55. This

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measurement was for the most part constant, even at 1/16 the original concentration. At a lesser concentration, OD decreased substantially until it was 1/128 the original concentration. At that point, the OD started to slowly level off towards 0.0. There was no additional binding to any other antigen, with the exception of pTau L2, which displayed a small amount of detection. This might have been caused by linker binding, but it is small compared to the binding to the Tau. In terms of clone D7, this titration ELISA was not performed as the signal back in the previous ELISA (see section 9.1.3) was not high compared to clone E4.
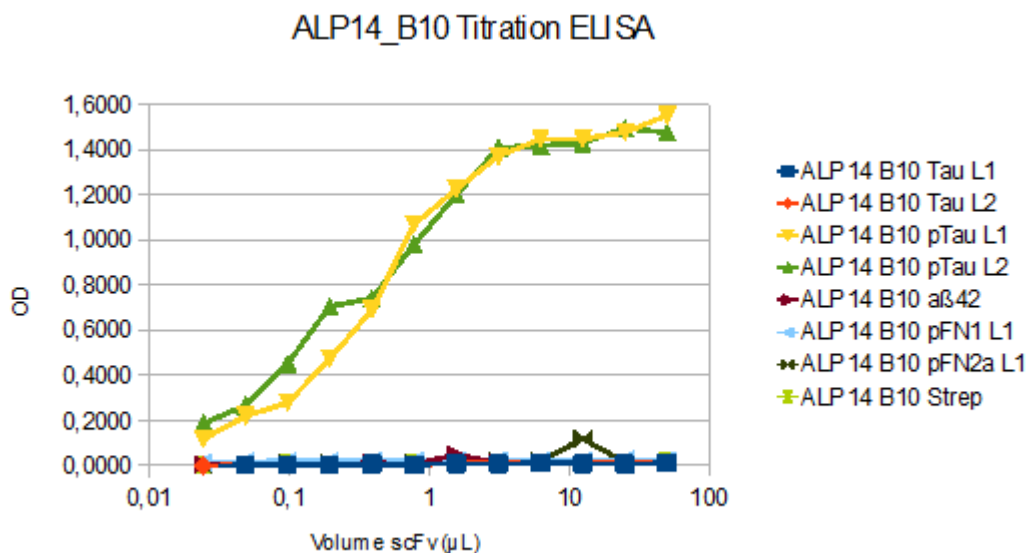


**Figure 9-23:** Titration ELISA for pTau scFv clone B10 against Tau (linkers 1&2), pTau (linkers 1&2), aβ$_{42}$, pFN1 (linker 1), pFN2a (linker 1) and Streptavidin

The ELISA that used the pTau scFv only bound to the phosphorylated Tau. At the highest concentration possible, the OD measured between 1.4 and 1.6. This measurement was for the most part constant, even at 1/16 the original concentration. At lesser concentration, OD decreased substantially until it was 1/1024 the original concentration. At that point, the OD started to slowly level off towards 0.0. There

was no additional binding to any other antigen.

To further compliment this, tables 9-9 and 9-10 below shows the fragment structure of these three antibodies.  This breakdown for the antibody fragments is as follows:  There are two types of chains: heavy and light.  On the heavy chain, there are three sections: variable (V), diversity (D) and joining (J).  There are also two types of light chains: kappa (K) and lambda (L).  On either the kappa or lambda chains, there are two sections: variable and joining.  Fragments can either have a kappa chain or a lambda chain, but not both.  The naming scheme uses the above letters and numbers to differentiate the segments

| Antibdy Name | ALP13-E4 | ALP13-D7 |
|---|---|---|
| Antibody Type | Non-Phosopho-Specific | |
| Heavy Variable | IGHV5-51*01 | IGHV1-46*01 |
| Heavy Diversity | IGHD3-22*01 | IGHD5-5*01 |
| Heavy Joining | IGHJ4*02 | IGHJ4*02 |
| Light Variable | IGLV3 | IGLV1-47*01 |
| Light Joining | IGLJ3*01 | IGLJ3*01 |

**Table 9-9:** Structures for the scFvs chosen that bind against Tau

| Antibdy Name | ALP13-B10 |
|---|---|
| Antibody Type | Phosopho-Specific |
| Heavy Variable | IGHV5-51*01 |
| Heavy Diversity | IGHD1-26*01 |
| Heavy Joining | IGHJ3*02 |
| Light Variable | IGLV2-8*01 |
| Light Joining | IGLJ3*02 |

**Table 9-10:** Structures for the scFv chosen that bind against pTau

Some of the similarities between the three fragments include:

- ALP13-E4 & ALP13-D7: Same joining segments for both the HJ & LJ

- ALP13-E4 & ALP14-B10: Same HV segments

- All three fragments have lambda structures for light chains

Other than these, these three are completely different fragments.

# CHAPTER 10

## CONCLUSION

### 10.1. Discussion

This project effectively found two sets of scFv antibodies that are able to bind to a certain Tau peptide. This was performed effectively by using phage display and by harvesting E. coli cultures in order to extract these antibodies. Because of the effects of phosphoylation, which is a side effect of Alzheimer's, these antibodies were created in order to bind to both Tau and pTau. These antibodies were also shown to have a high affinity to the antigen and could be detected in small amounts. Because of these reasons, it was shown that creating these antibodies was a success.

Furthermore, it was also shown that only one of the same antibodies could be formed based on the competition ELISA (pTau L1, not pTau L2). This is due to the fact that there are antibodies that also bound to the linker that were also discovered. In order to mitigate this, a fourth run would have to be performed with the same peptide, but with different linkers, so that the proper antibodies could be obtained. Other than this, it was shown that it was possible to obtain two different antibodies for Tau and pTau respectfully. The structures for each was shown in section 9.5, tables 9-9 and 9-10.

As mentioned in the results section, scFv clones for both Tau and pTau were found by using different antibody libraries and phage display. The majority of the clones that were tested during the ELISAs performed well, with the exceptions of

some clones during the DNA sequencing and the pTau – L2 with competition, where the scFvs only attached to the linker.  Based on the results found, scFv clones were found that were able to detect pTau antigens and clones were found that detected Tau antigens, both with specificity and with a high detection signal.  However during the course of these experiments, there were problems that had to be dealt with in order to accurately show these results previously shown.

The majority of these errors occurred during the panning and the screening of the scFvs.  These errors were mostly random errors that were caused by not following the protocols properly.  These were either a time-related or temperature-related error that resulted in the whole experiment being repeated over again.  For example, if a certain experiment was left out too long, the number of E. coli clones were either too big or too many.  One other problem occurred with temperatures, especially if a certain experiment had to run overnight.  As explained during the procedures, there were only three temperatures that each experiment had to be incubated at: 4°C, 30°C and 37°C.  While no problems occurred at 4°C, the biggest issue was at 30 and 37°C.  Incubation at 37°C meant bacteria growth during panning rounds, while 30°C was used to breakdown the E. coli to create new phages or to release scFvs into solution.  However during some experiments, samples that were supposed to be incubated at 30°C were actually incubated at 37°C.  This was either due to human error, or to other experiments being performed in the lab.  This caused problems because there was no detection or colonies after each experiment were completed, which meant that all of these experiments had to be redone.

But one of the biggest errors during each experiment was during the

amplification stage. In this stage, the newly made bacteria colonies had to grow from on OD of 0.1 to an OD of 0.5. This was performed by scraping off the agar plate the bacteria and placing between 0.3 and 0.5 mL in a solution of 2YT-AG. During this section, it was important that no agar was also placed in this solution, as it would hinder bacterial growth while in incubation. However, during this sequence there was one problem that could cause the experiment to start over again. In a normal bacterial growth, for every half hour elapsed, the OD would double. For example, at t=0, and OD=0.1, at t=0.5h, OD=0.2 and at t=1h, OD=0.4. However, during this stage, the growth was significantly different. What would happen is that there would be growth for the first half hour, but after that, the OD would decrease dramatically. It would decrease to a certain point either just above or just below the original OD. After that, it would remain stagnant for about a few hours, before it would gradually rise up again. Because of this, an experiment that could normally take between 1.5 and 2 hours, would actually take between 5 and 7 hours depending on the rise in the OD. While this phenomenon was normal during the experiments, the problems usually occurred when the OD fell below the original OD. When this happens, it shows that there was something wrong with the amplification, which meant that the experiment had to be redone.

Other errors in panning included measurements with the OD during incubation. This was crucial because the growth in OD was not linear with time, but more exponential. At times, if an experiment was being performed where incubation was necessary, if the incubation was even 10 or 20 minutes longer than expected, the OD would be too high for sufficient bacterial growth due to over-crowding and insufficient

supply of food for the E. coli. However, if an experiment was stopped too short, then there would not be enough E. coli to complete the experiment. To start each incubation process, the acceptable OD range had to be in between 0.075 and 0.1, with better results at 0.1. At the end of the incubation, the acceptable range had to be greater than 0.45, but no more than 0.65, with 0.5 being in the acceptable range. But since the bacterial growth during this time is not linear, it at times was hard to tell when it was appropriate to end this experiment.

One other important bit of information during the panning rounds was that two different protocols were used during the running of the experiments. The first involved bacterial growth on agar plates, while the second involved bacterial growth in micro titer plates. The first set of experiments was performed using the first method. This method had some positive aspects while performing each experiment. For instance, multiple samples after each panning round could be taken. This means that because 100 microliters were needed of phage to run the next experiment, the samples were collected in 1.5 – 2.5 mL samples. This meant that in case the experiment went wrong, there was always backup available, so that the experiment did not have to start over. On the other hand, this process was more labor intensive and took at times about 6-8 days to complete three rounds.

The remainder of the experiments was performed using the method with micro titer plates. This method not only cut back the time needed to create clones by 2 -3 days, but was also less labor intensive than the previously mentioned method. However, the results were not generated until the end of the third panning round. In the previous experiments, agar plates were used to grow bacteria colonies after each

panning round.  Since this occurred with larger volumes of solutions, separate vials were needed to store the results from the previous experiments.  This was to make sure that even if the next panning round was not a success, there was extra phage left over to redo the experiment so that it did not have to be started over again.  However, in this experiment, because small volumes of phage were used in each experiment, there was not any room for error and there were no checks in between to make sure that the experiments ran smoothly.  All the results had to be found at the end of the third panning round.  If no colonies were present at the end, then the whole experiment had to be repeated from the beginning.

In the previous experiments, it was shown that scFvs could be made for a specific region of Tau Protein, in both phosphorylated and non-phosphorylated forms. This is beneficial in that these antibodies can be used as a biomarker.  However, there can be more experiments that can further help the project.  These experiments can be explained below.

**10.2.   Further Work**

The first experiment that can be done can be to create an scFv that can detect both forms of this peptide of Tau.  As shown in the previous experiments, only the scFv made for non-phosphorylated Tau bound to non-phosphorylated Tau and the same can be said for phosphorylated Tau.  However, the reverse (Tau $\rightarrow$ pTau scFv and pTau $\rightarrow$ Tau scFv) cannot work.  This was shown with the final titration curves above.  A third antibody can be created that could detect both forms, but this could require a larger stock antibody library and more than 4 rounds of panning in order to successfully obtain these antibodies.

The second experiment would be to obtain IgG from these scFvs. This could either be performed by engineering the antibodies in a way that would allow for IgG creation, or by performing it in the usual manners to obtain IgG. This would be by introducing either the scFv or the peptide into a mammal and then extracting the antibodies. This would be beneficial for performing assays in which tau needs to be detected from a fluid and it needs something to attach to. IgGs work better because they have a stabilizer in which they are able to attach to the well, whereas scFvs only contain the parts which are supposed to attach to an antigen and maybe a detection antibody.

One final group of experiments would be to find antibodies for the entire Tau protein. As mentioned in the background, only about a dozen proteins exist that are known to connect with certain Tau peptides. While this project only focuses on one specific section, there are many different spots on the protein that have no antibodies associated with it. Because phosphorylated Tau is broken off from the microtubules, there are hundreds of peptides in spinal fluid that could be found. With this many different peptides, it should be known that any different amount of peptides could be in one solution and it is a lot more difficult to isolate one type. For this reason, it would be better to at least find a few more scFvs that would be able to effectively detect all known Tau peptides, both phosphorylated or not.

Further work will have to be performed in order to effectively either create IgG antibodies and completely map the entire protein, but this will happen in the upcoming years. However, it is safe to say that two new antibodies have been discovered that would aid in detecting not only Tau protein, but one that is known to undergo

phosphorylation.  In conclusion, based on the above experiments and data, two scFv

antibody fragments have been replicated in order to attach to both Tau and pTau,

which would aid as a biomarker against Alzheimer's disease.

# APPENDIX

A.1: Masks for Photo Resists for the Channel – Side Molds
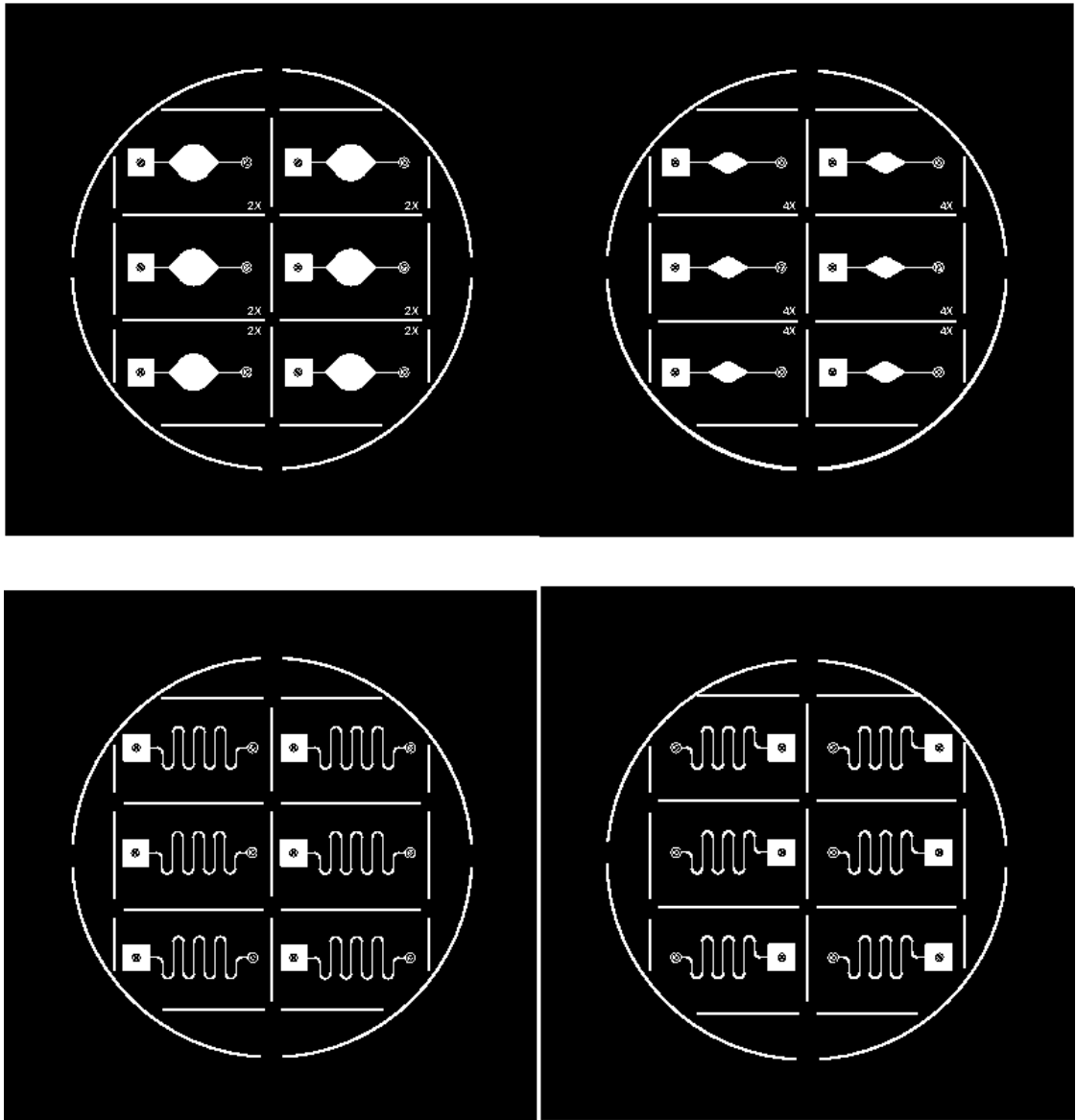


**Figure A-1:** Photo lithography masks for channel molds

A.2: Mask for Photo Resist for the Molds for the Filter Holder
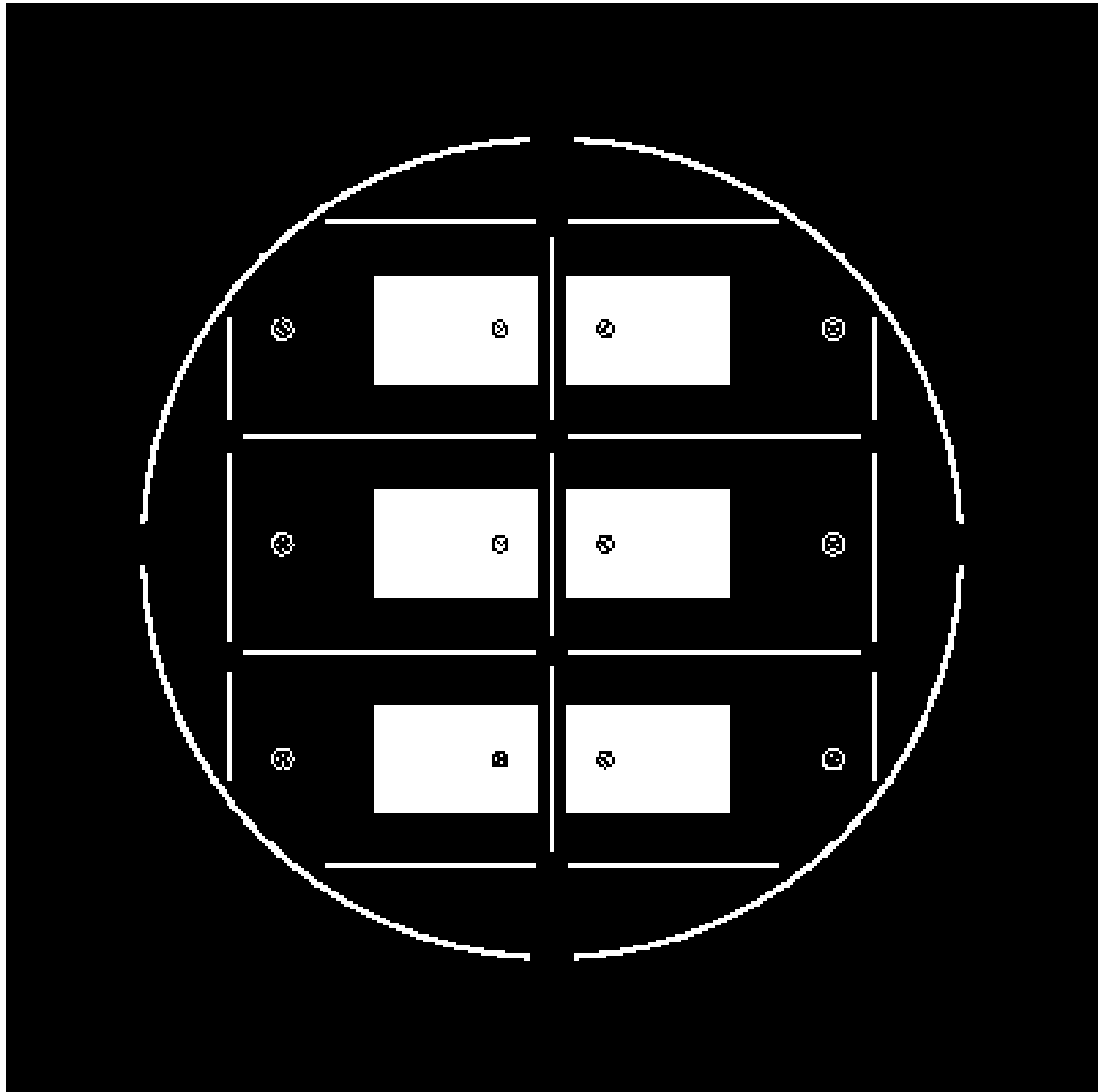


**Figure A-2:** Photo lithography mask for filter holder mold

A.3: Protocol for HAR Photolithography for Silicon Wafer Molds[146]

# Standard Operation Procedure for HAR Photolithography

*Experiment Date*:_____

## Hazardous Chemical: Sulfuric Acid, 96%

**Hazard Identification:** Corrosive.
**Health effects:** Causes severe skin burns. Causes severe eye burns. Causes burns of the mouth, throat, and stomach.
**Personal protection:** Safety goggle, nitrile gloves together with trionic gloves, face shield, wet chemical apron, and lab coat should all be worn.
**Engineering Controls:** Use the fume hood in the Wind Tunnel Lab in Kirk.
**Handling and Storage:** Keep container tightly closed in a cool, well-ventilated area. Do not store above 23℃.
**Stability and Reactivity:** Stable. Avoid exposure to excess heat, combustible materials, organic materials, moist air, water, oxidizers, amines, bases.

## Hazardous Chemical: Hydrogen Peroxide, 30%

**Hazard Identification:** Strong oxidizer. Contact with other material may cause fire.
**Health effects:** Corrosive to eyes, skin, nose, throat and lungs. May cause irreversible tissue damage to the eyes including blindness.
**Personal protection:** Safety goggle, nitrile gloves together with trionic gloves, face shield, wet chemical apron, and lab coat should all be worn.
**Engineering Controls:** Use the fume hood in the Wind Tunnel Lab in Kirk.
**Handling and Storage:** Keep container tightly closed in a cool, well-ventilated area. Do not store above 8℃. Separate from acids, alkalis, reducing agents and combustibles.
**Stability and Reactivity:** Stable. Avoid exposure to excess heat, combustible materials, organic materials, moist air, water, oxidizers, amines, bases, metals.

## Hazardous Chemical: Methanol

**Hazard Identification:** Flammable.
**Health effects:** Hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation. Slightly hazardous in case of skin contact (permeator). Severe over-exposure can result in death.
**Personal protection:** Safety goggle, nitrile gloves, and lab coat should all be worn.
**Engineering Controls:** Use the fume hood in the Wind Tunnel Lab in Kirk.
**Handling and Storage:** Store in the yellow cabinet for the inflammables.
**Stability and Reactivity:** Stable. Avoid exposure to excess heat, ignition sources, oxidizing agents, metals, and acids.

**Hazardous Chemical: SU8 Developer**

**Hazard Identification:** Flammable. Flash point is 460℃.
**Health effects:** May be harmful if swallowed, inhaled, or absorbed through the skin. Readily absorbed through the skin. No significant signs of any health hazard are expected to occur through skin absorption. Irritating to eyes and respiratory tract. May cause burning, tearing, redness or swelling. Vapor or mist is irritating to the eyes, mucous membranes and upper respiratory tract. Prolonged and/or repeated exposure can cause gastric or nervous system effects.
**Personal protection:** Safety goggle, nitrile gloves and lab coat should all be worn.
**Engineering Controls:** Use the fume hood in the Wind Tunnel Lab in Kirk.
**Handling and Storage:** Store in the yellow cabinet for the inflammables.
**Stability and Reactivity:** Stable. Avoid exposure to oxidizing agents, strong acids, and strong alkali.

1st day

1. **Cleaning:** Add 75ml $H_2O_2$ and then 75ml $H_2SO_4$ sequentially to form piranha solution. The substrate wafer is cleaned in the solution for 15 min (until no bubbles are observed on the surface of the wafer) with gentle shaking. The wafer is then soaked in distilled water for 5 mins (change to clean water and repeat 2 times ) with gentle shaking.

2. **Dehydration:** Dehydrate the wafer at 200~220 ℃ for a minimum of 30 mins on hot plate (No dehydration is needed when using AP300).

3. **Adhesion improvement:**

Method 1: After cleaning the silicon/glass wafer, spin SU-8 2002 for 25 sec. at 300rpm and 30 sec. at 3000rpm. The acceleration for low speed is usually set to 100rpm/sec, 300rpm/sec for high speed. The SU-8 2002 does not spread well on the wafer. Repeat for multiple times until a desirable coating is observed.
Prebake at 65 ℃ for 1 min and softbake at 95 ℃ for 3 min. Over-expose without photomask for 2 mins. Postbake at 65 ℃ for 1 min and 95 ℃ for 3 min. (Do not develop it afterwards, this contaminates the wafer.)
Hard bake at 150 ℃ for 30 mins on hot plate.

Method 2: Blow the surface using clean nitrogen. After puddling 2-3ml AP300 on the substrate, spin AP300 for 25 sec. at 300rpm and then 30 sec. at 3000rpm. Then, repeat the spin coating to air dry the substrate. **Make sure to put AP300 in perfectly sealed Zip-Loc bag cushioned with nitrogen to protect it against moisture!!!**

2nd – 3rd day

Temperature: _____ Humidity: _____

4. **SU8 preparation:** After getting a new batch, loose the cap and heat photoresist at an **upright** position at 50 ℃ in oven for 2 h. **Don't** degas the bottle. **Don't** pierce into the resist as this may induce extra stress.

5. **Spin coating:** Align the wafer carefully with the chuck of coater. Blow the wafer surface from center to edge with N$_2$ gun. Next, spin SU-8 2050 for 45 sec at 300rpm then 50sec at 600rpm (130-158 um layer). Remove large edge bead if necessary.

6. **Planarization:** Then, let it sit perfectly level for 1hr (relaxation time), so the photoresist will "smooth out". SU-8 has good self-planarization property.

7. **Softbaking[1]: Make sure the hotplate is perfectly LEVELED before baking the wafer.** Then, ramp heat from 50 degrees to 95 degrees for 23 min at 2 ℃/min. Bake for 3 hr at 95 ℃. Let cool to room temperature at 1 ℃/min.

Step 5-7 can be repeated if higher thickness film needs to be built. Add one extra hour for baking at 95C when baking another layer. For instance, bake for 4 h at at 95℃ when baking the second layer. Bake for 5 h at at 95℃ when baking the third layer, etc.

4th day

**Optional Step (only for high aspect ratio):**

Barrier Coating and Contrast Enhancement

1) Spin coat Barrier Coating 7.5 (ShinEtsuMicroSi, Pheonix, AZ) above the SU-8 at 1000rpm. **Use more than anticipated, as it doesn't spread well (this also prevent substrate from sticking on the photomask.** Air dry completely for 5 min. (**enough time** to spread at 300rpm, then **fast** ramp to 3000rpm, 60 s total).

2) Spin on Contrast Enhance Material 388SS (ShinEtsuMicroSi, Pheonix, AZ). Air dry completely for 15 min.

8. **UV Exposure:** The Karl Suss MJB3-Broadband (3.5mW/cm$_2$), 365nm i-line.

Make sure that there is no gap between photo mask and SU-8 surface. The gap would result in widening of the patterns due to diffraction! Pay attention to the bubbles which may also cause the gap.

UV exposure for 6 mins will work for 400um mold with barrier coat and Contrast Enhance Material, only 2min one time with 1 min interval to allow surface cooling. [2]

USE HARD CONTACT. DON'T PRESS SOFT CONTACT BUTTON.
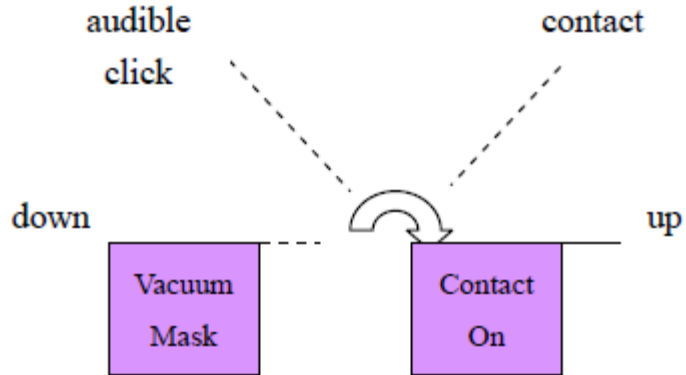
**Figure A-3:** Diagram for UV light exposure for soft lithography mold creation

Tips on adjusting the alignment stage:

 (1) the contact begin around 150 degree, fully contact at 170 degree. This is important to make sure that the wafer is being hold properly, not to loose to cause gap, or not to tight to stick on the photomask.

(2) Hold the outer silver ring when adjusting the stage. CW□lower, CCW□raise.

(3) Use razor to separate the wafer from mask carefully if wafer stick to mask.

Film Thickness:____300 μm____ ____100 μm___ _____50 μm_____

With CEM and BC: ___Y/N_____ _____ _____

Substrate material:___Silicon_____ _____ _____

Energy dosage required: _____ _____ _____

Estimated exposure time: 2.5+1+2.5 min ____2 min_____ ____90 sec_____

Rinse in water (dissolving the Barrier Coat and lifting off the Contrast Enhance Material) and air dry.

9. **PEB**[3]**:** ramp heat from 45 to 85 degrees at 2 ℃/min. Held for 40 min at 85 ℃. Let it cool gradually to room temperature at 1 ℃/min.

10. **Developing:** Place the wafer **face-down** in the glass container. Set the shaker to max speed 10 min in developer I (**take the wafer out every 5 min and gently blow dry with N2**), 30 min in clean developer II (**take the wafer out every 3 min and gently blow dry with N2**), and then 1min in IPA. Plasma could be used to clean undeveloped residues from the mold. (Make sure use fresh solution for high aspect ratio mold.)

Hard bake at 150 ℃ for 30 min (use 1 ℃/min as ramp to heat up and cool down).

4th day

11. **Channel Fabrication:** (5:1 prepolymer/curing agent for high aspect ratio mold)

50+5g PDMS is typically enough for making 4 chips and one substrate for all.
For high aspect ratio PDMS pattern, more curing agent could be added (e.g. 1:5) to the prepolymer.

Pour approximately 6g PDMS on wafer to form the first layer. Degas in the vacuum oven. Bake at 60 ℃ for 30mins.

Add another 6g PDMS on wafer to form the second layer. Degas and bake at 60 ℃ for 2.5 h.

**For HAR PDMS patterns**, degas 1 h for the 1st layer. Degas the 2nd layer as usual and bake at 60 ℃ for 4.5 h.

12. **Align PDMS to PDMS**[4]**:** Bonding PDMS to PDMS can be done by spray a liquid cushion of methanol after plasma treatment at 35psi for 3 mins. **Do not do the spray near a source of ignition, such as a hotplate.** The alignment may be helped by using a magnifier. Then, heat it on hotplate at 85 ℃ for 80mins. This method allows aligning of different PDMS layers without immediate bonding.

**Tips:**

[1] Ramped heating reduce the tension in the film during the baking. It's better to use hotplate than oven. Extend prebaking and softbaking time longer, use a Petri dish to cover the slide may avoid wrinkles and craters. The actual soft baking time ( e.g 1.5 hrs) is almost 2 times longer than the suggested soft baking time by the vendor in order to make the SU-8 dried completely.

[2] Never illuminate the SU8 for to long periods. Energies of 200 mJ/cm2, heat the SU8 at the interface with the photolithographic mask. This heating is responsible for a hard skin formation at the surface of the SU8. Therefore, for thick layers and for high exposure doses, the illumination should be done stepwise ( expose for 10 to 15 seconds, leave the system cool down for 60 seconds, illuminate again, etc ...).
It is important to note that the optimal exposure energy depends on the nature of the substrate.

[3] Post exposure baking must be done gradually to prevent micro-cracks of mold due to thermal tension.

[4] When aligning two PDMS patterns, grooves must be designed to allow methanol evaporates.

[5] Cross-linked SU-8 cannot be removed even by Piranha.

[6] The 2nd layer of photoresist will not level dimples on the 1st layer.

**Troubleshooting:**

**1. Dimples. –**a sign of **surface/thermal tension** (a) Caused by surface contamination, can be resolved by cleaning in Piranha; (b) Caused by bubbles or residual tension in SU-8, can be resolved by heating photoresist at 50 ℃ in oven for 2 h; (c) Presence of micro particle in the resist, likely from air; (d) Due to uneven heating of surface, this is most unlikely to happen; (e) **High humidity won't cause dimples, we have been successful in obtaining "mirrored" film from 20-60% humidity at 25C.**

**2. Poor adhesion.** Generally, glass has very poor adhesion to SU-8. Bare silicon has medium adhesion to SU-8. Patterns wider than 200 μm should be fine on silicon. For patterns <50 μm, adhesion promoting methods are needed, the following will help (a) clean the wafer thoroughly with Piranha, (b) Use AP300, sputtered chromium film, or SU-8 2002 as adhesion promoter, (c) over- expose the pattern.

**3. Soft mold**. (a) It could be that the resist film still "wet", increasing softbaking or PEK time. (b) under-exposure due to insufficient exposing time, less reflective substrate, or thicker CEM and BC film. (c) high humidity, SU-8 uptakes moisture.

<div align="right">

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Updated on Jun. 29, 2009

</div>

A.4: Protocol for CRP Detection Tests[147]

## Microfluidic Immunoassay Protocol for Detection of C-Reactive Protein

*Experiment date:* _____    *Temperature:* _____

**Objective:**

**Important Note:**    Equilibrate buffer to **RT** before experiments!

➢ Keep the channel wet by sealing Petridish with Parafilm during all processes.

➢ Don't wipe the chip with tissue, always dry it with long-fiber cloth and keep the surface free from particle.

➢ Always have a <u>negative</u> control and a <u>positive</u> control for each experiment.

➢ Make sure the surface <u>isn't</u> in contact with fluorophores. It causes high background.

➢ Flush the channels with PBS buffer and then aspirate them between steps.

**Surface Functionalization:**

1. All microchannels are coated with BSA (1.5mg/ml in **PBS**) for 244 min at RT.

2. The coated channels are then activated by 0.4% (v/v) glutaraldehyde in **pure water** for 1h at RT.

3. Protein A (50μg/ml in **PBS**) is incubated with the channel for another 1 h at RT.

**Storage:** stored at 4 °C in a buffer consisting of 10 mM Tris, 0.05% BSA, 0.05% Proclin 300, and 5% glycerol.

**Immunoassay:**    *ImmunoChips Functionalized on*: _____

4. Take pre-functionalized chips out from the package. Flush the channels with 1X PBS buffer.

5. Rabbit polyclonal IgG to C-reactive protein (CRP) in **Protein A/IgG binding buffer** with concentrations shown in the table below is injected into channels and incubated for 7 min at RT.

6. Flush the channels with PBS. Then, empty the channel. Make sure it is free of residual liquid.

7. Inject CRP in **PBS** with concentrations listed in the table into channels. Incubate for 5 min at RT.

8. Flush the channels with PBS. Then, aspirate the channel. Make sure it is free of residual liquid.

9. FITC labeled goat polyclonal IgG to CRP in **PBS** with concentrations shown in the table below is injected into channels and incubated for 5 min at RT.

10. Wash channels by **PBST** (1x PBS+0.5μl/ml Tween 20). Incubate 5 min during the wash.

11. Refill channels with PBS and take image using exposure time 2.5 s and 84%.

## Schematic of Sandwich Immunoassay

rIgG to CRP: <u>1 mg/ml</u>                          (**in Binding Buffer**).
CRP: <u>2.13 mg/ml</u>
gIgG to CRP: <u>1 mg/ml</u>
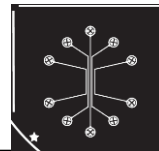
| | Concentrations in Micro-channels（µg/ml） | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| rIg to CRP | | | | | |
| CRP | | | | | |
| gIg to CRP | | | | | |

**Microscope Setup:**
0. Clean the glass slide and the 5x objective.
1. Brightness -0.50; Contrast 1.00; G.0.45 1.00.
2. Use Linearize for histogram (14 bit 16383).
3. Don't press White Balance in fluorescence mode. (Cyan 1.11 Red, Mag 1.41 Green, Yellow 0.48 Blue).
4. 1300×1030 RGB scanned color.
5. Index set to 0; Black reference enabled.
6. Best Fit set to 0%, disable Min/Max.
7. Push lever all the way in; shutter at 1$^{st}$ gear.

**Table A-1:** Capture antibody, CRP and detection antibody concentrations example for immunoassays

A.5: Example Graphs Taken from Fiber Optics for CRP Detection
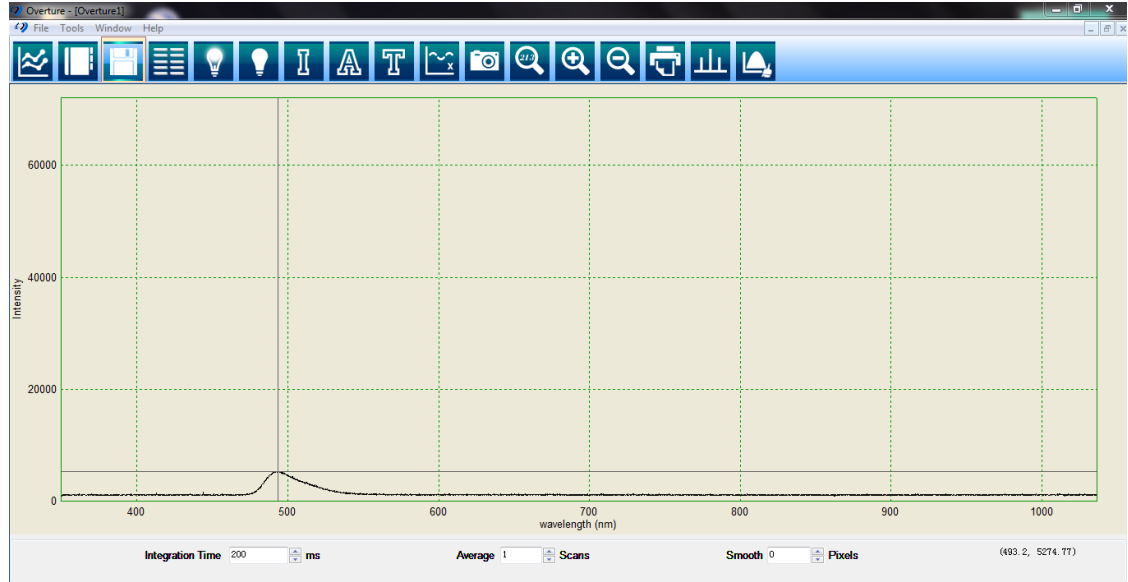


**Figure A-4:** CRP detection at intensity = 5274 (concentration ~ 1.3 µg/mL in blood)
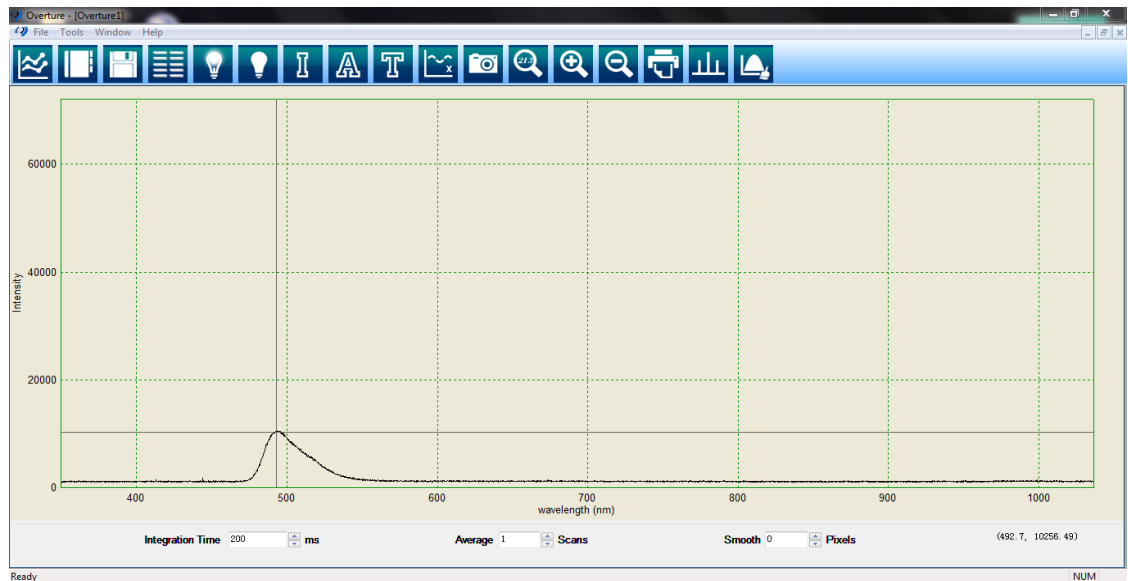


**Figure A-5:** CRP detection at intensity = 10256 (concentration ~ 3.4 µg/mL in blood)

B.1: Protocol for Phage Display with Agar Plates (Translated from German)[143]

**Panning Ab-libraries: Panning mit biotinylierten Oligopeptiden**

Dissolve the Oliogpeptide in PBS with 5%DMSO.

Coat 100 ng Streptavidin in 100 µl PBS per Well overnight at 4°C. For each panning round coat <u>two</u> wells on <u>two separate</u> Maxisorb Stripes! One well is for the biotinylated Oligopeptide binding und second well is for the  pre-incubation of the Ab-Phage library on top of streptavidin, in order to remove streptavidin binders!

*<u>Alternative</u>: Coat 100 ng biotinylated Oligopeptides in PBS or NaHCO$_3$ Buffer per well overnight at 4°C direct on the plate. Check beforehand the Streptavidin-HRP Conjugae, if the Oligopeptides bind on the plate!*

Wash with PBST in the 3xELISA-Washer (Program "3 N"), to remove the unbound Streptavidin.

Place 100 - 500 ng biotinylated Oligopeptides in 150 µl PBS in a Streptavidin Well geben and incubate for 1 h at RT (*<u>not</u> when the Oligopeptides are coated directly on the plate!*).

Wash with PBST in the 3xELISA-Washer (Program "3 N"). Fill the wells to completely block with 2%MPBST. Incubate at RT for 2 h.

At the same time, block an empty Streptavidin well (without Antigens) per round with Panning block for1 h. (*when the Oligopeptides are coated directly on the plate, block an empty well with panning block*)

After pre-incubate the Ab-library ($1x10^{11}$-$1x10^{12}$ Ab-Phage, depending on the library), or the amplified Phage in the previous panning rounds in 150 µl panning block for 1 h.

Transfer the Phage from the pre-incubation wells to the wells with the antigen, add 100 ng streptavidin (competitive assay from the previously used  Streptavidin binders) and incubate for 2h at RT.

Remove the unspecific bounded phage in the ELISA-Washer (Program "10OBN" with Nunc Maxisorbplatte, "10BONS2" with Nunc Maxisorb Stripes).

Elute the bounded Phagen with 200 µl 10 µg/ml Trypsin for 30 min at 37°C [with basic Elution 150 µl 100 mM Triethylamin, 5 min at RT on the shaker (too long of an Incubation reduces the infectionality of the phage). Neutralize the Phage with 150 µl 1M Tris HCl pH7.5. With acidic Elution: 150 µl HCl/Glycin 0,1 M pH 2,2, incubate 15 min on the shaker. Neutralize with150 µl Phosphate buffer pH 7,6].

Innoculate 50 ml 2xTY-T mit XL1-Blue . Take the colony up O.D.$_{600}$ 0,5. Take 20 ml and infect with 190 µl Phage, Incubate for 30 at 37°C. Centrifuge for 10 min at 4000 rpm (3220xg).

Titration Setup:

Infect 50 µl der XL1-Blue Kultur with around 10 µl $10^{-2}$ -$10^{-8}$ (after each panning round) Phage elution. Incubate for 30 min at 37°C and plate up the LB-Amp und incubate overnight at 37°C (see Protocol Titration Setup).

--------------

Dissolve the pellet in 250 µl SOB-GA und coat on an SOB-GA Agarplatte (15 cm diameter). Incubate overnight at 37°C.

On the next day, clean the Bacteria off with around 5 ml TY-GA (use Drigalski-Spatula) and place in a Falcon.

Innoculate 50 ml 2xTY-GA mit 50-200 µl Bacteria (O.D. $_{600}$<0,1). Incubate the colony at 37°C until the O.D.is between 0,4-0,5. Infect with 5 ml (~2,5*$10^9$ bacteria) M13K07 a 1:20 ratio (5*$10^{10}$ pfu) and incubate for 30 min at 37°C without shaking and 30 min at 200 rpm.

Centrifuge the resulting bacteria at 4000 rpm (3220xg) for 5 min and place in Glycerin stocks with 20 % Glycerin.

In order to remove the glucose, (Phage will not build, if the Lac-Promotor is blocked), pellet the bacteria at 4000 rpm (3220xg) for 10 min (Eppendorf 5810R, Rotor A-4-81).

Carefully resuspend the pellet in about 2xTY-AK  and add in 30 ml 2xTY-AK in a 100 ml Erlenmeyer flask. Incubate overnight at 30°C and 250 rpm in the shaking incubator.

Centrifuge the bacteria for 10 min at 4000 rpm (3220xg) at 4 °C in a falcon.

Place the supernatant in a new falcon (if the supernatant is still cloudy, centrifuge the suspended bacteria again!) and precipitate the Phage particles with an additional 1/5 Volume PEG/NaCl. Mix the supernatant and incubate for 1 h on a shaker in ice.

Centrifuge for 1 h at 4000 rpm (3220xg) (Falcon) and 4 °C.

Remove the PEG-dissolved efficiently (Tube above roll of papertowels).

Resuspend the pellet in 500 µl Phage-Dilution Buffer and transfer to a 2 ml cap. Centrifuge the rest of the bacteria at 13,000 rpm for 1 min and transfer the supernatant to a 2 ml vial and incubate at 4°C or to begin the next panning round.

Chemicals and  Compounds:

- PBS pH 7,4 (8,0 g NaCl, 0,2 g KCl, 1,44 g $Na_2HPO_4*2H_2O$, 0,24 g $KH_2PO_4$ per 1 l)
- DMSO
- 50 mM $NaHCO_3$, pH 9,6
- Streptavidn (Applichem)
- PBST (PBS+ 0,1% Tween 20)
- 2 % MPBST (2 % milk powder in PBST, kept fresh)
- Panning block (1 % milk powder + 1 % BSA in PBST, kept fresh)
- 10 µg/ml Trypsin in PBS

- 100 mM Triethylamin

- Phosphate buffer pH 7,6 (6 mM $NaH_2PO_4$, 50 mM $Na_2HPO_4$)
- HCl/Glycin 0,1 M pH 2,2

- 1 M TrisHCl pH 7.5

- 2xTY-Medium pH 7,0 (1,6 % Tryptone, 1 % wheat extract, 0,5 % NaCl)

- 2xTY-T (2xTY + 50 µg/ml Tetracyclin)

- 2xTY-GA (2xTY + 100 mM Glucose + 100 µg/ml Ampicillin)
- 2xTY-AK (2xTY + 100 µg/ml Ampicillin + 50 µg/ml Kanamycin)

- Mg-solution 2 M (1 M MgCl, 1 $MgSO_4$)

- SOB Medium pH 7,0 (2 % Tryptone, 0,5 % wheat extract, 0,05 % NaCl, sterile after autoclaving, before using 20 mM Mg-solution addition)
- SOB-GA (SOB + 100 µg/ml Ampicilin + 100 mM Glucose)
- 2 M Mg-Lösung (1 M $MgCl_2$ + 1 M $MgSO_4$)
- Ampicillin (100 mg/ml Stock)
- Kanamycin (10 mg/ml Stock)

- 2 M Glucose (filtered sterile)
- Glycerin 87%, autoclaved

- PEG/NaCl (20% (w/v) PEG 6000 und 2,5 M NaCl)

- Phage Dillution Buffer (10 mM TrisHCl pH7,5, 20 mM NaCl, 2 mM EDTA)

Organisms:

- VCS-M13 or M13K07 Helper phage or Hyper phage (Kanamycin-Resistance)
- XL1-Blue MRF' (Tetracyclin Resistance) (Amber Suppressor Mutated (sup E))
- Phage bank Glycerol stock

Hint: XL1-Blue can be grown up to O.D. 0.5 und then kept on ice until infection (F-Pili remains at 4°C)

Materials and tools:

- Filtertips
- 50 ml Falcon
- 2 ml screw on vial
- Immunotubes or Microtiter 8 row plates (250-300µl per well) (Nunc Maxisorb)
- Microtiter plates or Stripes with bound Streptavidin
- Petridish 15cm diameter
- Eppendorf 5810R, Rotor A-4-81

Work schedule for a panning round:

Day 0: Antigen coating

Day 1: Panning, Titration of the eluted phage, overnight growth of the eluted Ab-phage

Day 2: Overnight growth and amplification of the eluted phage

Day 3: PEG-Precipitation of the phage, Titration of the amplified phage + antigen coating

Day 4: Evaluation of the titration, next panning round

M.Hust 11/16/05

B.2: Protocol for Phage Display with Micro Titer Plates (Translated from German)[144]

## Panning Ab-libraries: Panning in Immunotubes (MTPs)

Coat the well with the antigen (5 µg Protein should be used for the first panning round, 0,5-5 µg or a constant 5 µg for the following rounds): Dissolve the protein in 150 µl 1xPBS or $NaHCO_3$-buffer (preferred) and place in a well. Incubate overnight at 4°C. (with biotinylated antigens, coat 200 ng Streptavidin in 150 µl $NaHCO_3$-buffer overnight at 4°C and wash away the next day the Streptavdin with the Washer and incubate the biotinylated Antigen for 45 min at RT in 50 µl PBS).]

Remove the antigen solution.

Completely fill in order to block the wells with 2%MPBST. Incubate at RT for 2 h. Wash with PBST in a 3xELISA-Washer (Program "3 N").

At the same time, block completely an empty well (without antigens) per round with panning block for1 h.

Then pre-incubate $1x10^{10}$-$1x10^{12}$ scFv-Phage from the library (with HAL7/8 around $2,5x10^{11}$), or the amplified Phage from the previous panning round in 150 µl panning block for 1 h.

Transter the phage from the pre-incubation wells into the wells with the antigen and incubate for 2h at RT.

Remove the not or partially bound Phage in the ELISA-Washer (Program "10OBN" with Nunc Maxisorb plate, "10BONS2" with Nunc Maxisorb Stripes). The number of the washing repeats is based on the number of the panning round (ex. 2x wash with 2nd panning round).

Elute bound scFv-Phage  with 200 µl 10 µg/ml Trypsin for 30 min at 37°C.

Innoculate 50 ml 2xTY-T mit XL1-Blue. Let the colony grow until O.D.$_{600 =}$ 0.5. Add 20 ml and infect with 200 µl eluted scFv-Phage in a 50 mL Falcon, incubate for 30 min at 37°C for 10 min.

Centrifuge at 4000 rpm (3220xg).

Titration setup of the eluted phage:

Plate 200 µL of the infected bacteria ($10^{-2}$) und plate again 20 µL for the dilution in PBS und around 100 µL ($10^{-4}$ - $10^{-6}$). Incubate overnight at 37°C (see protocol Titration setup).

--------------

Dissolve the pellet in 250 µl 2xTY-GA und spread on a 2xTY-GA Agar plate (15 cm diameter). Incubate overnight at 37°C.

The next day, suspend the bacteria with around 5 ml 2xTY-GA (use Drigalski-Spatula) and transfer into a falcon.

Innoculate 30 ml 2xTY-GA in a 100 mL flask with 50-200 µl bacteria (O.D. $_{600}$<0,1). Let the colony grow at 37°C until the O.D. is around 0,4-0,5. 5 ml (~2,5*$10^9$ bacteria) infect with M13K07 in a 1:20 ratio (5*$10^{10}$ pfu) incubate for 30 min at 37°C without shaking und 30 min at 200 rpm.

Centrifuge the suspended bacteria at 4000 rpm (3220xg) for 5 min and place in Glycerin stocks with 20 % Glycerin.

*This step is common, but not necessary!*

--------------

In order to remove the glucose, (Phage will not build, if the Lac-Promotor is blocked), pellet the bacteria at 4000 rpm (3220xg) for 10 min (Eppendorf 5810R, Rotor A-4-81).

Carefully resuspend the pellet in about 2xTY-AK and add in 30 ml 2xTY-AK in a 100 ml Erlenmeyer flask. Incubate overnight at 30°C and 250 rpm in the shaking incubator.

Centrifuge the bacteria for 10 min at 4000 rpm (3220xg) and 4 °C in a Falcon (if the supernatant is still cloudy, centruge the suspended bacteria again!). Take twice 1 mL supernatant (~1x10$^{12}$ Phagen/mL), store at 4°C, or bzw. Directly add 100 µL + 50 µL panning block for the next panning round. Titrate the amplified phage to the control (see protocol: Titration steps).

*This step of the process is optional:*

*Place the supernatant in a new falcon (if the supernatant is still cloudy, centruge the suspended bacteria again!) and precipitate the Phage particles with an additional 1/5 Volume PEG/NaCl. Mix the supernatant and incubate for 1 h on a shaker in ice.*

*Centrifuge for 1 h at 4000 rpm (3220xg) (Falcon) and 4 °C.*

*Remove the PEG-dissolved efficiently (Tube above roll of papertowels).*

*Resuspend the pellet in 500 µl Phage-Dilution Buffer and transfer to a 2 ml cap. Centrifuge the rest of the bacteria at 13,000 rpm for 1 min and transfer the supernatant to a 2 ml vial and incubate at 4°C or to begin the next panning round (see protocol: Titration steps).*

Chemicals and solutions:

- PBS pH 7,4 (8,0 g NaCl, 0,2 g KCl, 1,44 g $Na_2HPO_4*2H_2O$, 0,24 g $KH_2PO_4$ per 1 l)
- 50 mM $NaHCO_3$, pH 9,6
- PBST (PBS+ 0,1% Tween 20)
- 2 % MPBST (2 % milk powder in PBST, kept fresh)
- Panning block (1 % milk powder + 1 % BSA in PBST, kept fresh)
- 10 µg/ml Trypsin (Sigma T-0303) in PBS

- 100 mM Triethylamin

- Phosphate buffer pH 7,6 (6 mM $NaH_2PO_4$, 50 mM $Na_2HPO_4$)
- HCl/Glycin 0,1 M pH 2,2

- 1 M TrisHCl pH 7.5

- 2xTY-Medium pH 7,0 (1,6 % Tryptone, 1 % wheat extract, 0,5 % NaCl)

- 2xTY-T (2xTY + 50 µg/ml Tetracyclin)

- 2xTY-GA (2xTY + 100 mM Glucose + 100 µg/ml Ampicillin)
- 2xTY-AK (2xTY + 100 µg/ml Ampicillin + 50 µg/ml Kanamycin)

- 2xTY-GA Agar plate (2xTY-GA + 1,5% Agar)

- Ampicillin (100 mg/ml Stock)
- Kanamycin (10 mg/ml Stock)

- 2 M Glucose (filtered sterile)
- Glycerin 87%, autoclaved

- PEG/NaCl (20% (w/v) PEG 6000 und 2,5 M NaCl)

- Phage Dillution Buffer (10 mM TrisHCl pH7,5, 20 mM NaCl, 2 mM EDTA)

Material und Tools:

- Filtertips
- 50 ml Falcon
- 2 ml screw on vial
- Immunotubes or Micro titer 8 row plate (250-300µl per well) (Nunc Maxisorb)
- Streptavidin Immunotubes/MTPs/Stripes (Nunc)
- Petri dish 15cm diameter
- Eppendorf 5810R, Rotor A-4-81

- Tecan Columbus ELISA Washer
- MTP shaker Vortemp56 (Labnet) oder Thermo Shaker PST-60HL-4 (Lab4You)

Organisms:

- M13K07 Helper phage oder Hyper phage (Kanamycin-Resistant)
- XL1-Blue MRF' (Tetracyclin Resistant) (Amber Suppressor Mutated (sup E))
- Phage bank Glycerol stock

Hint: XL1-Blue can be grown up to O.D. 0.5 und then kept on ice until infection (F-Pili remains at 4°C)

Arbeitsablauf für eine Panningrunde:

Work schedule for a panning round:

Day 0: Antigen coating

Day 1: Panning, Titration of the eluted phage, overnight growth of the eluted Ab-phage

Day 2: Overnight growth and amplification of the eluted phage

*Day 3: PEG-Precipitation of the phage, Titration of the amplified phage + antigen coating (optional)*

Day 4: Evaluation of the titration, next panning round

M.Hust 09/12/11

B.3: Number of Colonies Obtained for Each Panning Round

| | $10^4$ | $10^5$ | $10^6$ | Average* |
|---|---|---|---|---|
| **Tau Colonies** | Too Many | 153 | 7 | 153 X $10^5$ |

**Table B-1:** Tau E. coli colonies after third panning round (panning in micro titer plates)

| | $10^4$ | $10^5$ | $10^6$ | Average* |
|---|---|---|---|---|
| **Tau Colonies** | Too Many | Too Many | 330 | ---- |

**Table B-2:** pTau E. coli colonies after third panning round (panning in micro titer plates)

| | $10^4$ | $10^5$ | $10^6$ | Average* |
|---|---|---|---|---|
| **Tau Colonies** | 31 | 1 | 1 | 20.5 X $10^4$ |

**Table B-3:** pTau L1 with competition from Tau L1 E. coli colonies after third panning round (panning in micro titer plates)

| | $10^4$ | $10^5$ | $10^6$ | Average* |
|---|---|---|---|---|
| **Tau Colonies** | 2 | 0 | 0 | 1.0 X $10^4$ |

**Table B-4:** pTau L2 with competition from Tau L2 E. coli colonies after third panning round (panning in micro titer plates)

* The average values were taken from the average between the $10^4$ and $10^5$ counts. This was performed with the formula (($10^4$ coefficient) + 10 x ($10^5$ coefficient)) / 2. The resulting coefficient is multiplied by $10^4$ to obtain the average result.

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