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DEVELOPMENT OF A PAPER BASED LATERAL FLOW DEVICE FOR THE DETECTION OF IαIP VIA COMPETITIVE ELISA

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BY

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ABSTRACT

This research has the focus on developing a paper-based device capable of sequentially flowing three separate fluids. Different assembly methods are investigated and compared to develop a more streamlined approach for the prototyping and testing of biological reagents. A faster method of fabrication is developed followed by an investigation of the potentials to detect the presence of Inter α Inhibitor Protein (a biomarker associated with sepsis). This is primarily achieved by applying the techniques of an Enzyme Linked Immunosorbent Assay (ELISA) onto a paper based platform. The research presented shows the steady progression toward and optimization of a lateral flow device capable of achieving the above and eventually presenting the capability of a competitive ELISA to detect IαIP. This platform allows for the possibility to generate results within a fraction of an hour and furthermore with smaller fluid volumes as compared to current bench top tests, such as ELISA which is typically conducted in 96 well plates.

ACKNOWLEDGMENTS

Over the course of the last year I have had the tremendous opportunity to pursue a Master's Degree in Mechanical Engineering at the University of Rhode Island. Through this endeavor I have had the pleasure to meet and to work with a number of outstanding people who have contributed both to my success and enjoyment within the program.

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LIST OF ABREVIATIONS

A3CS	Allyltrichlorosilane
AB	Antibody
ALP	Alkaline Phosphatase
ASSURED	**A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free, and **D**eliverable
B-IαIP	Biotinylated Inter-Alpha Inhibitor Protein
DPI	Dots per Inch
DAB	Diaminobenzidine
NBT/BCIP	Nitroblue Tetrazolium/ 5-Bromo-4-chloro-3-indolyl phosphate
ELISA	Enzyme Linked Immunosorbent Assay
FC-72	Perfluro-Compound
HA	Hyaluronan
HRP-Strep	Horse Radish Peroxidase-Streptavidin
IαIP	Inter Alpha Inhibitor Protein
LFA	Lateral Flow Assay
LFD	Lateral Flow Devices
LFTS	Lateral Flow Test Strips
LOD	Limit of Detection
MAB 69.26	Monoclonal Antibody 69.26 (specific to IαIP)

mIU/mL	Million International Units per Milliliter
PDMS	Polydimethylsilaxane
PBD	Paper Based Device
POC	Point of Care
RGB	Red Green Blue
SHAP	Serum-derived Hyaluronan-Associated Protein
TRIS	tris(hydroxymethyl)aminomethane
vol.%	Volume Percentage
wt.%	Weight Percentage

1 CHAPTER 1 – Introduction

Within the medical research community there has been growing popularity to develop laboratory diagnostic tests for application into point-of-care settings. Point-of-care settings can include frontlines, communities without a direct connection to medical facilities, or even accident scenes. They are areas where on-hand tests would be beneficial to return fast and reliable diagnostic results. The demand for point-of-care devices has largely been driven by their advantages in cost, ease-of-use, speed of detection, spectrum of application, and disposability. Many researchers are striving to meet these characteristics. The sensitivity and accuracy within such tests has proven beneficial to settings both in developed countries and to lower-resourced settings [1]. Many research institutions have realized the potential impact of point-of-care devices, which were forecasted by a growth rate of 85% and a revenue increase of $2.13 Billion in 2009 to $3.93 Billion in 2016 [2]. With such a large number of resources being pushed toward development and improvement POC devices, breakthroughs are certainly on the horizon line.

The first chapter of this master's thesis serves to introduce point of care devices and describe the current state of their development.

1.1 Point of Care Devices an Introduction

Point of Care (POC) devices are medical devices developed for use outside of laboratory settings and are most commonly applied to disease detection. These devices are fast becoming a popular area of research as they offer improvements in

scalability, cost, detection times, and access [3]. Although POC devices are typically associated with resource limited settings, the benefits they offer are proving to be advantageous for a variety of applications including: hospital rooms, emergency response scenarios, at home, and military settings [1]. The common characteristic that is sought after POC diagnostics is “simplicity”; which is defined by the Food and Drug Administration in table 1-1:

Table 1-1: Describes the FDA Qualifications for a "Simple Test" [1] [4]

• Is a fully automated instrument or a unitized or self-contained test.
• Uses direct unprocessed specimens, such as capillary blood (fingerstick), venous whole blood, nasal swabs, throat swabs, or urine.
• Needs only basic, non-technique-dependent specimen manipulation, including any for decontamination.
• Needs only basic, non-technique-dependent reagent manipulation, such as “mix reagent A and reagent B.”
• Needs no operator intervention during the analysis steps.
• Needs no technical or specialized training with respect to troubleshooting or interpretation of multiple or complex error codes.
• Needs no electronic or mechanical maintenance beyond simple tasks, e.g., changing a battery or power cord.
• Produces results that require no operator calibration, interpretation, or calculation .
• Produces results that are easy to determine, such as ‘positive’ or ‘negative,’ a direct readout of numerical values, the clear presence or absence of a line, or obvious color gradations.
• Provides instructions in the package insert for obtaining and shipping specimens for confirmation testing in cases where such testing is clinically advisable.
• Has test performance comparable to a traceable reference method as demonstrated by studies in which intended operators4 perform the test. If a reference method is not available for a test you are proposing for waiver, please contact OIVD to discuss your proposed plan prior to submitting your application.
• Contains a quick reference instruction sheet that is written at no higher than a 7th grade reading level.

In an effort to meet these criteria, corporations have begun to develop and market POC tests which make a simpler device including or exclude instrumentation for detection [5].

Instrumented Devices

- **Permanent integrated instruments**- Designed for high-throughput to return results quickly and accurately within a laboratory setting. However, they cannot be considered “simple tests” due to the trained personnel needed. [6]
- **Permanent Instruments using disposable components**- Included in this category are disposable components on which results are developed to be processed by an analyzer (ie. handheld device or laptop). The analyzer may also serve as a source of power to move fluids within the system, for thermal control, or for detection purposes [5]. These can interpret multiple tests over a short period of time with the aid of a processor. Additionally, results may be transmitted to doctors via internet for further examination. This is an ideal scenario for POC settings, as it brings the expertise of a hospital to the frontlines of disease. The downside is that the processor can drive up the cost of such a test, and may require special components for repair when damaged. [6]

Un-Instrumented (Autonomous) Devices

- **Pure disposables**- Usually develop a detection scheme on a disposable platform (ie. paper) which integrates many inexpensive components and reagents. A major drawback of many disposable platforms is that they lack the sensitivity that lab tests provide and cannot provide quantitative results. [6]

1.2 POC Microfluidic Devices

Although POC tests have also been developed in many forms to utilize advantages of different materials, the greater majority do not fall into the category of microfluidic devices [7]. Microfluidic devices, allow for a manipulation of small volumes of fluid, which reduces the consumption of reagents and of sample fluids. This means smaller sample fluid volume is needed from patients; depending on the sample needed this could cause discomfort to the patient upon extraction. Also, for detection of disease in infants, larger volumes of samples may not be possible. Infants cannot provide as high a volume of blood as compared to an adult.

Microfluidic devices may further be manipulated by an Instrumented Device, which can incorporate pumps and channels to direct fluid toward analysis sites in the device. Channels can be made from glass, silicon, and polymers (ie. polydimethylsilaxane PDMS). The integration of pumps and chips increases complexity and correspondingly, increase the chance for mechanical malfunction [8]. In a resource-limited setting replacement parts may be hard to find.

In turn, as more research groups are focusing on the development of POC diagnostics, they are turning to paper-based or fibrous-based materials. These materials are generally cheaper to manufacture and fall within the constraints for a successful POD device: **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free, and **D**eliverable to end users (**ASSURED**) [8; 7].

1.3 POC Diagnostic Applications

Concerning Global Health Issues, POC devices have been the turn-to solution as they are rapid, simple, and inexpensive, making them more readily accessible [5]. POC devices are designed to detect the presence of any chemical substance or compound that can help to indicate the presence of disease, infection, or other abnormality. This is called an analyte. Most POC tests utilize blood, but depending on the application, saliva or urine may also be used. Arguably, saliva is the most preferred solution for POC testing, as it is the least invasive and is least hazardous to both patients and health care workers; the problem is that it may have a varying consistency of analyte concentration when compared to that in blood or urine [5]. Depending on the target, any of these fluids may be used in a range of diagnostic applications, including but not limited to: Virus detection, diabetes, bacterial Infection, pregnancy, allergy, agriculture and disease [6; 8; 9; 10].

1.4 POC Diagnostic Materials

1.4.1 Dipsticks

One of the simplest paper-based mechanisms is a Dipstick Assay or a Testing Dipstick. Two examples are shown below in Figure 1-1.

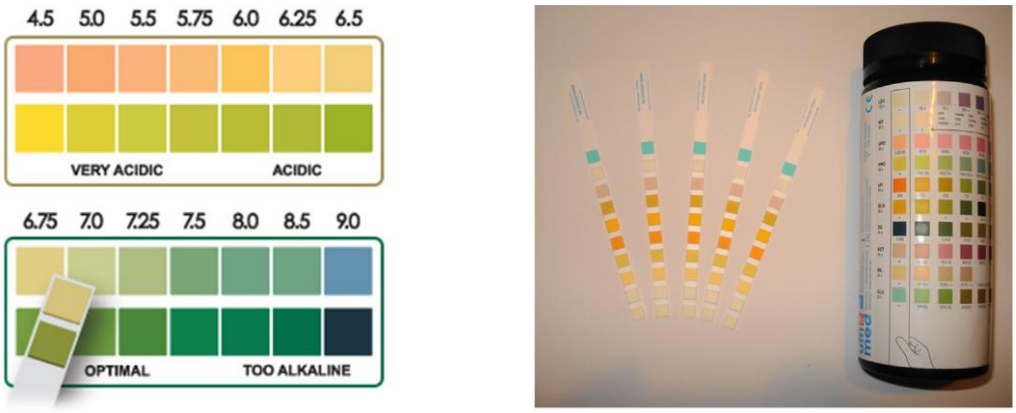


Figure 1-1: Dipstick Assays. Diagnostic pH Strip (left) [11]. Urine Test Strip (right) [8].

These test strip or pH strips are pieces of paper that are treated with various concentrations acid-alkali solutions. After being exposed to a test sample, these dried reagents react with the in-sample hydrogen ions (H^+) to develop a color change. Depending on the number of H^+ different color reactions will occur, which can be quantified by comparison to an included chart. [8]

Urine Test strips can detect metabolic products such as leukocytes, nitrates, urobilinogen, protein, pH, haemoglobin, specifi Gravity, ketornes, bilirubin, and glucose. Diabetes, renal and urinary tract issues, liver diseases or haemolytic disorders are common applications [12].

1.4.2 Lateral Flow Assays

Another class of paper-based tests is lateral flow assays use fibrous materials to take advantage of their autonomous flow characteristics (capillarity). Additionally, they integrate various biological reagents to create the necessary reaction schemes for detection of virus, or infection. Lateral flow immunoassays (LFIA) have become widely accepted for a broad range of diagnostic needs.

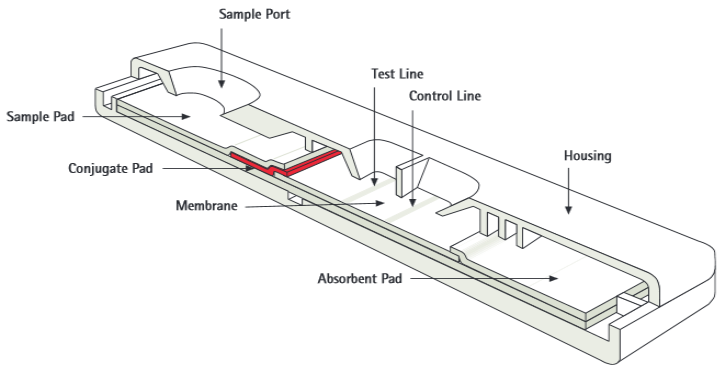


Figure 1-2: An Example of a Lateral Flow Device.

LFIA generally consist of a sample pad, a conjugate pad, an absorbent pad and a detection area. The detection area produces two indicator lines (test line and a control line) when a sample runs through it. The absorbent pad provides driving force through capillarity to pull a sample fluid along the test strip [8]. A membrane is incorporated ahead of the absorbent pad to serve as the testing platform, where diagnostic schemes are developed. Capture molecules such as enzymes or antibodies may be isolated on this membrane; which eventually help to develop the detection scheme [8]. Just before the membrane, lays a conjugate pad which stores detection molecules, which would provide signal or color change at the end of the test. These labeled-molecules have a high affinity to bind the target. Eventually, the label will provide the visual proof for presence of a target analyte.

As a sample fluid is applied, it passes first through the conjugate pad, where the target analyte binds to labeled antibodies; this process develops the top half of what is commonly called an 'Assay-Sandwich'. This complex is then pulled by capillary flow toward the membrane, which has on it anchored molecules designed only to capture the top half of the sandwich compound. When this biding occurs the Assay-

Sandwich is complete and consists of an anchored capture molecule, an analyte, and a labeled detection molecule. The accumulation of such complexes forms the test line on the device and is the basis for any Lateral Flow Immunosorbent Assay (LFIA) [13]. Most commonly, this type of reaction scheme utilizes antibodies and to capture a virus and develop diagnostic results.

1.5 Biology of a Lateral Flow Immunosorbent Assay

Antigens and antibodies are the two major biological components necessary to effectively run an immunoassay. The term immunoassay, combines the word “immuno”, referring to an immune response, and “Assay”, referring to a test; thus, an immunoassay combines antibodies (generated in correlation with an immune response) and antigens (responsible for the immune response) to develop a diagnostic test.

Antigens are foreign or toxic entities that elicited a response by an immune system. This response is characterized by a development of antibodies, which bind to foreign entities (virus or bacteria), effectively marking them for elimination.

Antibodies, also known Immunoglobulin (Ig), are large multifunctional proteins created by the immune system to identify and to help eliminate antigens. As glycoproteins they are composed of four polypeptide chains which covalently link to one another through disulfide bonds to form the characteristic Y-shape [14]. As depicted by the below figure, antibodies are made from two light chains and two heavy chains. The antibody structure is sub-divided into two regions: variable region

and a constant region.

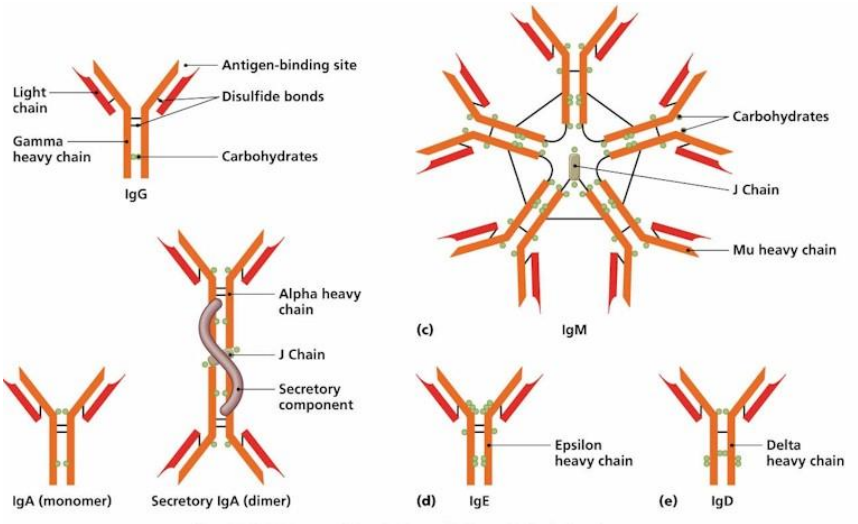
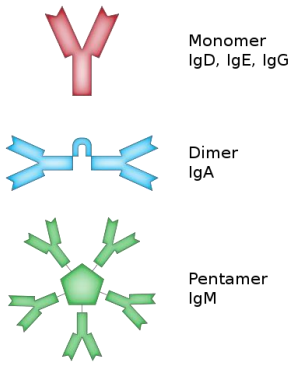


Figure 1-3: Structure of an Antibody Along with Various Isotopes [15]

Constant regions (the bottom half of the Y-shape) have the same amino acid composition. Between different antibodies variable regions (the top-half of the Y-shape) make up the antigen binding site and between different antibodies will have a different amino acid composition. Antibodies come in five isotopes in mammals; these are discussed.

Table 1-2: A Description of the Isotopes and Functions of Antibodies. [16; 17]

Antibody	Description	Complexes
IgA	Can be found in areas that line the gut, respiratory tract, and urogenital tract.	
IgD	Serves as an antigen receptor on B-cell, unexposed to antigens. B-Cells are part of the immune system; they are responsible for making immunoglobulins.	
IgE	Involved in the histamine release associated with allergens, also protects against parasitic worms	
IgG	Most commonly found isotope and is responsible for majority of antibody-based immunity.	
IgM	This is responsible for the immune response during early stages of immune system development (before sufficient IgG is created)	

IgG is the most common isotope found in human serum, it represents 85% of the antibodies that can be found in serum [17].

Antibodies may also be present in Polyclonal and Monoclonal forms. The primary difference resides in these forms inherent ability to bind to epitopes. Epitopes are binding sites on a surface of an antigen to which antibodies, B-Cells, or T-cells bind. This region is also called a *determinant* or *antigenic determinant*. There may be many different epitopes on the surface of an antigen available for an antibody [18].

Antibodies are isolated within sheep, rabbit, or goats. An immune response is elicited by injecting these animals with an antigen (this may be done a number of times). After an immune response develops, blood is extracted and serum is isolated. This serum which contains the molecules created in response to an antigen is called antiserum; it may contain a mixture of antibodies, each may have an affinity to bind to a different epitope on the antigen (see below) [18].

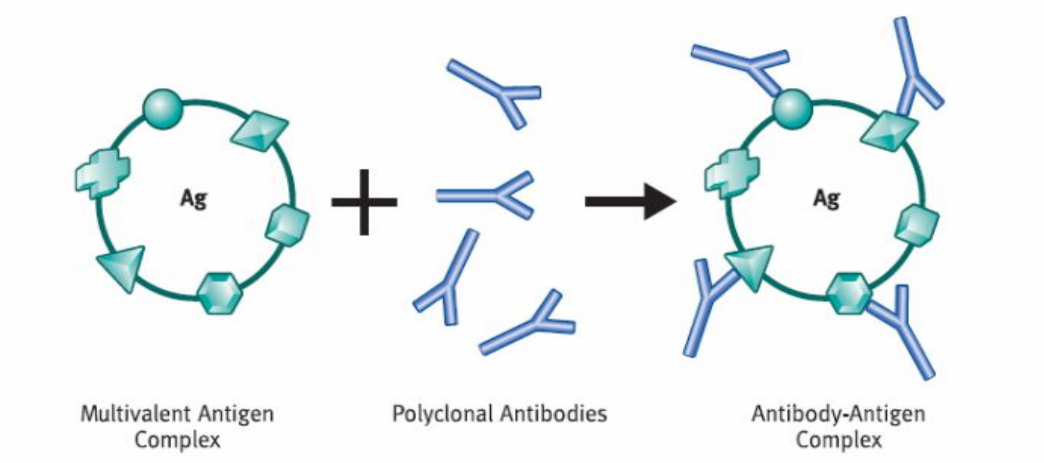


Figure 1-4: Depiction of a Polyclonal Antibody [18].

On the other hand, monoclonal antibodies are more specific, in that they will bind only to a specific epitope on an antigen (see below). They are isolated from a hybridoma cell line, which are tumor cells that produce many copies of a particular antibody. Tumor cells can produce antibodies indefinitely and consistently [18].

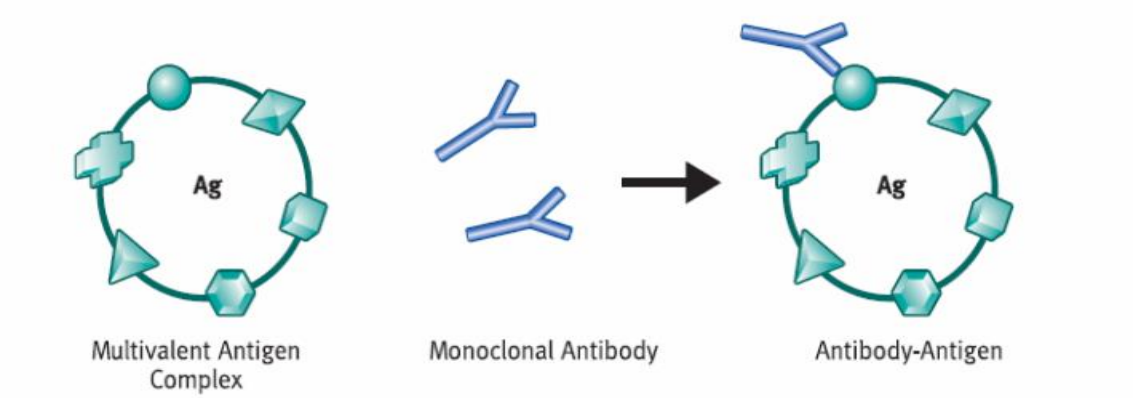


Figure 1-5: Depiction of a Monoclonal Antibody [18].

By taking advantage of this chemistry and binding affinity between antibodies and antigens, diagnostic tests can be developed.

1.6 Enzyme Linked Immunoassay (ELISA)

As it stands, Enzyme Linked Immunoassay (ELISA) is the gold standard for antigen detection within a laboratory setting. It offers superior sensitivity and specificity and is robust and easy-to-use, but trained person is needed to effectively run a test.

An ELISA is conducted by placing a sample fluid onto a functionalized surface to make it easier for antibodies to bind. As the solution incubates, the antibodies' affinity to a particular analyte helps to develop antibody-antigen complexes. After a certain amount of time, the surface is then washed to remove any unbound proteins,

and a second fluid containing detection antibodies is added. As mentioned previously, detection antibodies are labeled with an indicator that eventually induces a signal. In the case of ELISA, detection antibodies are labeled with an enzyme. When an "ELISA Sandwich" complex is formed it includes an anchored antibody, the analyte and labeled detection antibody. Following a second wash step to further remove any unbound proteins, a substrate is added. The substrate interacts with the enzyme bound to the detection antibody to create a measureable indication through color change or electrochemical signal [5]. The figure below provides a visualization and comparison between the reaction schemes used in a Lateral Flow Assay and an Enzyme Link Immunosorbant Assay.

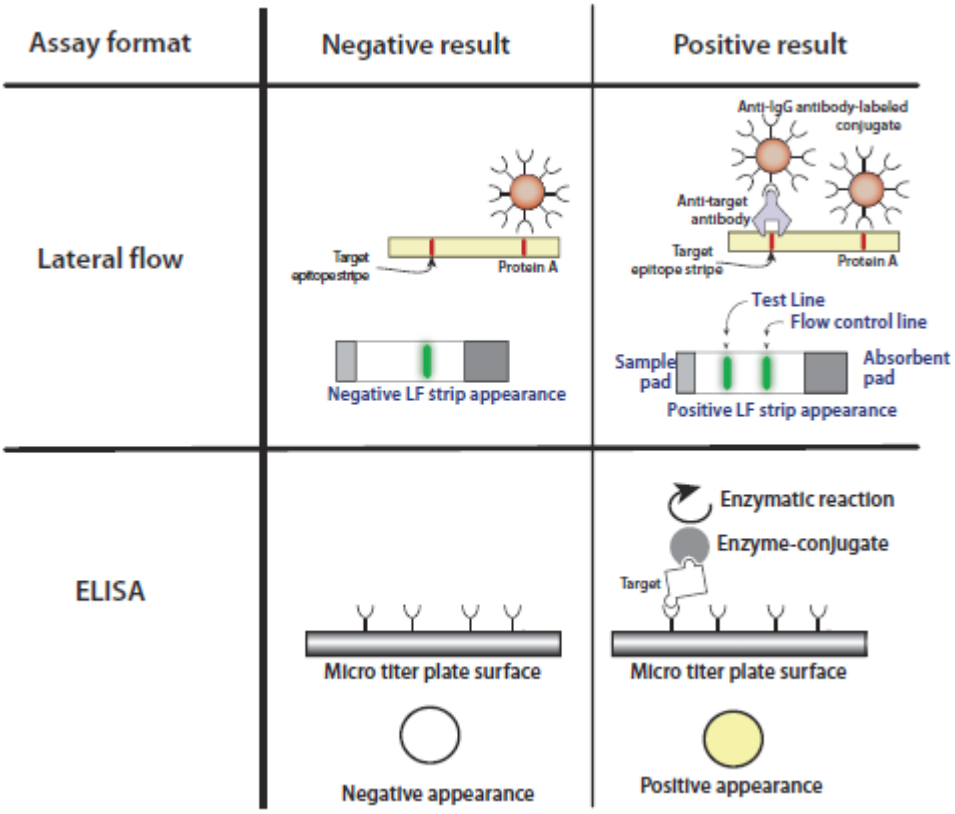


Figure 1-6: Biological Mechanisms Involved in a LFA and an ELISA [5]

A major challenge of adapting ELISA to a POC platform is the need for multiple fluid steps. Additionally many of the necessary reagents needed for ELISA become ineffective if stored at room temperature. None the less, the superior sensitivity of ELISA is the driving force for many research groups in their endeavors to bring ELISA into a Lateral Flow Setting.

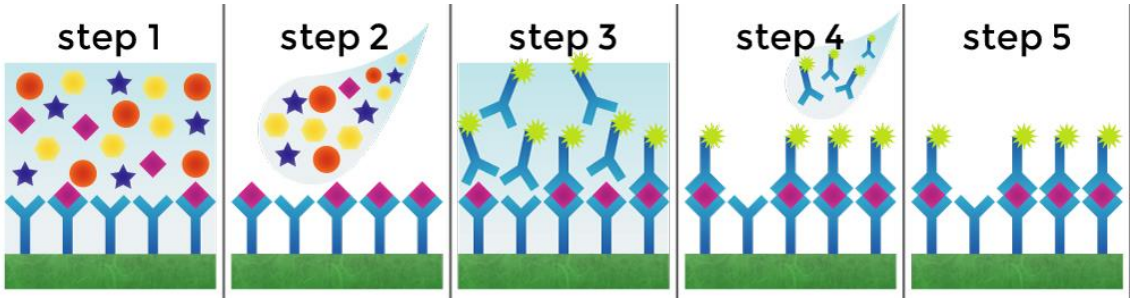
1.7 Lateral Flow Assay Reaction Schemes

Lateral Flow Assays may be conducted within two formats or reaction schemes, through a Non-Competitive Assay (or "Sandwich Assay" as described in section 1.4) or through a Competitive Assay. It is important to note that solid phase immunoassays have played a large role in assay development in the last 70 years. Solid Phase refers to the solid surface (i.e. microtiterplate or a membrane) on which proteins (ie. capture antibodies) may be adhered. This surface functionalization is the key functional unit within an ELISA reaction scheme.

1.7.1 'Sandwich Assay' or Non-Competitive Assay

The sandwich principal was briefly described in section 1.4, but a review and comparison will serve to thoroughly translate the concept. The first step in a non-competitive set up is to functionalize a surface for the anchoring of a capture antibodies. The surface may be within a 96 well micro-titer plate or similarly may be a strip of nitrocellulose, the most common detection area in paper based devices. Once the surface has been functionalized, a particular capture antibody may be anchored through incubation in a microtiter well or by dropping a small volume of

solution onto the fibrous membrane. A sample containing analyte may then be added to develop the bottom of a so called 'Assay Sandwich.' A wash step is then introduced by adding a buffer solution to remove any unbound complexes. Subsequently, a solution containing labeled antibodies is added, these will complete the assay sandwich and furthermore will serve to provide the necessary indicator for binding activity and thus the presence of analyte. After analysis, higher signal intensities will be indicative of a greater presence of detection antibodies and thus a higher concentration of the analyte. The below figure further illustrates this process [19].



- **Step 1**: Sample is applied after capture antibodies have been immobilized
- **Step 2**: Sample fluid is removed or washed to remove unbound proteins
- **Step 3**: Labeled detection antibodies are added
- **Step 4**: Unbound detection antibodies are removed or washed away
- **Step 5**: "Assay Sandwich" is completed and signal analysis may be conducted.

Figure 1-7: Illustration of a 'Sandwich' Assay.

1.7.2 Competitive Assay

The Sandwich or non-competitive assay may not always be a favorable choice due to the limitation that smaller particles (compared to antibodies) may not elicit the same binding affinity. These particles include analytes such as drugs or environmental pollutants; in these cases, a competitive assay may be used.

Antibodies specific to a particular target antigen are anchored under the same methods for a non-competitive assay. The major difference between the schemes resides in the sample fluid. Within a competitive platform, detection antibodies are not used, but rather the sample (which allegedly contains an analyte) is prepared with a labeled version of the analyte in question. When the sample containing defined quantities of labeled-analytes and un-labeled analytes is introduced to a pre-defined set of capture antibodies, they compete for limited binding sites. Another difference in the competitive scheme is that signal intensity is inversely proportional to the concentration of target analyte. When there are fewer analytes present, more labeled analytes will bind to the capture antibodies producing a stronger signal [19]. The following figures illustrate the two scenarios, one with higher concentration of labeled analyte and one with a lower concentration of labeled analyte in relation to the target analyte.

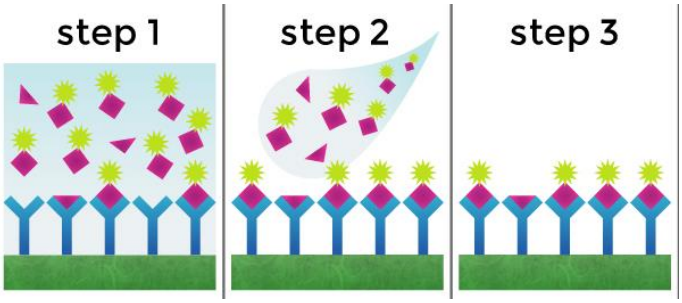
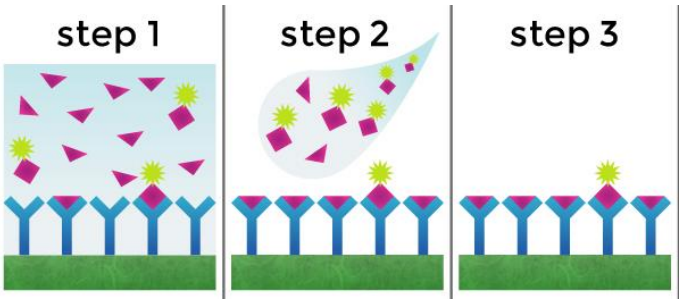


Figure 1-8: Illustration of a competitive assay with higher concentration of labeled analyte.



- **Step 1**: Sample containing labeled and un-labeled analytes is added a platform containing capture antibodies
- **Step 2**: Sample is removed or wash away to remove unbound analytes
- **Step 3**: Signal analysis is conducted.

Figure 1-9: Illustration of a competitive assay with lower concentration of labeled analyte.

1.7.3 Assays and Antibody Use

When designing an assay it is important to consider the clonality of antibodies to choose an appropriate capture protein and for detection. As mentioned previously, monoclonal antibodies may bind to only one epitopes or binding site on a particular analyte. Within the "Assay Sandwich" the capture antibody is generally a variation of a monoclonal antibody, for the reason that it is desirable to have the highest possible specificity, capturing only the target analyte. By using an antibody that can recognize and bind only to epitopes on a specific analyte, the chance to capture erroneous proteins is reduced.

Once the detection antibody has been captured, the desire remains to generate the highest possible signal. Thus, polyclonal antibodies are used as the detection antibody in order to increase the chances for binding. Since polyclonal antibodies can bind to different epitopes on an analyte, thus the chances for binding are increased subsequently increasing the signal intensities that can be generated.

1.7.4 Common Detection Methods

Detection antibodies may have a range of labels providing the visual confirmation of target analyte presence. The label may come in different forms and may produce direct or indirect signals. The most popular labelling schemes are listed in the table below.

Table 1-3: Popular Labeling Methods [20] [21].

Labeling-Detection System	Examples
Radioactive nuclides	^{125}I, ^{32}P, ^{35}S, ^{3}H
Fluorescence	Fluorescein, Rhodamines, Phycobiliproteins, Rare-earth chelates, Ethidium, Quantum dots
Luminescence	Luminol derivatives, Acridinium esters, Dioxetane derivatives, Bacterial or firefly luciferace
Color	Latex beads (blue color), nanometer sized gold particles (red color)
Enzymatic	Alkaline phosphatase (ALP), Horseradish peroxidase (HRP), beta-Galactosidase

An ELISA utilizes an enzyme as a detection method because of the color change that occurs when a substrate is introduced. Enzymatic reaction schemes generally offer a signal with high contrast leading to a favorable signal-to-noise ratio and also have low limits of detection [22].

1.8 Sepsis an Introduction, Definition, and Motivation

The principals above explain the basic components and basic mechanisms that may be applied to point of care devices for to disease detection. In the past ELISA has been applied to HIV and to Malarial detection, but the proposal of this research two-fold, to bring ELISA to a paper based platform and to develop a test for the inflammatory disease sepsis.

1.8.1 Introduction to Sepsis

Sepsis has become a major health problem not only for developing countries, but also within industrialized societies [23]. Additionally, with a mortality rate precedence of 39.6%, sepsis ranks 13th on the list of international classification of disease [24]. Within the US, there are more than 1,000,000 cases each year [25].

Sepsis is characterized by an inflammation in response to a foreign microbial infection. Although it is easy to recognize pathologically, it has long remained difficult to define and to diagnose. American physician, William Osler, once said that patients appear to die as a result of the body's response to an infection rather than from the infection itself [25].

If the early onset of symptoms is left untreated, the body's inflammatory response could progress to a state of shock and eventually could cause organ failure resulting in fatalities. Sepsis can develop from a number of sources including: pneumonia, blood-stream infections, intra-abdominal infections, urological infection and furthermore by surgical infection. The bacterial infection is the most common source, but symptoms can arise from fungi, virus, or even parasites [26]. A septic infection can start in the abdomen and urinary tract, but most often originates in the lungs with a 50% incidence [27].

Symptoms that arise from a septic infection are similar other infection, but may additionally include high fevers, hot/flushed skin, elevated heart rates, hyperventilation, altered mental status, swelling, and low blood pressure [28].

The medical community has struggled to define the illness holistically. In 2001, a system similar to the one that defines cancer progression was proposed. It included 'Predisposition, Infection, Response, and Organ Dysfunction' or PIRO [29]. This system has been updated to reflect the progression of sepsis from early onset to stages of potential mortality. See the below table for a more details.

Table 1-4: Symptomatic Progression of Sepsis [23]

Systemic Inflammatory Response Syndrome (SIRS)	Two or more of the following: • Increase body temperature (38.5°<T<35.0°) • Increased heart rate (90 BPM) • Increase respiratory rate (20 BPM) • Lower white blood cell count
Sepsis	Systemic inflammatory response syndrome and documented infection
Severe Sepsis	Sepsis and one sign of organ hypo-perfusion and organ dysfunction: • Molted skin • Lower urinary output • Change in mental status • Acute lung injury • Cardiac Dysfunction
Septic Shock	Severe Sepsis and one of the following: • Low systemic mean blood pressure • Need for dopamine
Refractory Septic Shock	Need for dopamine or norepinephrine or epinephrine to maintain average blood pressure above 60 mmHg

Symptoms may progress quickly to a state of septic shock which has nearly a 50% rate of mortality. As the incidence of sepsis increases in hospital settings, early recognition and treatment are necessary to effectively combat the onset of life-threatening symptoms. Although there has been much research on sepsis, it remains the most lethal affliction in the modern hospital [23].

1.8.2 Sepsis and the Role of Inter-α Inhibitor Protein

Sepsis has been difficult to diagnose because of the variety of sources that can be responsible for the onset of symptoms. This in turn makes it challenging to isolate and characterize a clear biomarker. Recent research has shown promise for such a biomarker with the protein called Inter-α-inhibitor-protein (IαIP), which has been correlated with many physiological and pathological activities including tumor invasion, metastasis, stabilization of extracellular matrix, inflammation, and wound healing [30] [31] [32]. IαIP falls within the category of plasma-associated serine protease inhibitors which are synthesized in the liver. These proteins can be been found in the synovial fluid within inflamed knee joints for example. Additionally, the levels of IαIP in blood serum have been shown to decrease by 20-90% in patients showing signs of sepsis. The levels of IαIP have proven to be inversely correlated with mortality. Studies have shown that the presence of neonatal sepsis results in a 62% decrease in the plasma levels of IαIP, which an average of 322 mg/L in non-septic patients decreases to a level of 121 mg/L in patients with neo-natal sepsis. [33].

1.8.3 Inter-α Inhibitor Protein and links to Mortality

In the past IαIP was thought only to inhibition of many proteases[1], however a group headed by Dr. Yow-Pin Lim at Brown University has shown that it may be also involved with inflammatory response. His group conducted experiments to measure

[1]**Proteases** are any enzyme that performs proteolysis or the breakdown of proteins into their composite components or amino acids, which are the building blocks for all proteins.

the correlation between IαIP activity and LT-induced cytotoxicity[2] (lethal toxin induced cytotoxicity). Mouse peritoneal macrophages were induced with inflammatory ascites[3]. Results from this experiment are displayed below.

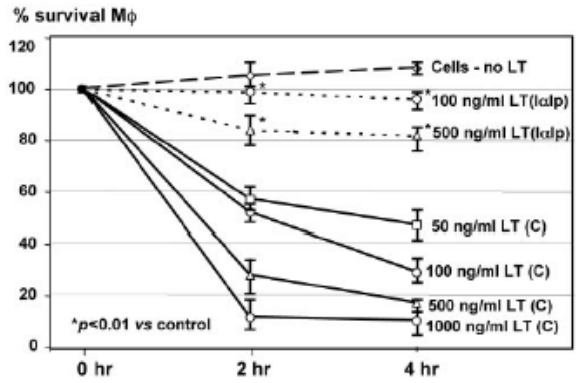


Figure 1-10: Survival rates of peritoneal macrophages with varying levels of LT exposure [34].

The results from this study show groups of cells which have been exposed to varying levels of lethal toxin. The cells show a decreased survival rate with increased levels of lethal toxin. However, when cells are in the presence of additional IαIP, they show increased resilience to the toxin (this is denoted by the two dotted lines in figure 1-11). The group suggests that IαIP inhibits the activity of Furin, which may be involved in the pathway that transports the toxin into a cell's cytosol. In infections where IαIP is consumed or down-regulated, resulting a decrease in the activity of Furin and an acceleration of toxin transport into cells, fatal outcomes could result. Similarly to toxic infection, bacterial infections also result in decreased IαIP levels within a similar physiological framework.

Further studies have shown that adult patients with severe sepsis show

[2] **Cytotoxicity** is a quality of a chemical or compound making it toxic to cells (ie. venom).
[3] **Ascites** is the buildup of fluid in the space between lining of abdomen and abdominal organs.

decreased levels of IαIP and increased mortality rates. This was presented by Dr. Yow-Pin Lim, who is a leading researcher in the field of IαIP.

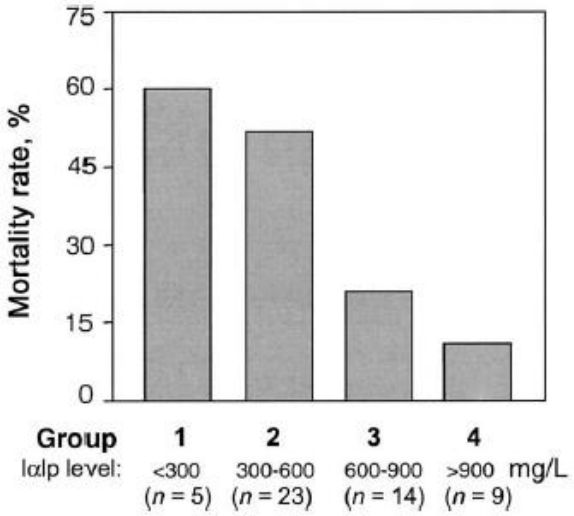


Figure 1-11: Correlation between IαIP levels and mortality rate [35].

The reports from this research show that decreased levels of IαIP are correlated with higher chances for mortality. The data evaluated from 51 patients showed a range of 20-90% decrease in plasma IαIP levels compared to healthy patients. This phenomenon has also been supported by research from Balduyck et al [36]. Although IαIP may only be a precursor in the steps that induce inflammation and furthermore the onset of sepsis, it offers a method for treatment, and furthermore a target around which to develop an ELISA-based platform. More information about the inherent structure and role of IαIP is provided in the appendix.

1.9 Motivation and Objective Outline

The main aims of this thesis work are to develop a functional test that is capable of running a Lateral Flow Assay for the diagnostic application of sepsis. In

collaboration with Dr. Yow-Pin Lim's group a, competitive assay will be used to detect levels of IαIP. Further aims of this research are to create an autonomous device that may run multiple fluids.

For the purposes of providing a framework for this research, Chapter 1 has served to provide basic background knowledge for the technology and mechanisms at play within ELISA, LFA, and biological targets for septic diagnosis.

Chapter 2 elaborates on these basic principles introduced in chapter 1 and will also discuss the current state of paper based platforms through a literature review.

Chapter 3 describes the methodology of LFA fabrication as it applies to the focus of this study. Included here is a description of materials used, fabrication processes, necessary tools, and preparation protocols of competitive ELISA biologicals.

Chapter 4 explains the results and offers implications of particular experiments conducted over the course of this study.

Chapter 5 offers concluding remarks and summarizes the progression of this study along with recommendations for future research.

2 CHAPTER 2 - REVIEW OF LITERATURE

Within this chapter the current state of research on paper-based technologies is reviewed along with their application to microfluidic devices and LFAs. To begin, the components that commonly make-up such devices are reviewed, specifically including research on preparation techniques, design advantages, and advances in fluid flow control. Following this, the applications to biological experiments including ELISA are further discussed with application to the biological target for sepsis.

2.1 Components of LFAs

The components and materials were briefly described in chapter 1 but a more detailed view of these materials will help the understanding Lateral Flow Test Strips (LFTS). Millipore is a major manufacturer of LFTS and they provide a comprehensive manual about the materials and mechanics of such devices. Figure 1-2 was provided in this manual, but its components are commonly used in many other test strips; another example is shown in the below figure.

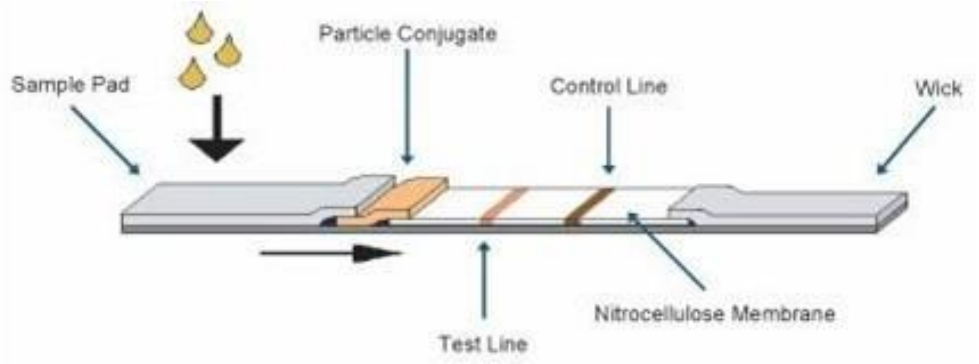


Figure 2-1: A LFTS and its Integrated Components [37]

2.1.1 Nitrocellulose

Again, the major components of a lateral flow test strip include a sample pad,

a conjugate pad, a nitrocellulose membrane, and a wicking area. The most important functional unit of a LFD is the nitrocellulose membrane, as it allows for anchoring of capture proteins and serves as their detection area. The membrane plays an effect in reagent deposition, assay sensitivity, assay specificity, and consistency in test lines. As mentioned nitrocellulose is the most common membrane and it uses electrostatic forces to anchor proteins. This interaction takes place between the strong dipoles of nitrate esters in the membrane and the strong dipoles of the peptide bonds in the proteins. Their chemical structure is provided through graphical images below. [38]

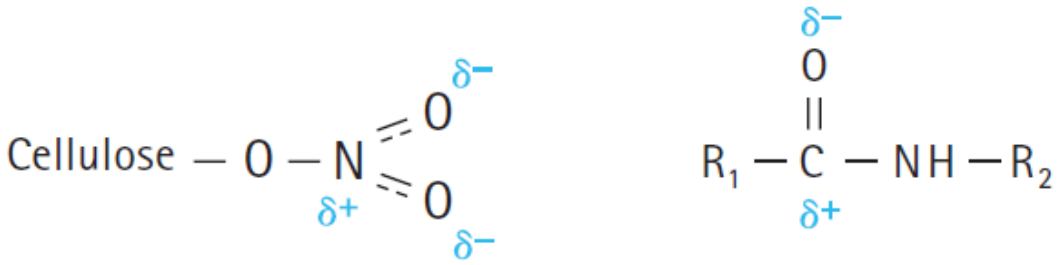


Figure 2-2: Structure of Nitrocellulose Ester and Protein dipoles [38]

Depending on the interactions and molecules within a test strip, other materials may take advantage of alternate binding mechanisms.

Table 2-1: Membrane Binding Properties of Different Polymers [38]

Membrane Polymer	Binding Mechanism
Polyvinylidene Fluoride	Hydrophobic
(Charge-modified) Nylon	(Ionic) Electrostatic
Polyethersulfone	Hydrophobic

2.1.2 Flow Rates in Nitrocellulose

As the membrane will be integrated into a LFTS, it is also important to understanding its flow properties of such a material. The speed at which fluids may flow through a material is referred to as 'capillary flow-rate'. Capillary flow is a

measured in units of cm/sec and is largely dependent on the material properties of the porous structure. Pores are the empty spaces between the fibers of the membrane through which fluids to pass. The pore size, pores size distribution, and porosity may change the flow characteristics. [38]

Pore size is a measurement of the largest pore that may be found along direction of flow. Larger pore sizes will allow for higher flow rates, but smaller pore sizes may offer better filtration options. Membranes are often specified by their pore sizes and are on the μm scale [38]

Pore distribution may be highly variable within the same nitrocellulose. This distribution of pore sizes varies from membrane to membrane and can play an effect in the capillary flow rate. [38]

Porosity is the volume of air that is occupied by the three-dimensional space of the membrane. It is also referred to as the bed volume or the air volume; it is directly proportional to pore size and membrane thickness. [38]

2.1.3 Conjugate pad

Before coming into contact with the nitrocellulose membrane, a sample fluid must interact with proteins which are stored in a conjugate pad. As mentioned, this is an important component for storing the elements that would make up the top half of the assay sandwich. When sample flows to the conjugate pads, theoretically analytes will incubate with the stored detection reagents. Millipore describes the important characteristics for the conjugate pad:

- **Low non-specific binding**- this property describes the ability of the material to release dried reagents. If detection antibodies are not allowed to flow to the nitrocellulose, no signal will be produced.
- **Consistent flow characteristics**- there must be a consistent deposit of reagent onto the membrane to produce a clear signal
- **Consistent bed volume**- this refers to the amount of volume that may be contained in the material. Variable volumes may produce inconsistent signals.
- **Low extractables**- the material should not contain any particles that can interfere with the biology of a test or interfere with flow of the device.
- **Good web handling characteristics**- the materials must be easy to handle in order to be incorporated into the device.

Fiber glass filter paper is a common material used for the conjugate pad. Other common materials used are described below:

Table 2-2: Conjugate Material Properties [38]

Material	Description	Key Characteristics
Glass Fibers	100-500µm thick, sometimes contains binders to hold fibers together	● Large volume, low nonspecific binding ○ Poor tensile properties difficult to handle
Cellulose Filter	300-1000µm thick, consistent density of fibers	● Low nonspecific binding, uniform quality ○ Becomes weak when wetted
Surface-modified polyester	100-300µm thick, hydrophilic polyester filters	● Low nonspecific binding, excellent tensile strength ○ Low and variable hold volumes.

Thickness, basis weight, tensile strength, extractables, dimensions, packaging, labeling and certification should be considered in choosing the correct conjugate pad include as they may play a role in developing the LFA.

2.1.4 Sample Pad and other materials

The materials used for the sample pad are cheapest and easiest to manufacture in the scheme of the LFTS. They can be made from glass fiber, cellulose fiber, or a filter paper.

The most important characteristic of a sample pad is to evenly and consistently distribute a sample fluid to the conjugate pad and subsequently to the detection zone. Sample pads may have other uses as well:

- To increasing sample viscosity
- To increase ability to solubilize detection reagents
- To prevent non-specific binding of reagents to downstream materials
- To modify sample to be more compatible with immune-complex [38]

Additionally, woven meshes have been introduced to LFDs as they can more evenly distribute the sample volume. They also may be more expensive and difficult to process. [38]

Just as with the conjugate pad, thickness, basis weight, tensile strength, extractable and of course dimensional variables can affect the flow and effectiveness of the device [38].

The absorbent area's main function is to create a gradient of force through capillarity to maintian flow of fluid.

2.1.5 Housing

A housing or casing is typically the last component included in a LFTS. It may

often be beneficial to the test, but are certainly not necessary. Housings or cases may offer better preservation capabilities for reagents, and can store fluids until they are needed for a test. The housing can also offer a platform on which to include labels or instructions to aid in the use of the device. Also instructions for interpreting test results may be provided. Lastly, the housing can provide a force to ensure better proper flow of fluids from one membrane to the next.

2.2 Fluid Flow Characteristics within LFD

Fluid flow and timing can be controlled though various mechanisms and design techniques. The fundamental theories that describe fluid flow through a medium along with methods to characterize LFD design and functions are explained below.

2.2.1 Fluid Flow Characteristic Equations

Edward Washburn pioneered the physics of capillary flow and followed in the footsteps of Richard Lucas. They each published papers that explain the physics of capillary flow. From a theoretical standpoint capillary flow can be explained by the Washburn Equation, which assumes a material to have parallel cylindrical tubes. [39]

$$L = \sqrt{\frac{\gamma D t}{4\eta}}$$

- ***L*** = distance traveled by fluid
- ***γ*** = surface tension of the liquid
- ***D*** = average pore diameter
- ***t*** = time
- ***η*** = viscosity of the fluid

Equation 1: Washburn Equation

As water is the most commonly used fluid, its surface tension (***γ***) at room temperature is estimated to 0.0728 N/m and viscosity (***η***) is 1.002×10^{-3} Ns/m^2. This

equation takes into account two assumptions: one, that the fluid traveling through a membrane originates from a non-limiting source, and two, that the material through which the fluid flows is dry.

Fully wetted flow, that means to say flow through a wet material, is described by Darcy's Law.

$$Q = \frac{kA}{\eta L}\Delta P$$

- ***Q*** = volumetric flow rate
- ***k*** = permeability of paper to fluid
- ***A*** = cross Sectional area perpendicular to flow
- ***η*** = viscosity of the fluid
- ***L*** = length of travel
- ***ΔP*** = pressure change along the direction of flow *L*

Equation 2: Darcy's Law [40]

This equation may be simplified by assuming an equal cross-sectional area along the whole length of a fluidic channel.

$$q = \frac{k}{\eta L}\Delta P$$

- ***q*** = flow rate of a fluid
- ***k*** = permeability of paper to fluid
- ***η*** = viscosity of the fluid
- ***L*** = length of travel
- ***ΔP*** = pressure change along the direction of flow *L*

Equation 3: Darcy's Law normalized for equal cross-sectional area [21]

Also by dividing the volume of a fluid by the flow rate, one can estimate the flow rate across a pre-wetted channel.

$$t = \frac{V}{Q} = \frac{\eta L^2}{k\Delta P}$$

- ***t*** = time
- ***V*** = volume of fluid
- ***Q*** = volumetric flow rate
- ***η*** = viscosity of the fluid
- ***L*** = length of travel
- ***k*** = permeability of paper to fluid
- ***ΔP*** = pressure change along the direction of flow *L*

Equation 4: Timing Equation for Wetted Channel [21]

Assumptions can be made for the viscosity of water at room temperature

(1.002×10^{-3} Ns/m^2 [41]), for the change in pressure ΔP which is constant with capillary flow (4,560 N/m^2 [42]), and for the permeability of paper (3×10^{-13} m^2 [42]). This leaves only the variable of the fluid channel geometry to be determined. This set of equations come in aid during the initial set up and design of a fluidic chip.

2.2.2 Controlling the Direction of Fluid Flow

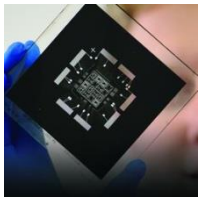
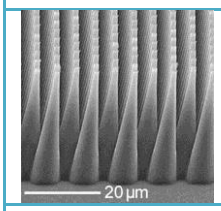
The previous equations merely serve as a point of reference from which to build on and to develop the geometry to run a LFA. Different geometries may be often desirable to adequately control fluid flow within a LFD. There are many techniques which may be employed to fabricate paper-based devices to control fluid flow characteristics.

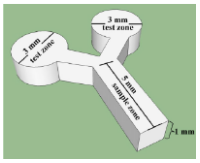
The foremost problem in this field has been to make paper hydrophobic to create the boundary to guide fluid flow. The fabrication methods for creating hydrophobic areas fall within two categories:

- **Selective hydrophobization** – involves treating selective areas with a hydrophobic agent to prevent hydrophilic activity. Of course, areas that have not been treated by these agents may still channel fluids because they remain hydrophilic. This may be achieved through wax printing or ink jet printing.
- **Entire hydrophobization followed by selective dehydrophobization** – rather is a process of hydrophobizing a whole area followed by a selective dehydrophobization of particular areas needed for fluid flow. This may be achieved through photolithography, ink jet etching, or plasma oxidation

Details of various fabrication methods and their relative advantages are provided in the below table.

Table 2-3: Hydrophobic Area Treatment and Fabrication Methods [43; 44]

IMAGE	METHOD	DESCRIPTION	● ADVANTAGES ○ DISADVANTAGES
	**Photolithography**	Using a screen or mask, sensitive material may be selectively broken down by light or chemical exposure.	● High resolution may be achieved (200µm) with clean sharp barriers. ○ Necessary equipment may be expensive, more time is needed for washing, and paper may become more brittle and hard to handle.
	**PDMS**	Using a negative, PDMS plastic may be allowed to cure to a mold that contains a necessary pattern for fluidic control.	● Cheap, easy to fabricate, devices are also flexible. ○ Barriers are not sharp, and cannot be put into high throughput fabrication.
	**Inkjet Printing**	Patterns may be developed on the computer and simply printed on a desired paper platform.	● Cheap and simple to make patterns, good for prototyping ○ Need for a printer or other means to print channels, not suitable for mass production
	**Plasma Etching**	By using plasma, hydrophilic areas may be created..	● Patterning is very cheap ○ Multiple masks and steps are needed to create hydrophobic area
	**Wax Printing**	Using a printer wax may be used in a similar manner to ink in order to create the fluidic channel framework. Wax must be melted through depth of paper.	● May be able to create many devices in short amount of time. ○ Extra heating steps are need, and modified printer needed.
	**Wax Screen Printing**	Using a screen wax may be selectively transferred to paper to develop channels	● Simple fabrication process. ○ Low achieved resolution, and new screens must be made for each new pattern.

	**Wax Dipping**	A metal part may be clapped to paper to block application of wax while paper is dipped into a wax solution.	● Fast fabrication and sharp channel resolution ○ A new metal part must be made for each new channel design.

Although the above are the most popular methods for fabrication cutting of paper to form channels is often used as well. A high level of precision, accuracy, and resolution may be achieved with the application of a laser cutter. Paper may then be combined or layered with other materials to increase stiffness and support for the fragile material. This has proven to be the simplest method for fabrication [45]. Additionally, wax printing has been more widely used since printing equipment is more affordable, easy-to-use, and is favorable for creating variations in fluid channel geometry. After printing wax onto paper and subsequent melting to create hydrophobicity through the entire thickness of the paper, edge resolution may be sacrificed. For prototyping and research, this method offers great advantages in the realm of PBD development as design adjustments for experimentation can be made quickly and easily.

These two methods can be combined to create more complicated patterns to expand capabilities of PBD by construction within a three dimensional framework. This will be discussed in a following section.

2.2.3 Unlimited and Constricted Flow Characteristics

As described by the Washburn and Darcy equations, theory can predict flow characteristics, however, there are many variables (i.e. variation in the fibrous

structure of the paper, temperature and humidity) that may change how fluids flow in paper. Another prominent variable is fluid volume supplied to a channel. For example, the case of a limited or constricted fluid source may be modeled by a thinner channel geometry feeding into a thinner channel. An example has been provided below.

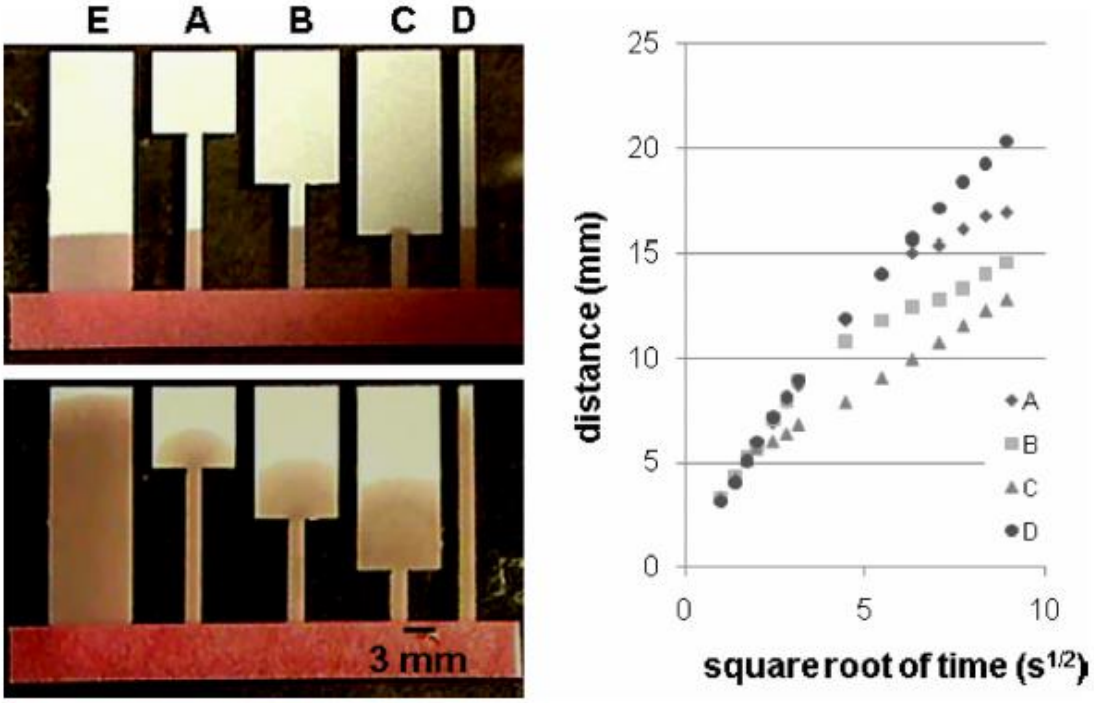


Figure 2-3: Fluidic Channels that Exhibit a Contraction along the Direction of Flow [42]

Channels that exhibit a limited source or an expansion show a slower fluid flow after the expansion, in comparison to a channel that has been designed with a constant width. The limiting source provides some resistance against the flow and prevents it from reaching a further distance.

On the contrary, when channels exhibit a contraction, quite a different phenomenon is observed. In the following figure, channels have been built to exhibit

a contraction along various points along the length of the channel.

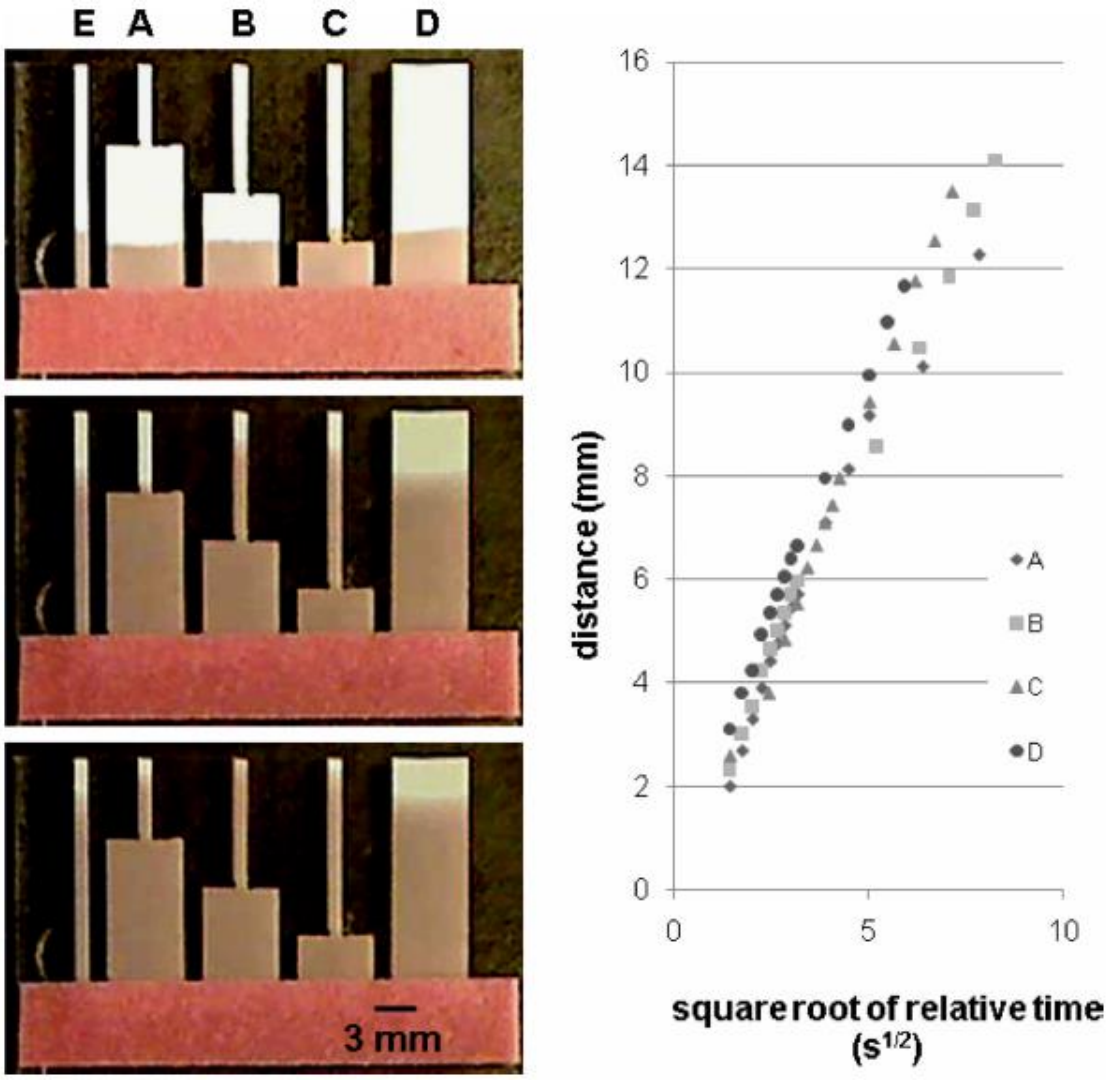


Figure 2-4: Fluidic Channels that Exhibit an Expansion along the Direction of Flow [42]

The contraction in flow seems to have no effect on the distance that the fluid may travel along the channel. By a non-limiting source, the fluid may travel an equal distance or a slightly further distance along the direction of flow. As seen in the data, there is not a significant difference between the variations, and therefore it can be assumed that the contraction does not limit the fluid flow characteristics.

Furthermore, this supports the flow characteristics of the Washburn equation, as the relative slope of flow/time$^{1/2}$ in the thinner channel agrees with the flow in the thicker channel [42].

The resistance created by the case of expansion offers a potential to better control the direction and timing of flow. This for example may be used to create a one-way resistance, can be especially useful in a 3-D fabrication.

2.2.4 Designing Fluidic Circuits by Electrical Analogy

As LFDs on a paper platform have incorporated more complex designs, analogies have been made to electric circuit design. This has been a useful method with which to plan the functions and fluidic timings needed within a microfluidic device.

In the case of the expansion and contracting channels, these have been equated to resistors within electrical diagrams.

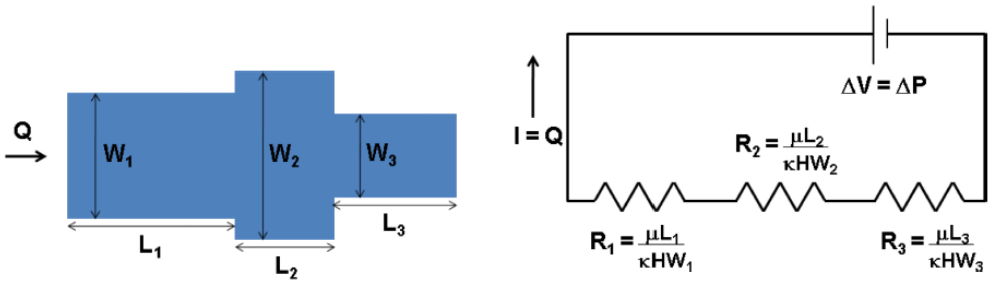


Figure 2-5: Resistance Displayed as Resistors in an Electrical Circuit Diagram [42]

Resistance to flow resulting from changes in channel width can be modeled using the above diagram and furthermore with Darcy's law along with Ohms law. When resistances are taken in parallel, the resistances may be summed by the

following equation:

$$Q = \frac{\Delta P}{\frac{\eta}{k}\sum_{i=1}^{N}\frac{L_i}{W_i H_i}} = \frac{\Delta P}{Req}$$

- ***Q*** = volumetric flow rate
- ***ΔP*** = pressure change along the direction of flow
- ***η*** = viscosity of the fluid
- ***k*** = permeability of paper to fluid
- ***i*** = segment in a sequence of resistors
- ***L*** = length of the segment
- ***WH*** = cross-sectional area perpendicular to flow

Figure 2-6: Darcy's Law Incorporated with Ohms Law [21]

2.3 New Components of Lateral Flow Devices

In addition to the geometric design of a fluidic circuit, advances have additionally be made in the realm physical construction, assembly techniques, and reagent pretreatment to offer more complex design solutions for microfluidic devices. In fact, moving construction of PBDs into a 3D format allows for more complex fluidic control.

2.3.1 3 Dimensional Fabrication of PBD

As PBDs have become a more researched topic, the complexity in construction and use has been developed. One of the first developments transitioned such devices from a 2 dimensional to a 3 dimensional assembly. A group at the University of Texas at Austin [46], published a method that utilized the principals of origami to translate fluids to different areas through a multilayered device. In a 2-D format, fluid flow only travels in one plane and is directed by the patterning of wax walls. In 3-D format, these 2-D patterns are layered upon each other to allow for the capillary flow to continue across multiple layers.

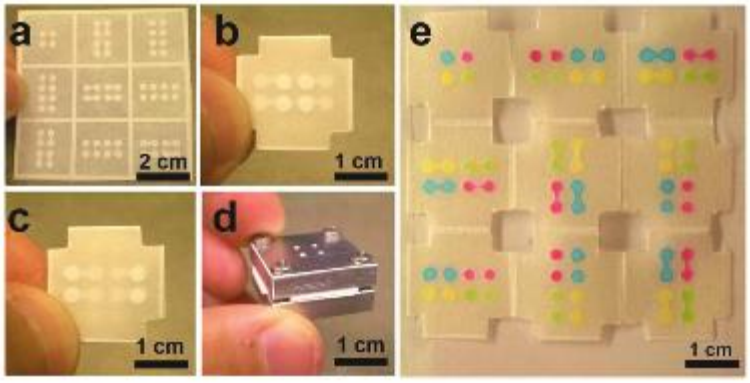


Figure 2-7: 3-D Construction of Paper-Based Device Assembled with Origami Method [46]

In the above figure, the various layers are displayed in a 2D layout in (a), this layout is subsequently folded with (b) showing the inlets of the device. After it has been folded, the device is placed into an aluminum housing, which has four drilled inlets for fluidic input. The largest photo (e) shows the results after an input of four colored fluids and how they have traveled through each layer.

Other research groups have adopted similar methods of fabrication, but have expanded this to suit the various needs of PBDs.

2.3.2 The Mechanical Switch

A group at Harvard University [47] has presented a method by which a PBD may be constructed with multiple layers to generate more complex flow patterns. They present a button that prevents for a continual fluid flow until pressed. This "mechanical switch" or button takes advantage of a disconnection in paper channels to prevents fluid flow. This disconnect may be created by a wax barrier between two channels or also by an air gap. In the case of an air gap, the fluid will not flow until two paper portions have been forced together. This works very nicely in a 3D format

as shown by the image below.

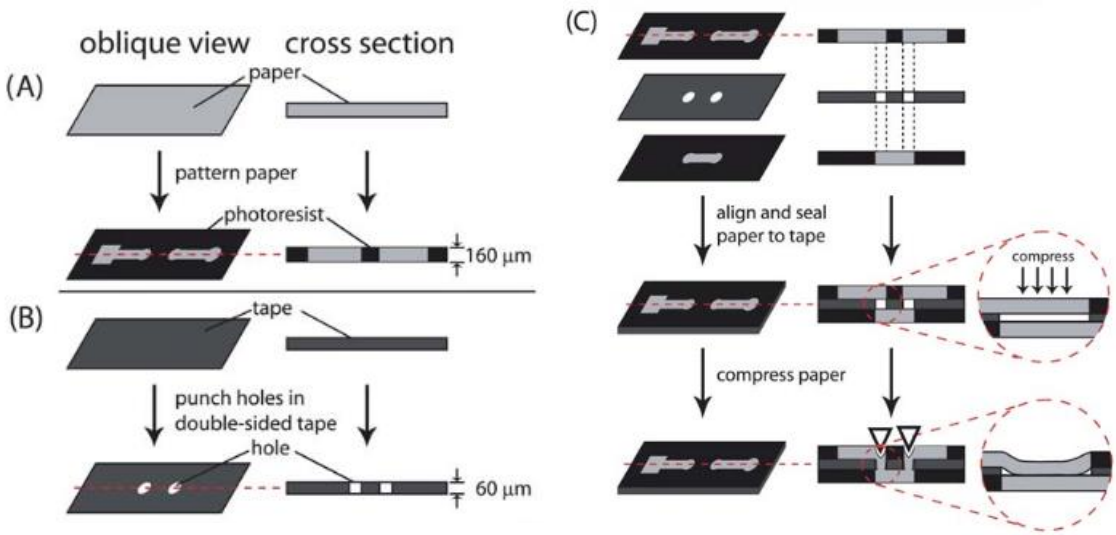


Figure 2-8: Construction and Cross-Sectional View of a PB Mechanical Switch [47]

In the above diagram, an air gap prevents fluid flow until a mechanical force connects the overlapping channels. When the area is pressed, the two channels become connected, thus allowing for fluid flow to continue.

Initial experiments showed that fluid would sometimes bypass the air gap, so two air gaps were incorporated into the design for more reliable function.

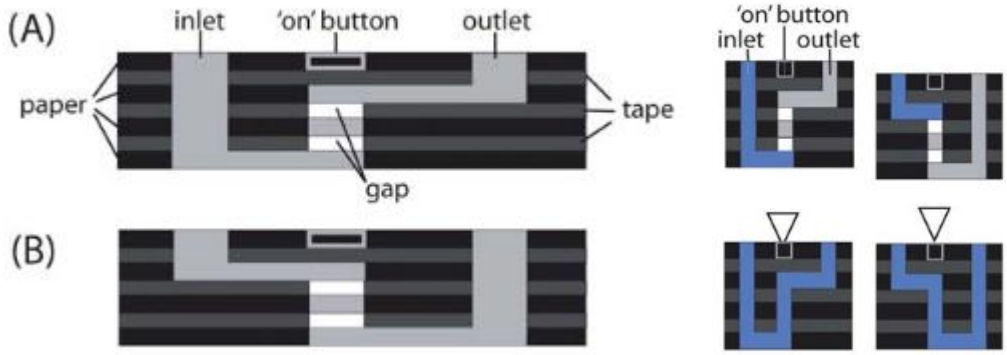


Figure 2-9: Mechanical Switch with Two Air Gaps [47].

Two variations of the mechanical switch are displayed along with the corresponding fluid flow. Flow may only continue into subsequent channels when the

mechanical switch has been pressed.

An important component in the construction is the incorporation of a double-sided tape which is needed to adhere the two layers of patterned-wax. This layer of double-sided tape has gaps cut out in order to provide an area through which fluid may travel to the subordinating layer. Another proposed method to adhere each layer is the use of spray adhesive. Other devices take advantage of hydrophilic solutions or materials in order to prevent fluid flow through a particular area.

2.3.3 A One-Way Fluidic Valve

A novel technology for PBDs was developed by Hong Chen at the University of Rhode Island. His research serves as the basis for what is called a “one-way valve” [48]. This valve was developed on a paper platform which has been treated with hydrophobizing agent. Under most circumstances this treatment will not break down until a surfactant agent is introduced to the area. When a solution comes to a hydrophobic rendered barrier, it will not be able to pass that barrier. On the other hand, if the solution has the surfactant dissolved within, it may break down the hydrophobicity and continue to flow further along the microfluidic chip. The figure below helps to illustrate this.

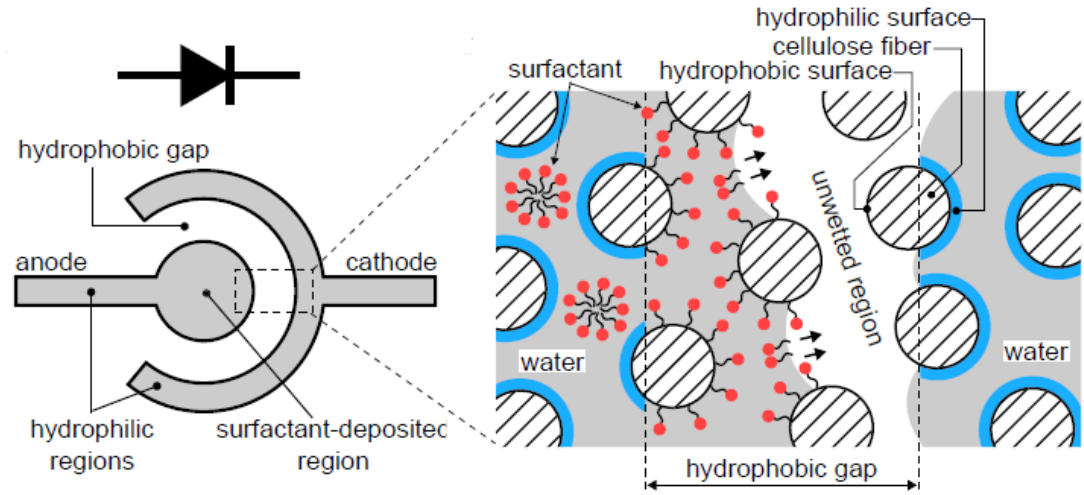


Figure 2-10: Mechanism of a Microfluidic Valve or Diode [48]

This concept may be incorporated into a two way circuit and in this layout follows diode notation borrowed from electrical engineering.

In the diagram above, paper is soaked with a compound called Allyltrichlorosilane (A3CS), rendering this area hydrophobic. The diagram above has this hydrophobic area between an anode and a cathode area, along with a magnification of the chemical principals of the hydrophobic treatment. The circles in the diagram to the right represent the fibers of the paper channel. Some fibers have a hydrophobic coating (blue outline), while others are represented without one. Those are the hydrophobic fibers which are in need of a surfactant (red) to bind to the hydrophobic fibers and therefore providing the necessary hydrophilic path for a fluid to continue along the intended direction. The surfactant is a solution of Tween 20, which has been previously dried onto anode area of the valve.

Using the principals presented here, one can design fluid reservoirs which may only be triggered when another fluid comes from the anode side of this

component.

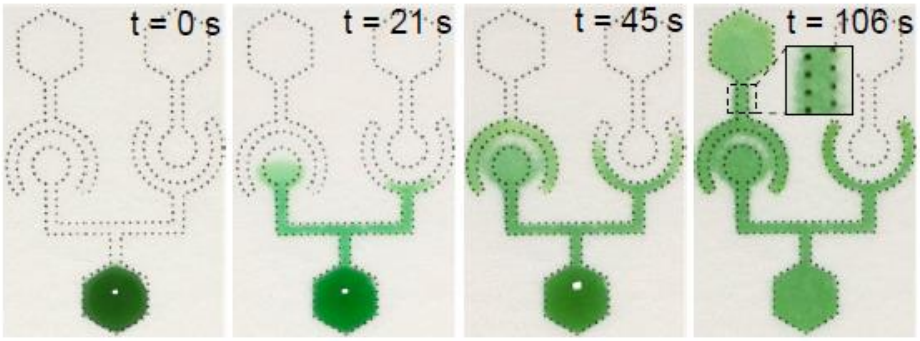


Figure 2-11: Real Time Depiction of the microfluidic valve [48]

The image above shows an experiment using colored water to illustrate the principals of this fluidic valve. When the green fluid reaches the anode on the left, it dissolves the surfactant which then bridges the hydrophobic gap to the cathode side. On the right hand side, the fluid is not allowed to pass any further because of the lack of surfactant on the cathode side.

This component was further developed into a 3D platform for incorporation into diagnostic tests. For demonstration purposes the figure below again only displays functions with the use of colored water.

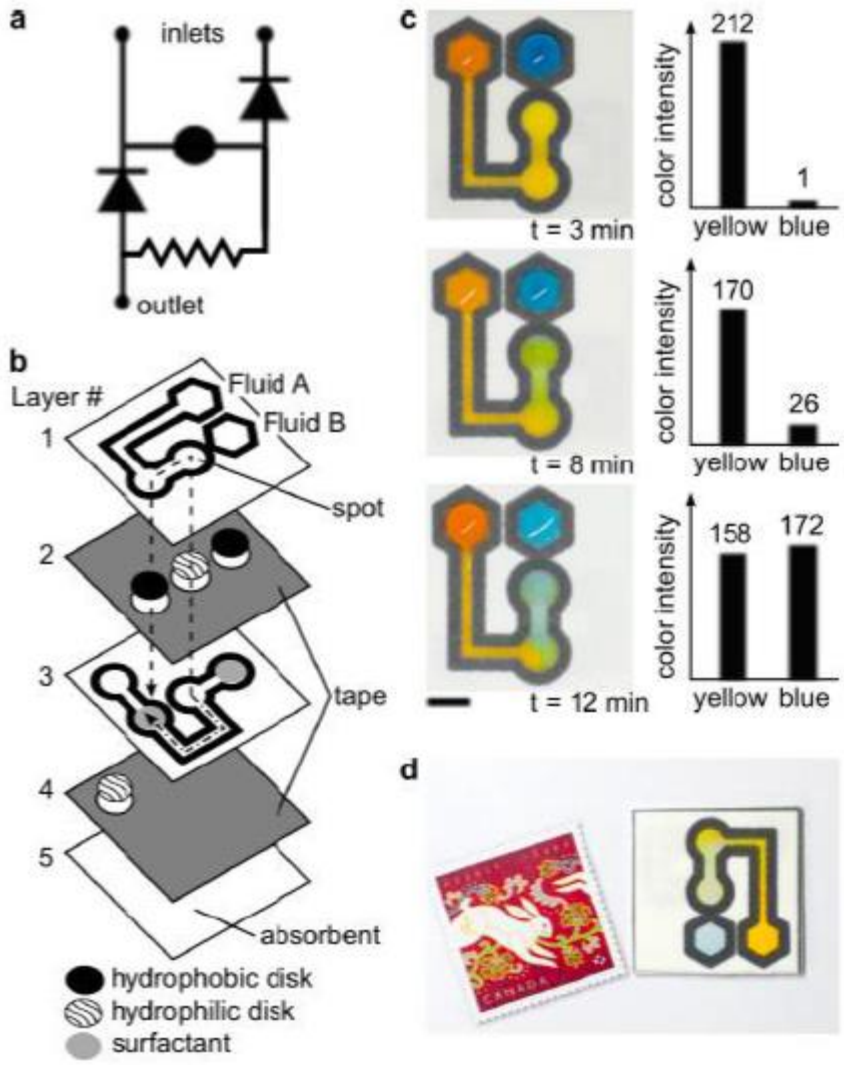


Figure 2-12: Microfluidic Valve Displayed in a 3D Application [48].

Again, the symbols from electrical engineering are used to describe the fluid flow of the circuit; they may be found in the top left corner of the diagram. In the bottom left portion one can see the typical assembly process along with the components of the two valves which have been incorporated into this particular circuit. The circuit was assembled with three layers of paper and two layers of doubled sided tape which included inserts for the valve components. In this case, the valve components are disks of paper sized to fit perfectly within the areas of the

double sided tape. The disk is treated with A3CS, and surfactant is incorporated into the second layer of the chip. Photos have been provided from an experiment using colored water. One valve is used to prevent Fluid B (blue fluid) from entering the circuit until Fluid A travels along the full distance of the circuit until it reaches the areas labeled as “spot”. Here it travels to the second layer and over to the area underneath the reservoir of Fluid B. Here the surfactant is dissolved and the hydrophobic area is broken down allowing the blue fluid to enter the circuit. A simplified valve assembly is shown below.

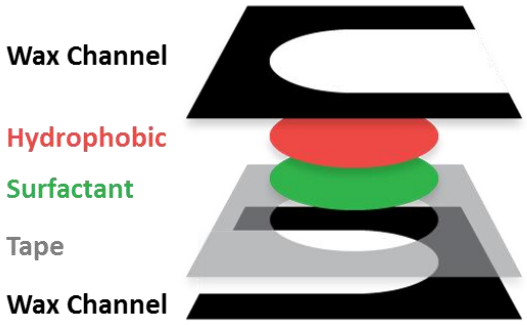


Figure 2-13: A Basic Illustration of the Structure of a 3-D Fluidic Valve

Although paper disks were used in this layout, there are many other mediums which may be used to incorporate the valve into a device. For example, cellulose powder can be used as a paste to be spread into the cutouts of the double sided tape.

Aside from the steps to hydrophobize paper, other solutions may be needed to assure a proper outcome when biological components are incorporated into a paper based device.

2.3.4 Fabrication with Adhesive Spray

Another method for fabrication has been presented by a research group at Pennsylvania State University, who used a spray adhesive to assemble the multiple layers of each 3-D chip. By spraying the individual layers with 3M's Super 77™ Multipurpose Adhesive spray a higher throughput for the assembly was achieved [49]. The spray adhesive can replace the function of the double-sided tape which requires more time for assembly, needs to be precisely aligned, and requires a filling (paper or paste) to connect the functional paper layers of the device. This method for fabrication shows promise to save time and resources, especially in the prototyping and testing stages where many chips need to be fabricated and tested while certain parameters are optimized. The group reports that for every two sheets of paper that need to be adhered only one needs to be sprayed to leave 0.9mg of spray per cm^2. To achieve this paper was sprayed for approximately 1 second at a distance of 24cm [49]. With this method, a 50% reduction in the time for fluid to travel through multiple layers was achieved in comparison to using double-sided tape and paste [49].

In a fabrication that included double-sided tape, there are many inconsistencies with alignment and more so with the connection between individual layers of the device. By switching to a spray adhesive many of these inconsistencies could be eliminated resulting in more consistent and predictable functionality. This would be especially significant when incorporating other components into the chip,

such as one-way valves and biological assays.

2.4 Adding Biology to a PBD

As biology is integrated into a paper based platform, there are a few key preparation steps that need to be considered in order to produce a chip that results with the best possible result. In the following section methods to improve this resulting signal are discussed.

2.4.1 Surface Treatment

When comparing traditional ELISA run in a 96 well plate to ELISA on a paper based platform clear differences and advantages are presented. As mentioned previously, tests generally completed on paper need far less time and smaller fluid volumes for the necessary reagents. Furthermore, color enhancers or substrates may be used to create a signal that can be interpreted by the naked eye. This in turn cuts out the need for analysis equipment as needed by a traditional ELISA test. One of the major drawbacks of running such tests in paper is the lower Limit of Detection (LOD). In fact, the LOD for ELISA run in a 96-well plate is about three times lower [42]. Research groups have developed a few methods with which to increase the value for experiments run on paper.

In the same way that 96 well plates can be treated with a compound to promote binding of proteins or enzymes to the surface, a compound that blocks further binding to that surface may be used as well. This step is referred to as a blocking step, it usually follows the anchoring of capture antibodies. Bovine Serum

Albumin (BSA) is a popular method for blocking areas in the 96 well plates which have not been occupied by capture proteins. BSA may also be used on paper, specifically on the nitrocellulose detection area in order to maximize the detection signal. Other methods include blocking of paper with amino (NH3) and carboxyl (COOH) altered silicon dioxide (SiO_2), have shown to increase the signal intensity on paper by a factor of 7 for specific concentrations of detection protein [50].

Washing steps are another method that improve signal by removing unbound proteins. This is commonly used for ELISA run in 96 well plates to remove the free labeled-proteins. On a paper platform, it is not possible to perform multiple washing steps because fluid cannot be removed from the testing setup. It is however possible to incorporate one wash step to clean up background noise on such a device so that more contrasting signal may result. A group at the University of Washington incorporated a wash of TRIS-buffered saline with Tween 20 in order to remove any unbound proteins [51]. Once a signal is obtained signal amplifiers can be used to make detection more readable for devices and for the naked eye.

2.4.2 Signal Amplification

The most preferred method for detection within a paper-based device is through visual confirmation; therefore a signal must be visible to the naked eye and must not rely on the computing power of a reader. The same group from Washington University also described how detection may be enhanced through the use of gold nanoparticles [52]. The below figure depicts the methodology to enhance the signal

produced by the formation of an LFA Sandwich.

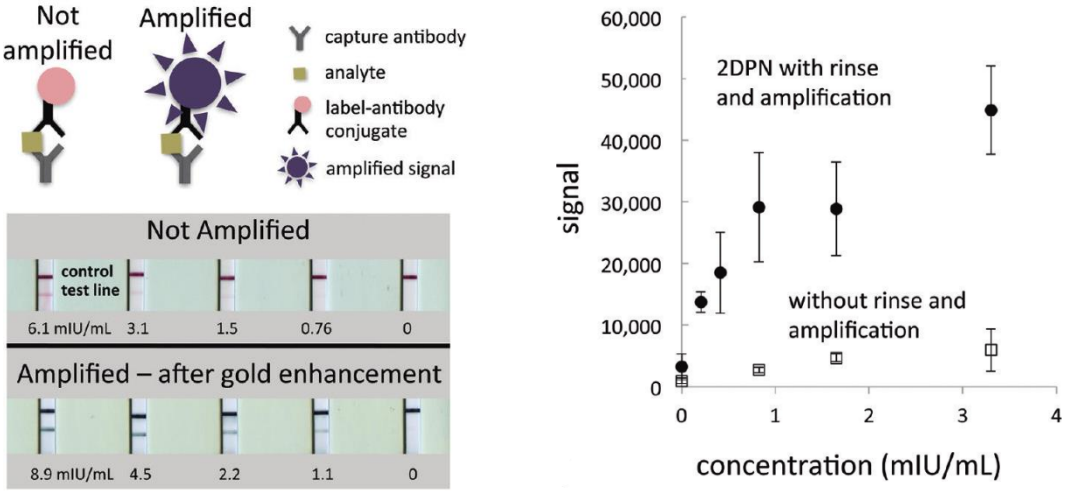


Figure 2-14: An illustration of how gold nanoparticles function to enhance signal and a plot of the signals that were measured using gold nanoparticle enhancement. [52]

By adding the gold nanoparticle to the label-antibody conjugate, a stronger more intense signal is generated. Below the illustration of this process, there are 5 test lines that represent various concentrations of the analyte. In this case, the gold nanoparticle increases the size of the detection particle and changes the color of detection from pink to purple. The limit of detection is shown to go below the range of 1 mIU/mL (million international units per milliliter). A following experiment showed, furthermore, how the signal intensity could be increased using the amplification particles along with a wash step. The results from this experiment at displayed in the chart in the right side of Figure 2-14. This signal was increased by 4-fold when gold nanoparticles were used for detection.

2.5 Combining Technologies and Building off Previous Work

As research is conducted in the development of paper-based devices for the application to diagnostics, the techniques and technologies described above may be

combined to serve in the ultimate goal to develop such a device. In the combination and development of this device, many considerations need to be understood and optimized independently in order to develop a fully functional and predictable device.

The development of a paper-based device with the capabilities for ELISA was pioneered by Roman Gerbers' and Wilke Föllscher who collaborated a Master's work. They made great strides to combine different materials and techniques fabrication to construct a device that sequentially flows biological fluids to model an ELISA. Their focus was on using a device to detect IgG using the substrate Alkaline Phosphatase. This provided a framework from which to build upon as research was conducted to develop a device for the detection of IαIP. Many of the figures seen throughout this thesis have been referenced to their work. Although, much of their work is reviewed and build upon in the Chapter 3, it can further be reviewed in their recently published article in Lab on a Chip, 'A New Paper-Based Platform Technology for Point-of-Care Diagnostics [53].'

3 CHAPTER 3 – METHODOLOGY

This chapter provides the methodology for LFA fabrication including a description of materials used, fabrication processes, necessary tools, and preparation of biologicals needed for a Competitive ELISA. Steps for optimizing and general protocols are also discussed.

3.1 Chip Fabrication Introduction

Designing a PBD for the application of an Assay requires 2 or more fluids. When designing and fabricating a multi-fluidic device (one with more than 2 fluids) appropriate timing and controlled release of these fluids is key. All the PBDs designed under Professor Faghri at the University of Rhode Island utilize a filter paper printed with wax. This is done in accordance to the work by Hong Cheng, Wilke Föllscher and Roman Gerbers [53].

The design presented in the following pages was developed in conjunction with Hanno Teiwes [54] that takes design queues from the previous work by Gerbers and Föllscher. The largest influence on this design was the incorporation of Föllscher's top layer as it allowed for a greater fluid storage within the secondary fluid. Secondly, a timing channel (second wax layer) was incorporated from Gerbers' design because of the greater control it offered in the timed release of fluids. However, instead of using surfactant paste and hydrophobic discs, a complete layer was dedicated for hydrophobic treatment, as it offered more control and a faster time for assembly. Below is an image of a previous chip design to serve as reference;

this image will be again called upon to describe ideal fluid flow sequence.

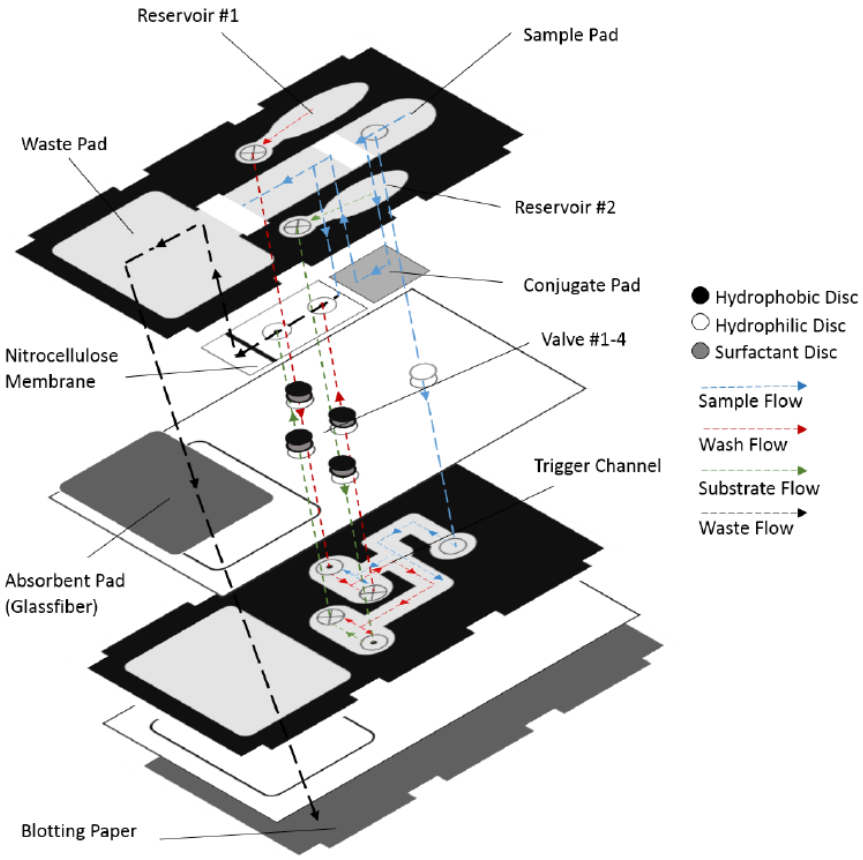


Figure 3-1: Chip Assembly and Fluid Flow Characteristics by Roman Gerbers and Wilke Föllscher [21] [55] [53]

3.2 Chip Fabrication

Materials and software needed to fabricate the above device include:

- Corel Draw® Software (Solidworks or CAD will also work)
- Xerox® ColorQube® 8570 solid ink printer and Xerox® Genuine Solid Ink Black
- Epilog® Mini 24 CO_2 laser cutter
- Isotemp® Model 280A vacuum oven
- Whatman® Grade 41 (9572190) filter paper (8x10in)
- Whatman® Grade GB003 Blotting paper (20x20cm by .8mm thick)
- Sterlitech® GA-55 Absorbent paper
- Ace Hardware® double-sided Carpet tape (No. 50106)

- Reynolds® parchment paper
- Whatman® 12µm Nitrocellulose 47mm Diameter (G3514143)
- Acros Organics® Perfluro-compound (FC-72)
- Acros Organics® Allyltrichlorosilane
- Sigma Aldrich® Tween 20
- Fisher Scientific® Cellulose Powder

3.2.1 Paper Materials

Wax channels can be printed on filter paper to create a hydrophobic barrier that can be used to guide fluid flow. Within the negative spaces, fluids are directed and additional functionalities may be added for greater complexity in the device. Unless otherwise stated, all chips are designed using the Corel Draw® software (Corel®), a vector based software not dissimilar from AutoCAD® or from Adobe's Illustrator®. Using this software, functional units of the PBD may be designed and printed. All designs are printed onto Whatman's® Grade 41 (9572190) filter paper which measures 8x10in. (203x254mm). To print the devices, the ColorQube® 8570 solid ink printer from Xerox® is used along with Xerox® Genuine Solid Ink Black.

After a design is printed it must be cut using a CO_2 laser cutter (Epilog® Mini 24) to remove all the excess paper and to cut out the individual components or layers of the device. On an 8x10 piece of paper, it is possible to print many layers for incorporation into the final device, but depending on the dimensions of the design multiple sheets may be needed. Typically, 6 layers are printed on one sheet of 8x10 in Filter paper. In order to maintain precision when assembling the chips, the laser cutter is used to cut out the individual layers.

3.2.2 Wax Paper Melting Process

Since wax is only printed on one side of the paper it must be heated to melt the wax through to the other side of the sheet. This is done by placing the components wax-side-up in a vacuum oven for approximately 25 seconds at a temperature of 130°C-140°C. In all constructions the Isotemp® Model 280A vacuum oven from Fisher Scientific is used. This process allows the wax to carry through to the opposite side of the filter paper. See the below images for the melting process.

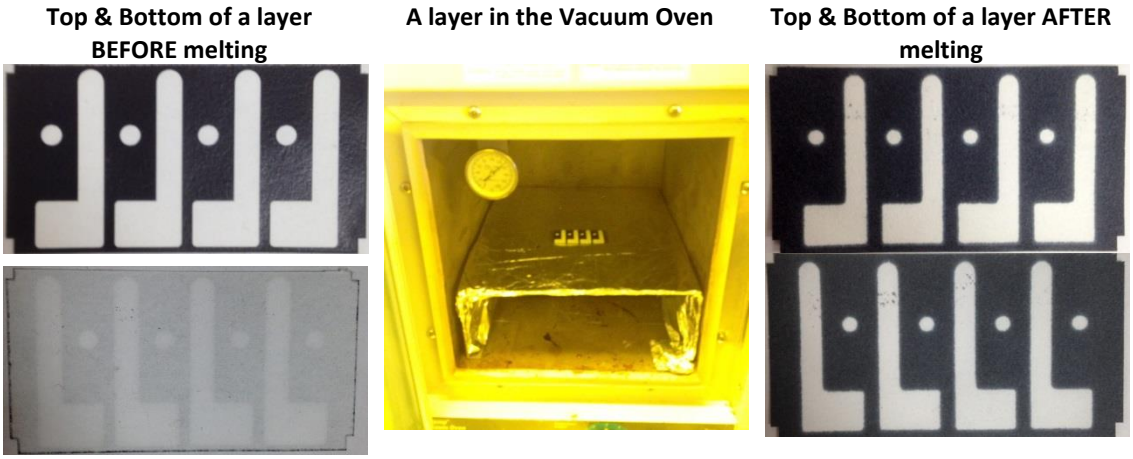


Figure 3-2: Process for Melting a Wax-Printed Layer

When, designing the chips it is important to note that wax will travel about 0.5mm toward the negative spaces. For example, if a 4mm circle is printed it will shrink down to a 3mm circle after melting. Also the longer a layer sits in the oven the further the wax will travel into the negative space. See below images for an example.

Circular Feature before Melting

Circular Feature after Melting

Figure 3-3: Effects of Melting on Wax-Paper Features

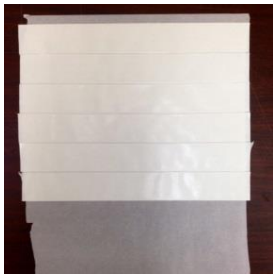
Also, it is important to note that the melting times are dependent on the size of the features, temperature of the oven, and placement within the oven. These conditions were the optimal standard, but there was some variation.

In addition to the filter paper, a blotting paper (Whatman Grade GB003 20x20cm by 0.8mm thick) and absorbent paper (Sterlitech® GA-55) are included to maintain connection between layers and create more consistent flow to waste areas.

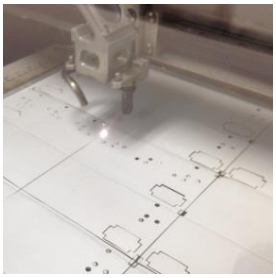
3.2.3 Double Sided Tape

In order to adhere each layer of the device, double sided tape is used as previously mentioned. According to previous protocols, Ace Hardware's® double-sided Carpet tape (No. 50106) is the best option as it is inexpensive ($3.99 for a 1.5inx42ft roll). Double sided tape is by applying segments to Reynolds® parchment paper in order to cut and to handle more easily during chip assembly.

Placing double sided tape onto the Reynolds parchment paper



Double sided tape and parchment paper. Two rows are double-sided tape are needed per layer.



Cutting of double sided tape in laser cutter.

Removing of unnessesary parts from double the cutouts.

Figure 3-4: Process for Preparing Double-Sided Tape

After the double sided tape has been cut out and cleaned up, it may be used to assemble the device.

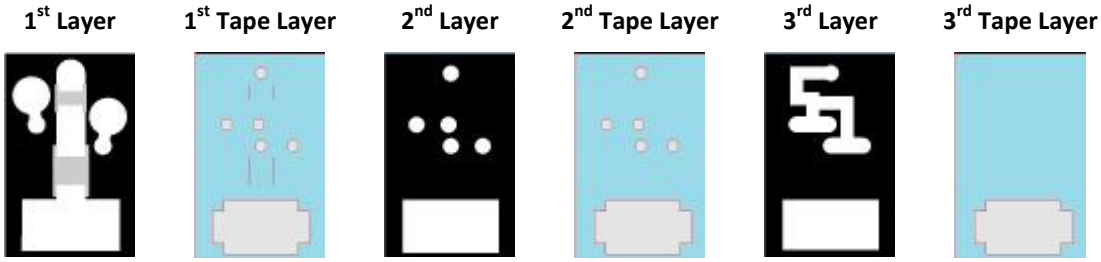


Figure 3-5: Tape and Wax Layers of Gerbers' Modified Chip

It generally follows that for every layer of filter paper one layer of double sided tape is needed. In the modified designs of Roman Gerbers by Hanno Teiwes, 3 layers of wax-printed paper and 3 layers of double-sided tape were used. The images above show the details of each layer (dimensions may be found in the appendix). In the 1st Layer, there are cut outs along the main channel for a conjugate pad and the nitrocellulose pad are placed. Also, there are lines on the 1st tape layer which indicate where to place the conjugate and nitrocellulose pads in order to ensure precise alignment to the top layer during assembly. On the bottom of each tape layer there are cut outs for absorbent pads. The absorbent pads bridge each layer to provide a

path for the waste fluid to reach the absorptions layer. The small square along the corners of each of these rectangles also help to properly align the absorbent squares. The 1st and 2nd Tape layers also have circular cut outs in which to fill hydrophilic material; again this can be in the form of a cellulose based paste or filter paper cut outs.

3.2.4 Laser cutter settings

As mentioned earlier Corel Draw is used for all design and cutting with the CO_2 laser cutter. Depending on the material being cut, different power and speed settings must be used. Specifications for each type of material are listed below.

Table 3-1: CO_2 Laser Cutter Settings for Various Materials

Material	Vector Speed	Vector Power	Frequency
Backing Paper (Blotting Paper)	40 %	60 %	5000
Double-sided tape	50 %	35 %	5000
Filter paper	40 %	15 %	5000
Glass fiber (absorption area)	50 %	10 %	5000
Glass fiber (conjugate pads)	50 %	10 %	3025
Nitrocellulose (cut 25x)	50 %	2 %	4250
Plastic PP- Layer 1.5mm	10 %	30 %	5000

Another important tool to aid in the cutting process is an alignment guide. When printing designs, they may not sit in perfect alignment to the edges of the paper. For example, when designs are printed the edges may not sit parallel to the edges of the paper. So in effect, the laser cutter is aligned to the design rather than to the edge of the paper on which the design is printed

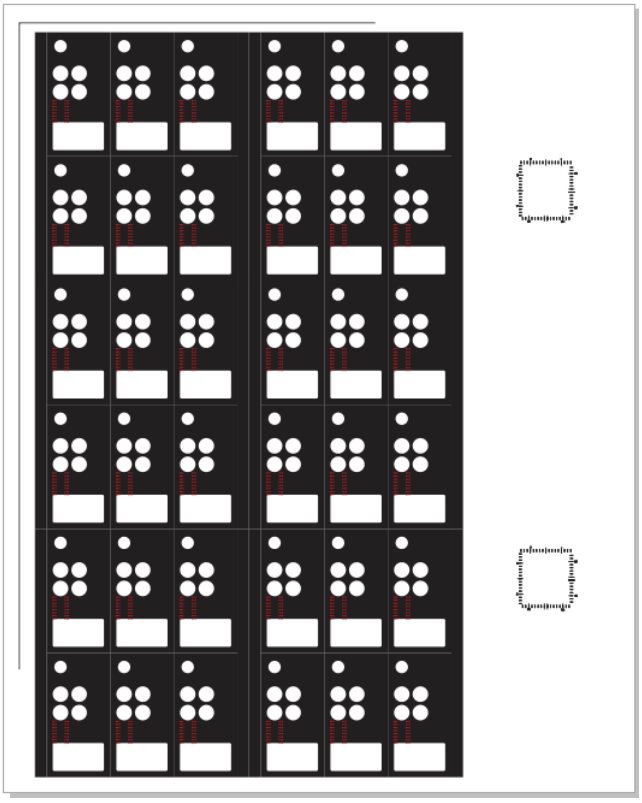


Figure 3-6: Cutting Guides

Alignment lines and alignment guides are printed in every design to increase precision during the cutting process. Alignment lines are printed and sit along the edges of the rulers within the laser cutter. Once the wax sheet is properly placed, preliminary cuts along the alignment guides are made.

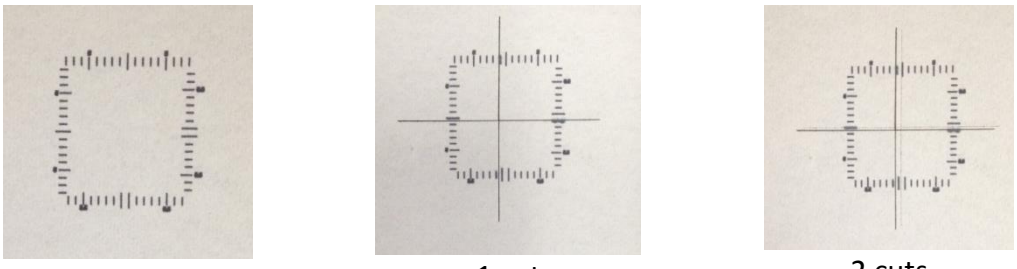


Figure 3-7: Alignment Guide for Cutting

In the second image above, a preliminary cut is made. From the first cut, the whole

cute must move 1mm in the x direction and 0.5mm in the y direction. The top and left dashes in the alignment guide start at 0 then go to 1mm, 2mm, 3mm, 5mm; while the right and bottom dashes are marked starting at 0.5mm and go to 1.5mm, 2.5mm, 3.5mm, 4.5mm, 5.5mm.

3.3 Chip Assembly

Once all the materials needed have been prepared, chip assembly can begin. The image below shows all the components needed for chip assembly.

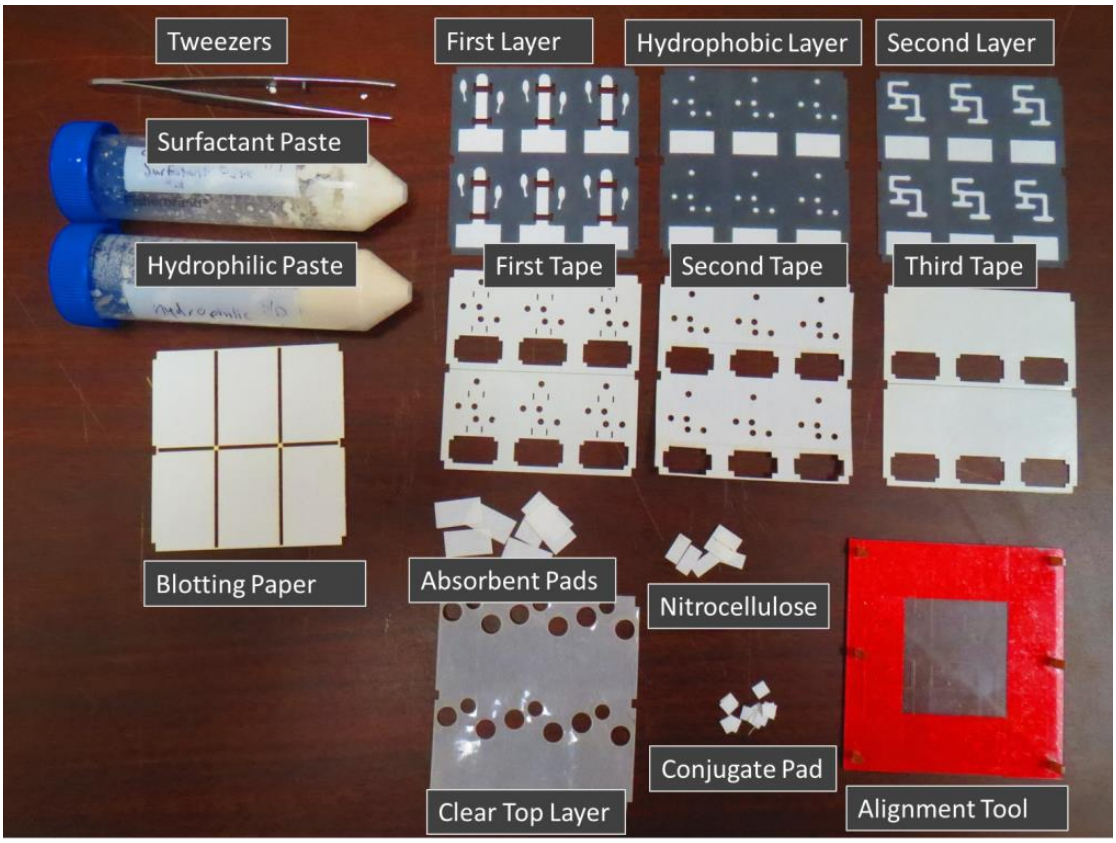


Figure 3-8: Materials Needed to Fabricate One Batch of 6 Chips [54]

The above materials include:

- 3 layers of wax-printed filter paper (to direct fluid flow)
- 3 layers of double-sided tape (to adhere wax layers)

- 1 layer of blotting paper (to provide a waste absorbent area)
- 6 Nitrocellulose Pads
- 6 Conjugates Pads
- Absorbent Pads
- 1 layer of clear packing tape (to prevent evaporation of fluid)
- An alignment tool (to ensure precise alignment of layers)

Using this method, 6 chips could be simultaneously fabricated within one batch. Within the above image, a red alignment tool is shown. This alignment tool is designed to fit into the cutouts along the sides of every chip. It serves as both a platform for assembly and also to ensure that each layer aligns perfectly with the layers above and below (dimensions are provided in the appendix). On the following page all the components of this previous fabrication is shown are illustrated.

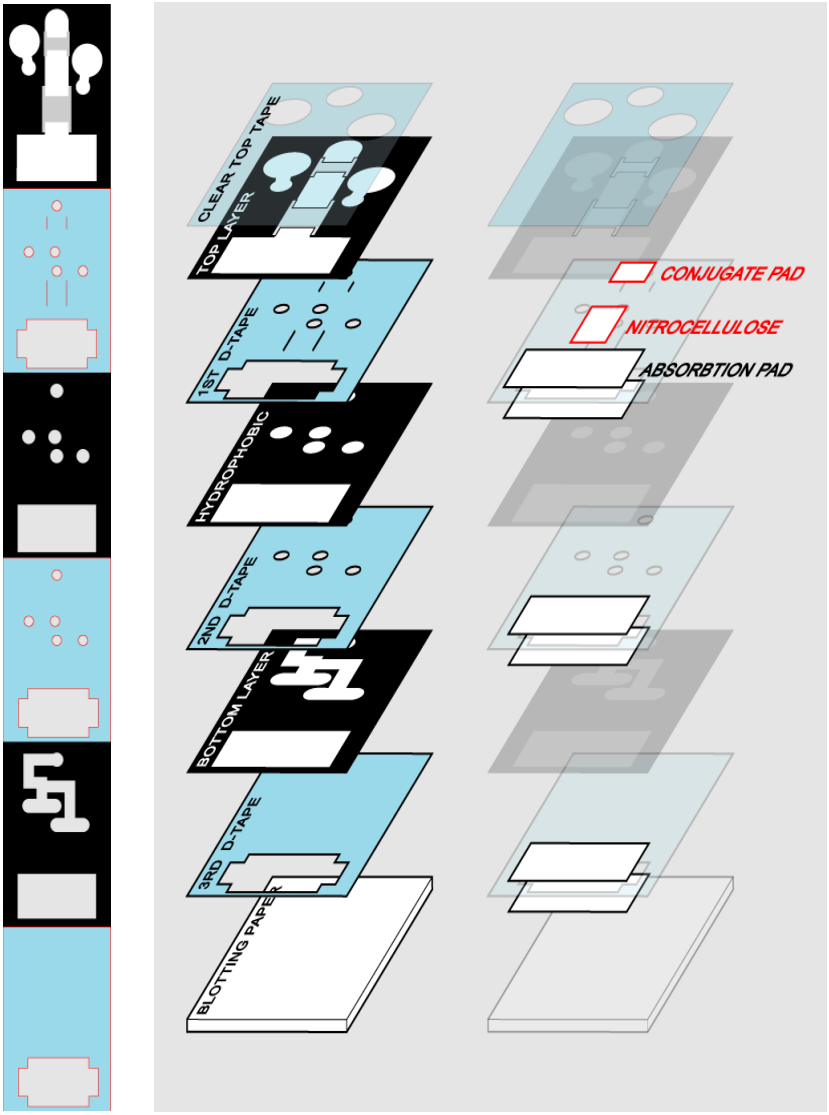


Figure 3-9: Illustration of assembly of the modified chip.

3.3.1 Hydrophobic treatment & Paste Application

Within this assembly the most important functional unit is the hydrophobic layer, which holds fluids in reserve until the hydrophobic areas are bridged by surfactant (this chemical process is described in 2.3.3). This particular design incorporates a whole layer of wax-printed paper treated with hydrophobic solution. Therefore, the surfactant needed to breakdown the hydrophobic barriers must be

placed in the layer below. This is done in the form of a cellulose paste which is mixed with a solution of surfactant.

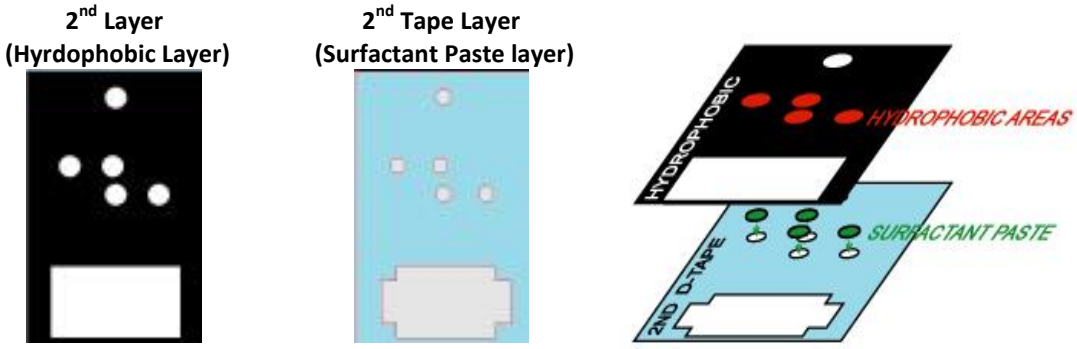


Figure 3-10: Hydrophobic and Surfactant Paste Layers

Each of the four hydrophobic areas (measuring 3mm) is treated according to the procedures laid out by Föllscher and Gerbers [21] [55]. Hydrophobic solution is prepared by mixing 50µL of Allyltrichlorosilane with 950µL of Perfluro-compound (FC-72) which is a 5% vol. solution. 2µL of this solution is pipetted onto each spot and allowed to dry for about 1 minute. This is done 4 times for a total of 8µL of hydrophobic solution. If the solution is added all at once it spills out into the surrounding wax area. The treated paper should be allowed to sit for a minimum of 12 hours within a petri-dish in order to fully develop hydrophobicity. Excess solution can be stored in a fridge for later reuse.

Surfactant paste is spread into the negative spaces of the 2nd tape layer. To create surfactant paste a surfactant solution is first prepared by adding a 35 wt.% of Tween 20 in Ethanol. This solution is next diluted with ultra-pure water at the ratio of 0.25mL/mL. Once that is completed, the water-tween 20-Ethonal solution can be added to cellulose powder at the ration of 0.5g/mL (cellulose/solution). This mixture

is next homogenized using a spinner and can then be applied to the cutouts in the 2nd Tape following its adherence to the 3rd wax layer.

Hydrophilic paste is a mixture of the same ratio of cellulose to ultrapure water (0.5g/mL). The pastes may then be applied with a metal spatula or with a popsicle stick wrapped in paraffin film. The paraffin film helps to prevent absorption of surfactant solution into the wood. The paste then needs approximately 10 minutes to dry. It should also be noted that surfactant past should not be applied directly to the hydrophobic layer as it will immediately render the hydrophobic areas ineffective. Rather, it can be applied to the 3rd layer and allowed to dry.

Another option is to use hydrophobic, hydrophilic, and surfactant treated discs of filter paper which can be applied directly into the cut outs of the tape layers. Each can be cut to the exact size of the cut outs in the tape layers.

As previously stated, the chips are assembled in batches of six. This is done to maximize the efficiency during assembly and furthermore, to have tests for the optimization of surfactant and ultimately of biological reagents.

3.4 Sequential of fluid flow

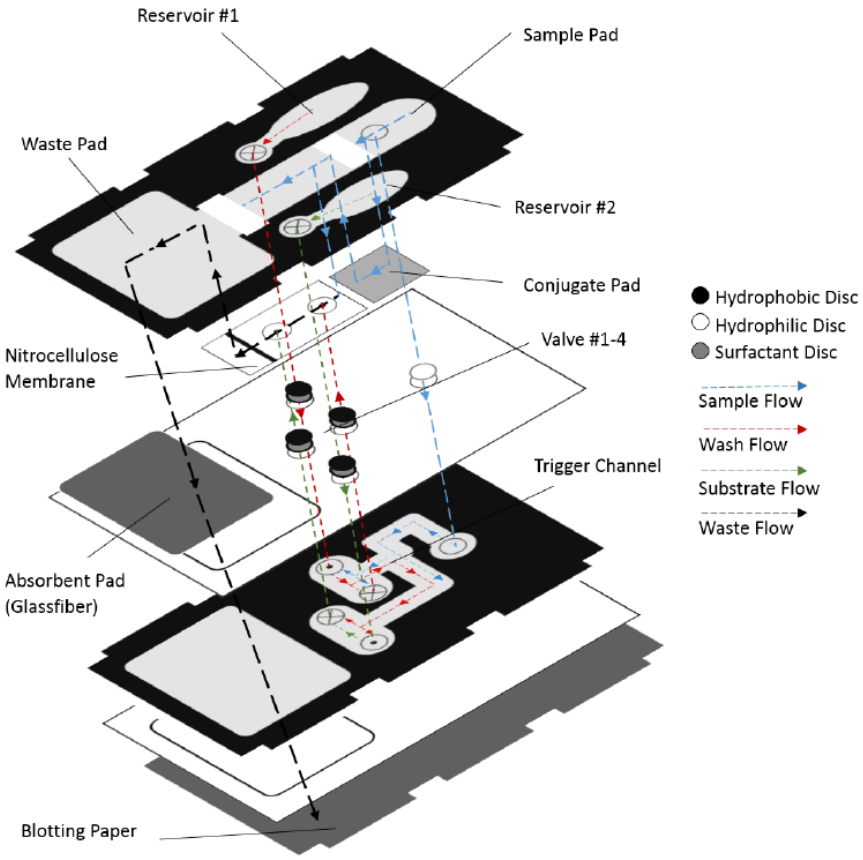


Figure 3-11: Fluid Flow Characteristics by Roman Gerbers and Wilke Föllscher [53]

In the designs from Gerbers and Föllscher, three fluids are applied to the chip for sequential loading. These include a sample fluid containing the analyte for detection, a wash-fluid which is used to clean up the signal on the nitrocellulose by removing any unbound proteins, and a substrate which creates the final color change and indication for the presence of an analyte. The wash fluid is stored in the first reservoir and will not interact with the chip until the hydrophobic discs below are inactivated through fluid interaction in the trigger channel. The substrate is stored in the second reservoir and again will not be triggered until the hydrophobic areas

below are inactivated by the fluid flow in the trigger channel.

Detection Antibody:	Anti-Rabbit-IgG (AP)	
Capture Antibody:	Anti-Rabbit-IgG (unlabeled)	
Analyte:	Rabbit-IgG	(1st)
Wash-Fluid:	SuperBlock® blocking buffer in TBS	(2nd)
Substrate:	BCIP/NBT	(3rd)

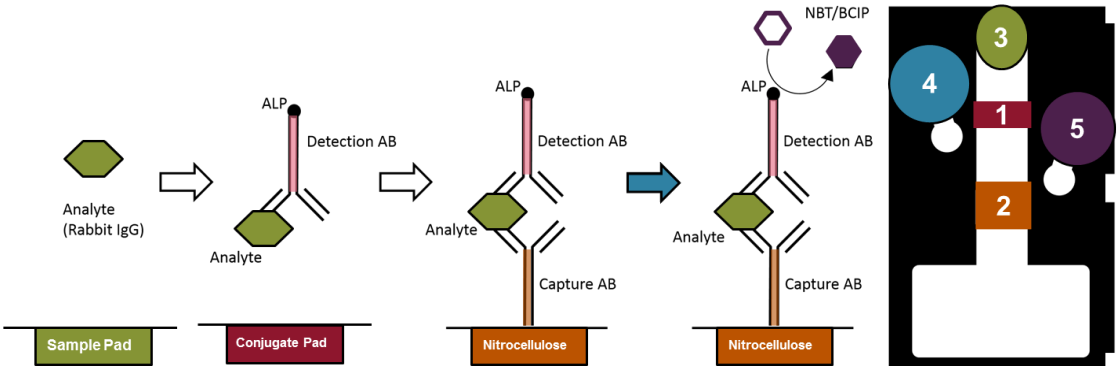


Figure 3-12: Mechanism of a 'Sandwich' ELISA within the Modified Design (adapted from [53])

The two fluids stored in reservoirs are applied first and then the sample may be introduced to the sample area. As the sample fluid flows down the main channel (in the top layer) it first interacts with a conjugate pad where detection antibodies are stored. If there are analytes in the sample fluid, they will bind to the detection antibody. As the fluid continues further down the channel it interacts with nitrocellulose on which capture antibodies are anchored. The capture antibodies will catch the analyte-detection antibody complex to form the ELISA sandwich described in 1.7.1. As this takes place, fluid has also travels down to the second layer (trigger layer) where it first triggers the wash fluid in reservoir 1. This wash fluid comes down to the second layer along the small bridge and up to the top layer where it will also be pulled along the main channel over the nitrocellulose to wash away any unbound proteins. Shortly thereafter, the substrate from the second reservoir is triggered and

it also travels along the small bridge to the top layer and to the nitrocellulose where it will induce a color change by interacting with detection antibodies. As explained in 1.7.3, the detection antibodies have a particular label that allows for an interaction with substrate.

This process is for an ELISA, however a chip to develop a competitive ELISA is needed. When designing and testing such devices, colored water are used for initial tests. After sequential flow is ensured, biological reagents may be used.

3.5 Protocol Development Chip

One of the first objectives of this project is to develop a chip on which to optimize various parameters. For example, during the initial stages of this research there were many boundaries encountered specifically with assembly, alignment, and surfactant & hydrophobic application. A less complex platform is designed to optimize many of the biological parameters independently from those of the fluidic valve. Also as there is always an interest to improve the assembly times for each chip, a platform is needed with which the assembly technique using spray adhesive could be studied.

In summary, a design was needed with which:

- Surfactant and Hydrophobic treatments could be further optimized, ideally in a 1-valve set up
- Spray adhesive assembly techniques could be investigated
- Biologicals could be tested independently from valve optimization

3.5.1 Design

Given the above goals and constraints, a 1-valve design was developed. Images of all components of an 8-batch chip along with each layer are provided below and dimensions are displayed in the appendix.

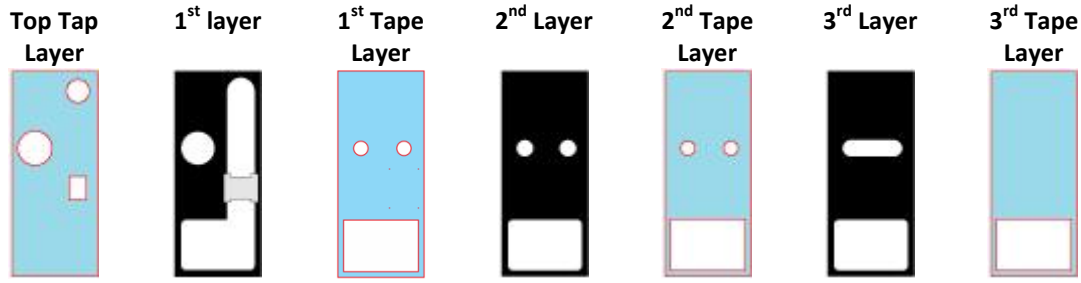


Figure 3-13: Protocol development chip layers

Each of the major components for this chip are included in the figure above. This chip incorporates 3 layers of printed-wax paper, 3 layers of double-sided tape, 1 clear top tape layer, and of course an absorbent pad (not pictured). This chip is designed to be slightly thinner than the modified design from Gerbers and Föllscher, thus 8 chips can be built onto the same area that 6 would fit. Also this design utilizes 1-valve to:

1. to compare the double-sided tape and spray adhesive methods of assembly
2. to re-optimize the concentrations and volumes for surfactant and hydrophobic treatment

This chip further serves as a platform to run biological experiments in the run up to a fully autonomous competitive assay. In the following section, the assembly process and components are illustrated.

3.5.2 Assembly with Double Sided Tape

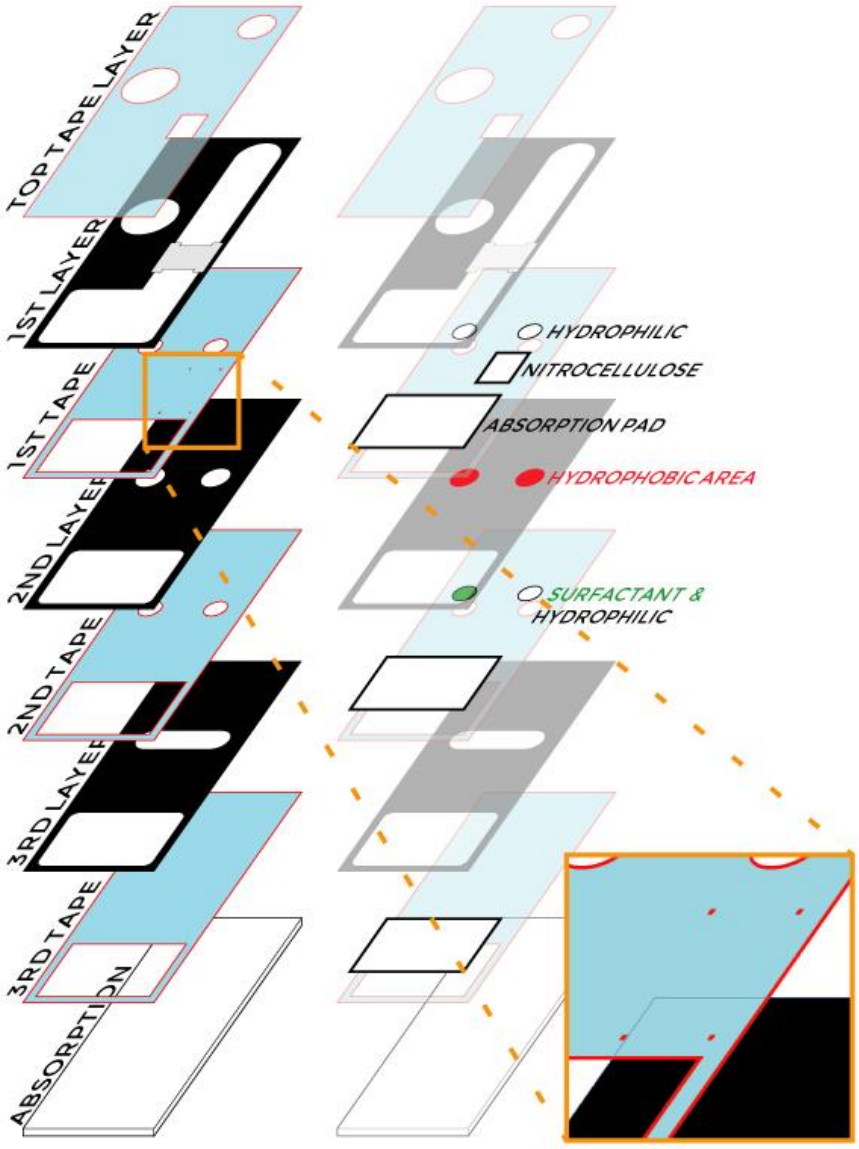


Figure 3-14: Protocol Development Chip Assembly with Double-Sided Tape

The area highlighted in orange shows small cutouts (red dots) designed to mark the placement of nitrocellulose for more precise alignment with the 1st layer. In the following image all the necessary for an 8-batch chip are shown. Included in the image are examples of the Popsicle sticks wrapped in paraffin film which can be used

to spread the paste into the tape cut outs. There are two popsicle sticks in order to keep the hydrophobic and surfactant pastes separate.

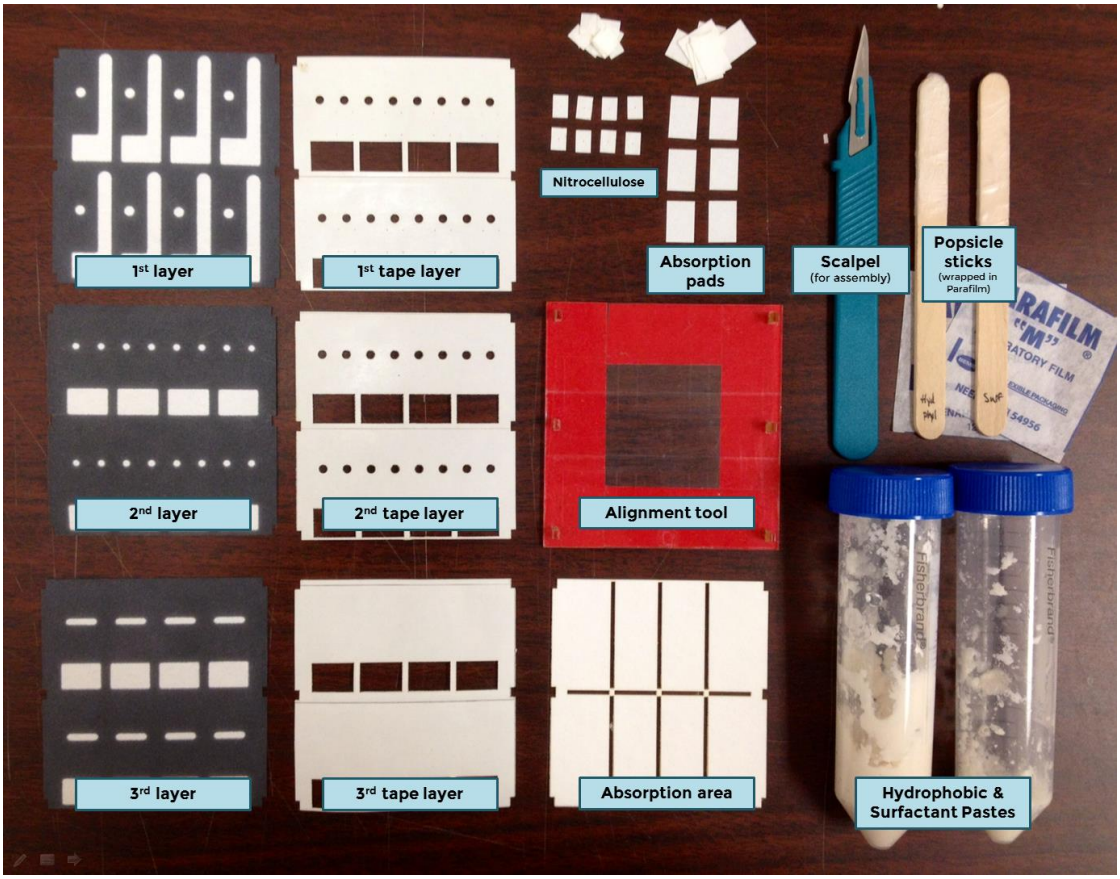


Figure 3-15: Materials Needed for Double-Sided tape Assembly

The top tape layer is the only component which has not been included in the above figure.

The assembly time and number of materials needed are the downfalls this assembly method. For example, when pastes are applied they must dry for at least 10 minutes. Additionally, during the assembly the double sided tape can be cumbersome to handle. For these reasons an interest developed to experiment with spray adhesive, introduced by G. Lewis et. al [49].

3.5.3 Spray Adhesive Method (5 layers)

In a spray adhesive assembly there are a few necessary changes. Since the double-sided tape layers are removed, a new wax layer is substituted on the bottom. As shown below, the 3rd layer is integrated and includes a smaller area for surfactant (the white circle on the left). Secondly, a complete layer of wax is needed to prevent fluid from flowing from the bottom layer or from the timing layer directly into the absorption pad. This was previously achieved by the 3rd layer of double-sided tape. Additionally, the geometries were slightly expanded in order to maximize the flow rate and decrease the resistance to flow from the fluid reservoir.

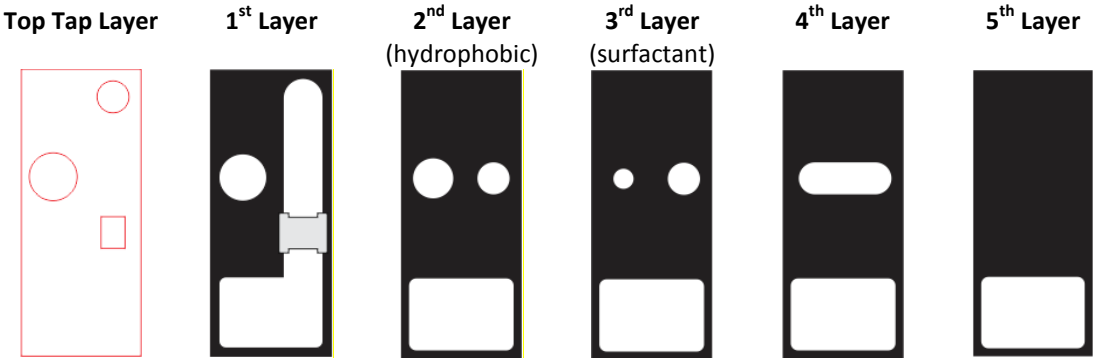


Figure 3-16: Protocol Development Chip Layers

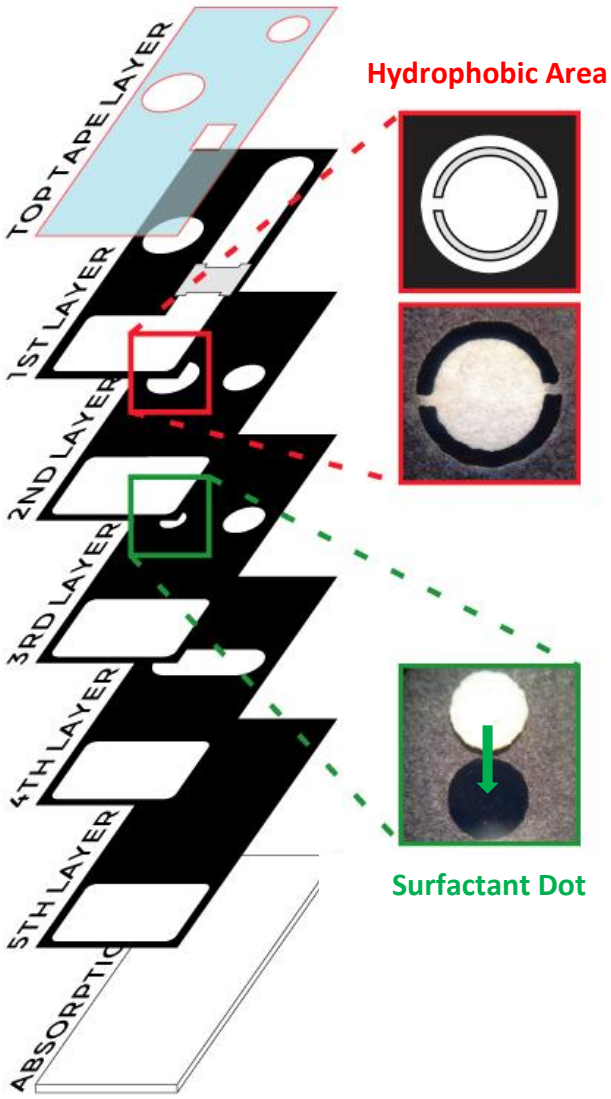


Figure 3-17: Assembly for Protocol Development Chip with Spray Adhesive (5 Layers)

In the illustration above, the hydrophobic area is slightly cut in a semicircular manner and is then melted. This is done to ensure that hydrophobic solution does not spread into the surrounding wax area during application. Also, the surfactant area may be treated directly or it can be cut out and replaced by a surfactant treated disk (this option is shown in the illustration). The discs are treated with surfactant solution in a petri-dish and allowed to dry at room temperature.

These two steps ensure that surfactant solution does not travel into the surrounding wax area. If this occurred, a less than intended volume would be left within the designated surfactant or hydrophobic area.

Once all materials are prepared, the chip may then be assembled using 3M's super 77 multipurpose adhesive. The adhesive is applied by spraying each side of the layers once for 0.25 second at a distance of approximately 24cm.

Figure 3-18: Materials Needed for Spray Adhesive Assembly (5 layers)

3.5.4 Spray Adhesive Method (4 layers)

The possibility of removing yet one more layer is investigated. Instead of dedicating a whole layer to surfactant dots, the 3rd layer (from Figure 3-17) may be removed leaving the 4th layer (the connection layer) to be treated with surfactant directly.

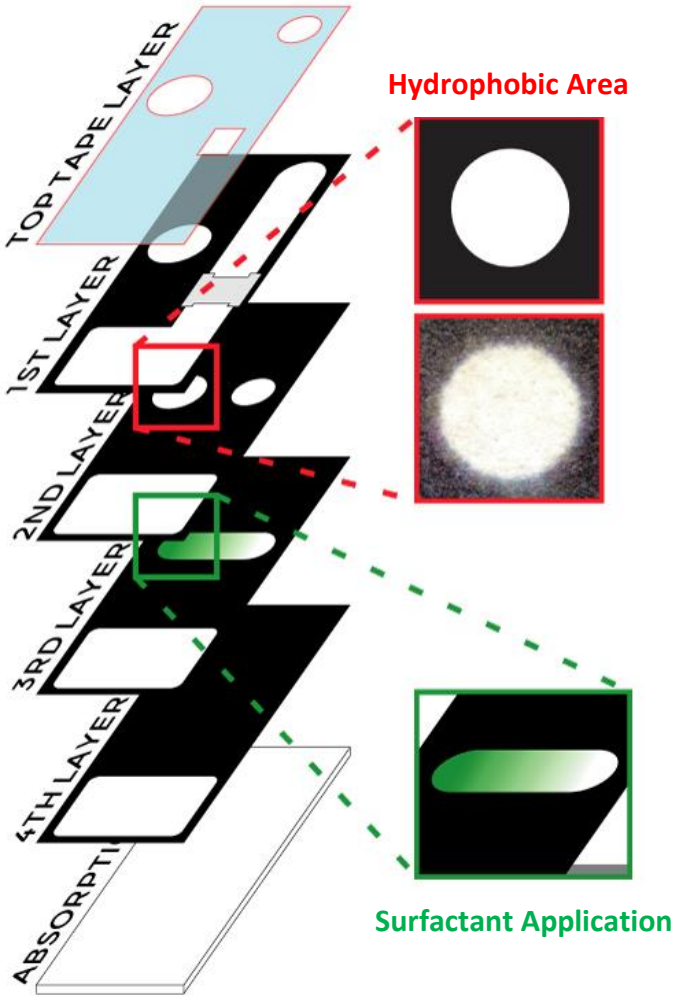


Figure 3-19: 4 Layer Assembly with Spray Adhesive

The cut is also removed from the hydrophobic area in order to prevent premature flow of fluid from the reservoir through the gaps. The hydrophobic area and surfactant area are slightly enlarged to be able to contain the same volume of reagent as previous tests. The same process follows for applying the spray adhesive. An image of all necessary materials is shown below. Although it is not shown in the image below, the 1st layer also has cutouts, which would serve as the spaces under

which the nitrocellulose pads would fit.

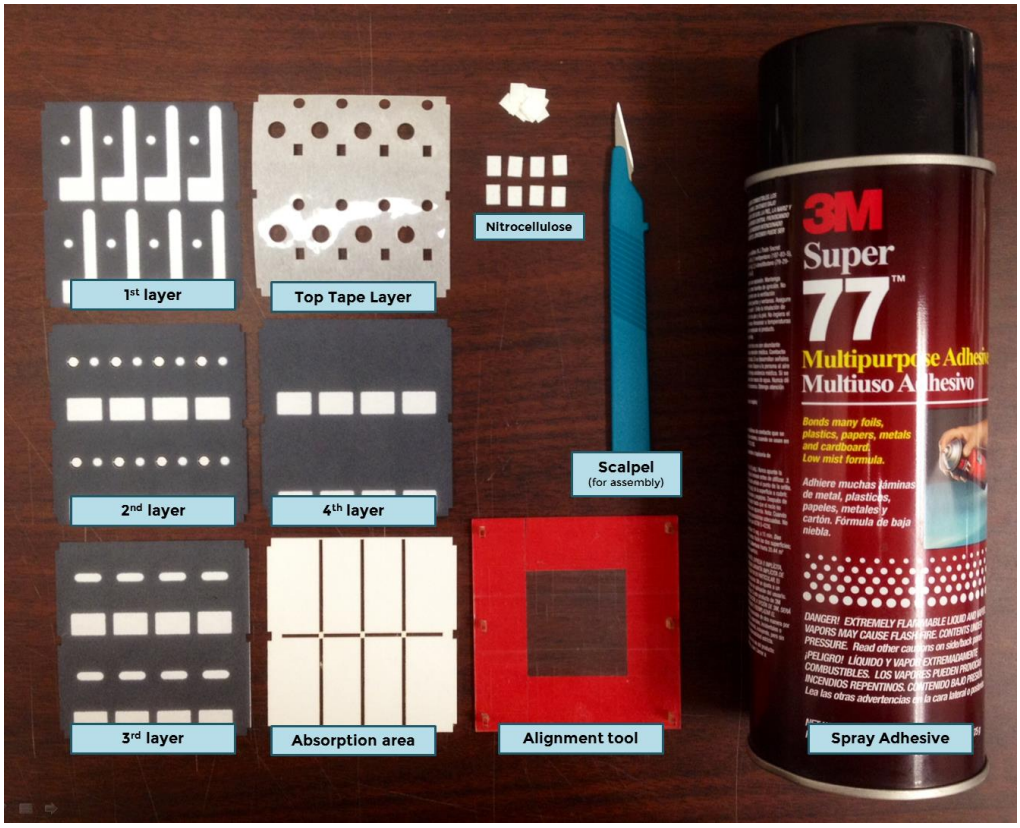


Figure 3-20: Materials Needed for Spray Adhesive Assembly (4 layers)

In this assembly the number of needed materials decreases. As discussed in 4.3, the assembly times also dramatically decrease.

3.6 Re-optimization of Hydrophobic and Surfactant Application

With new assembly methods, the hydrophobic and surfactant treatments need to be optimized to function properly, consistently, and predictably. To optimize the volumes and treatments the setup in Figure 3-19 is used. Starting with the previous methodology as a starting point the concentrations and volumes of treatments are varied in two phases. Each test is repeated in the 5 and 4 layer assemblies.

The hydrophobic treatment scheme from Gerbers and Föllscher (8µl treatments in steps of 2µl) serves as a starting point from which to optimize the concentration of surfactant. 'Surfactant dots' or circular cutouts of 1mm (shown in Figure 3-17) are treated with 1µl of surfactant solution in varying concentrations. Surfactant solution is created by mixing Tween 20 in Ethanol. This is done as a wt.%. For this particular experiment wt.%'s in the range of 30, 25, 20, 15, 10, and 5% are used. For example, to make a 30 wt.% surfactant solution, 0.3g of Tween 20 is mixed with 0.6g of Ethanol, making in total a 1g solution. These are then pipetted at 1µl volumes to the 1mm circular cutouts and are allowed to dry for at least 12 hours at room temperature.

3.6.1 Hydrophobic Optimization (5 Layers)

Once the optimal wt.% is determined, the volumes of hydrophobic solution are varied. This is still done in accordance with previous procedures. Using the same solution (50µl of Allyltrichlorosilane in 950µl of FC-72) the applied volumes are varied in the range of 8, 6, 4, 2µl, all in steps of 2µl. Hydrophobic treatments should be allowed to dry for at least 12 hours. Each variation is done in 3 batches of 8 chips, giving a total of 24 tests for the concentrations of surfactant and hydrophobic treatments.

3.6.2 Hydrophobic Optimization (4 Layers)

Within the 4-layer assembly (pictured in Figure 3-19), hydrophobic solution is applied in the same range (8, 6, 4, 2 µL) while surfactant solution is applied directly

onto the third layer at a 5 wt.% solution in 1 and 2 µL volumes.

3.7 Multi-fluidic Sequential Flow

In order to fully justify the use of a spray adhesive assembly, a chip capable of sequentially flowing three fluids is needed. A chip is designed under similar parameters as the protocol development chip. This is done to run tests with the same hydrophobic and surfactant solution volumes and treatments.

3.7.1 Design of a Multi-Fluidic Chip

Using the methodology of a 4-Layer chip, the following design is developed (dimensions may be found in the appendix). The first layer incorporates an 'H' shaped cutout under which the nitrocellulose pad could fit. On the second layer 6 columns of red dots are added. These serve as a guide with which to precisely place nitrocellulose in for proper alignment with the layer above. Many dots are incorporated in order to handle a range of nitrocellulose geometries for 5-3mm widths and 7-4mm heights.

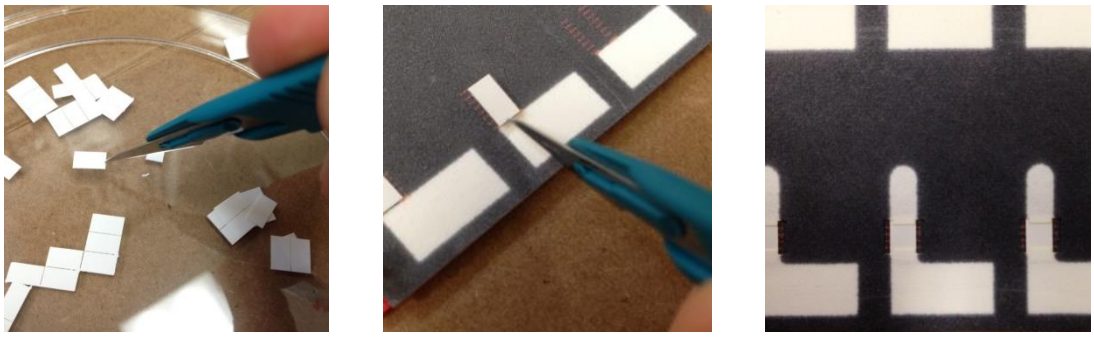


Figure 3-21: Using the Alignments Guides to Precisely Place Nitrocellulose

, A scalpel is used to apply nitrocellulose, as shown in the pictures above. The nitrocellulose should be handled as minimally as possible in order to preserve the

fibrous structure. When picking up nitrocellulose, it should be handled toward the top or bottom so as not to alter the functional detection area.

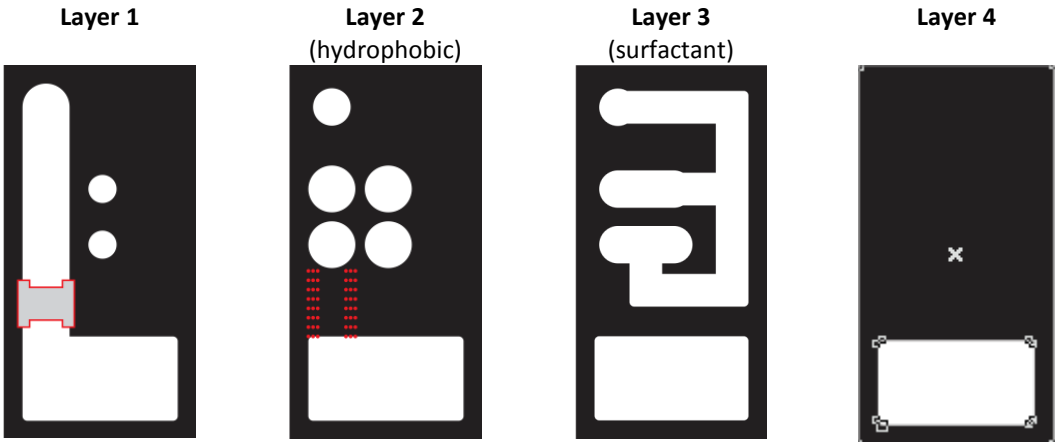


Figure 3-22: 4 Layers of a Multi-fluidic Chip Assembled with Spray Adhesive

Hydrophobic areas (4 larger circles) are treated with 2 µl under the same protocol as described in a previous section. Surfactant areas are treated with 3 µL of a 5 wt.% solution again under the previous protocol. After the chips are assembled, 70 µl of red and blue solution are added to the reservoirs. In order to make sure the hydrophobic treatments hold, the colored water is allowed to sit for 5-7 minutes. At this point 100 µL of clear fluid may be added to start the test. Times for each fluid are recorded.

3.7.2 Controlling Backflow in the Second Layer

After tests are conducted the chips may be disassembled to investigate the flow characteristics and determine sources of error. Previously, it was discovered that many of the secondary fluids exhibited a backward flow within the 3rd wax layer (timing/surfactant layer). Backward flow can be a problem especially when using biologicals. The fluid that returns backwards along the source path is lost and will not

be effective in the detection scheme. For example, if some volume of substrate is lost due to backward flow the resulting signal on the nitrocellulose may not be as distict.

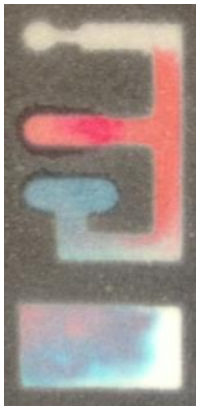


Figure 3-23: Backward flow in the 3rd Wax Layer

From the secondary fluid (red fluid) the flow returns along the path of the trigger fluid (clear fluid) and moves toward the point of entry and further toward the tertiary fluid (blue fluid). The tertiary fluid additionally exihibits similar backward flow characteristics.

According to Elain Fu et al, a contraction along a fluidic channel does not affect the distance a fluid can travel [42]. An expansion, however, will slow the progressive flow of a fluid along a channel [42]. In a contraction, the wider channel serves as an unlimited source of fluid, however, during an expansion, the amount of fluid that can continue down a channel is limited by the volume held in the thinner channel [42]. This can be seen in section 2.2.3.

Using this characteristic, 'kinks' are incorporated into the secondary layer for two reasons. The foremost reason is to test the geometry's ability to restrict backward flow and secondly to test if the valve opening times are delayed. Designs for the 3rd wax layer are shown below and dimensions have been provided in the appendix.

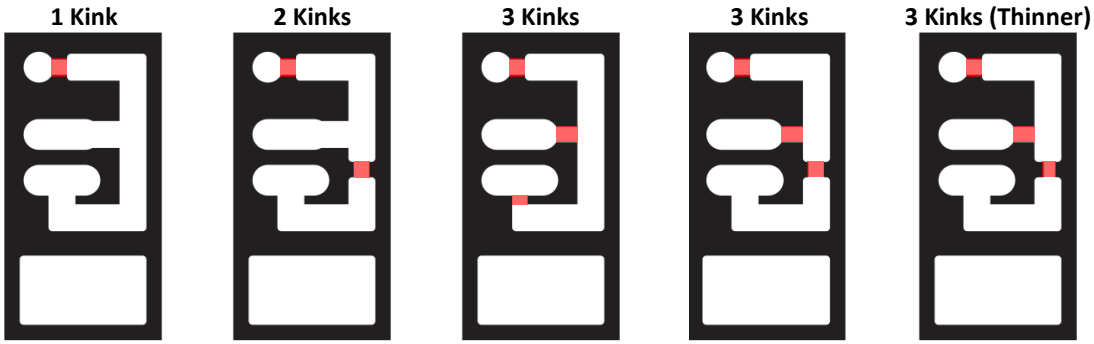


Figure 3-24: Second layer Incorporated with kinks Highlighted in Red

The kinks are designed to slow flow and impede backward flow. The narrower sections are designed to be about 2/3 the width of the main channel.

Figure 3-25: Assembly of 3-Fluid Chip (alternate 'Kink' layer is shown also)

3.8 Enzymatic Activity Test

The protocol development chip is also designed as a platform on which to test the activity of biological interactions. Since IαIP is detected through enzymatic

activity, a model experiment is developed to prove the feasibility of such as test. To show that the concept is possible, an enzymatic activity test is developed for the detection of Alkaline-Phosphatase (ALP). Alkaline Phosphatase is an enzyme that is responsible for removing phosphate groups from any type of molecule. These molecules can be nucleotides, proteins, or alkaloids. As its name suggests, ALP is effective in an alkaline environment [56]. This was the same substrate used by Roman Gerbers and Wilke Föllscher.

3.8.1 Alkaline Phosphatase Activity Test

Abcam® offers mouse monoclonal and polyclonal antibodies labeled with ALP (SB62a). BCIP®/NBT serves as a substrate to induce a color change when interacting with ALP. A purple color is produced when ALP interacts with BCIP/NBT.

To create a model enzymatic activity test, first a protocol development chip is assembled using the 4-layer design without any valves to prevent hydrophobic and surfactant solutions from interacting with the biology. After the chip assembly is complete, ALP labeled antibodies may be placed on the nitrocellulose in various concentrations. Since the protocol development chip is produced in batches of 8, 8 various concentrations of ALP labeled antibody are used. The Antibody is diluted in PBS to create concentrations: 0.5, 0.1, 0.1e-1, 0.1e-2, 0.1e-3, 0.1e-4, 0.1e-5, and 0.1e-6 mg/ml. Once the concentrations are made, they are pipetted at 0.4µL onto the 45µm nitrocellulose within the chip and are allowed to dry for 10 minutes at room temperature.

The substrate is purchased from SIGMAFAST™ (Lot. SLBD5142V) as a specific substrate to ALP. The substrate comes in tablet form and is dissolved in 10 ml of ultra-pure water to create the substrate solution.

At this point the chip is ready for testing. The test is started when 100µl of PBS are added to the top sample area in the main channel of the chip. As soon as this fluid reaches the nitrocellulose, 80µL of the substrate solution is added to the second input area from where it will flow to the main channel and through the nitrocellulose to interact with the ALP enzyme.

After the fluids have been completely depleted and the nitrocellulose dries, the chips are scanned at a resolution of 1200 DPI on a HP Photosmart 6510 scanner and the signal is measured using the ImageJ software to calculate the intensity of each spot on the nitrocellulose.

3.8.2 Nitrocellulose Shape Variation

The previous test is repeated with variations in the shape of nitrocellulose. After running the previous test, it was observed that there was a large amount of area on the nitrocellulose which was not covered with the label antibody; therefore no signal is produced on these areas. Also, these areas offer a path for the substrate to flow through without the chance to interact with ALP. In order to maximize the interaction between ALP and BCIP/NBT, the width of the nitrocellulose is varied along the middle from widths of 5.0, 4.0, 3.0, and 2.0mm. After running the test in the same manner, the chip is scanned and the signals are then processed with ImageJ.

Figure 3-26 Varying Nitrocellulose widths

3.9 Biologicals with Inter-Alpha Inhibitor

After working with Hanno Teiwes to complete a sandwich ELISA for the detection of IαIP a problem remained with the average signal/noise achieve with the platform. Within the previous set up, the maximum signal/noise ratio achieved was about 1.3. This value is far less than the capabilities of a bench-top set up. The results from the development of this platform may be seen within his thesis [54].

3.9.1 The DAB Substrate

When working with IαIP in both the 'Sandwich' & Competitive ELISA schemes, the DAB substrate is used to generate a signal. However, DAB will not develop a signal on its own, it must bind to HRP-Strep which first binds to Biotin (the label that is attached to the detection antibody in the 'Sandwich' ELISA and also to IαIP in the Competitive ELISA).

DAB & Streptavidin Poly-HRP Conjugate (HRP-Strep) are purchased from Pierce Protein Biology Products (Cat Nos. 34001 & 21140).

HRP-Strep is prepared by mixing 7µL of HRP to 500µL (concentration of .013 µL/mL) of HRP buffer solution. The HRP buffer solution is made by mixing 1.1g of Non-fat Milk Powder in 22g of PBST. The HRP/HRP buffer solution should be aliquot into centrifuge tubes and stored at -20°C.

DAB comes in powder form and is prepared under the protocol presented by IHC World [57]. DAB must be prepared in two components which are mixed only just before use; a DAB solution and a Hydrogen Peroxide solution (H_2O_2). 0.5g of DAB are mixed with 10mL of distilled water, then 250µL of 10N HCl is added to the solution. The final color of the solution should be light purple. The H_2O_2 solution is made by mixing 100µL of pure H_2O_2 in 10mL of distilled water. The DAB and H_2O_2 solutions should also be aliquot into centrifuge tubes and stored at -20°C.

Upon use, 50µL of the DAB solution and 50µL H_2O_2 solution are mixed into 1mL of PBS(x1) (these volumes may be increased depending on the total volume needed).

3.9.2 Reducing Background Signal

One of the problems encountered in the coinciding work of Hanno Teiwes was the strong background signal generated during the development of 'Sandwich' ELISA. Within both the 'Sandwich' ELISA and 'Competitive ELISA the signal is generated by the addition of the DAB substrate. However, a signal develops when DAB binds to HRP-Strep which binds to Biotin (the label that is attached to the detection antibody for 'Sandwich' ELISA and also to IαIP for Competitive ELISA).

This reaction scheme can be particularly problematic if there is extraneous HRP-Strep floating around in the background on the nitrocellulose. When DAB is introduced to the nitrocellulose, not only will it create a signal on the HRP bound to Biotin, but it will also create a signal on the free HRP that has not been washed off

the Nitrocellulose. In an effort to clean up this background signal, wash steps are introduced to remove some of the free HRP.

To test this, the detection antibody, Biotinilated-R21, is spotted onto 1.2µm nitrocellulose within the protocol development chip at a volume of 0.6µL. This is allowed to dry on a hot-plate at 37°C for 10 minutes. At this point the HRP-Strep and subsequently DAB may be added to chip at volumes of 30µL to develop a signal. All fluids were added to the cutout just ahead of the nitrocellulose in the of the top tape layer. To test the efficacy of wash steps, three methods for cleaning out the HRP are used:

Sequence 1 (0 washes)

- 40µL HRP solution added and allowed to flow through nitrocellulose
- 40µL of DAB solution added and allowed to flow through nitrocellulose

Sequence 2 (1 wash end)

- 40µL HRP solution added and allowed to flow through nitrocellulose
- 40µL of DAB solution added and allowed to flow through nitrocellulose
- 70µL of PBST added and allowed to flow through nitrocellulose

Sequence 3 (1 wash middle)

- 40µL HRP solution added and allowed to flow through nitrocellulose
- 70µL of PBST added and allowed to flow through nitrocellulose
- 40µL of DAB solution added and allowed to flow through nitrocellulose

Sequence 4 (2 washes)

- 40µL HRP solution added and allowed to flow through nitrocellulose
- 70µL of PBST added and allowed to flow through nitrocellulose
- 40µL of DAB solution added and allowed to flow through nitrocellulose
- 70µL of PBST added and allowed to flow through nitrocellulose

Following these tests, the chips are scanned at 1200 DPI with the HP Photosmart 6510 all-in-one printer. The images processed with ImageJ to determine if the signal/noise is improved.

3.10 Competitive ELISA

When working toward a paper-based device that can capture and detect IαIP the variables must be isolated to be optimized. The first step is to determine the correct amount of capture antibody to use in order to capture enough protein to generate a signal. Second, an effective concentration for the labeled competitor (Biotinylated IαIP) must be determined. Then finally the generation of the sigmoidal curve based upon a variation of IαIP concentration is developed. It is important to point out that reagents are pipetted into test chips during the optimizing of these parameters. These test chips are designed and fabricated without valves in order to isolate the biological parameters. Following a similar protocol to Hanno Teiwes, a simplified chip is developed, which forces fluids over a nitrocellulose upon their addition to the chip. An overview image has been provided below.

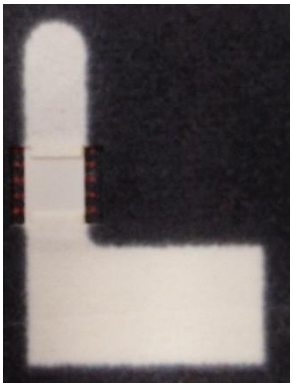


Figure 3-27: Image of the 'simple' Test Strip on which Reagents are Added by Hand.

Within this chip there is an absorption pad, a wax layer on which nitrocellulose sits, a top wax layer, and a top tape layer. This was assembled with the spray adhesive.

3.10.1 Optimization of Detection Antibody (MAB 69.26)

Within the Competitive ELISA scheme for the detection of IαIP, Monoclonal Antibody 69.26 (MAB 69.26) is used as a capture antibody. This protein was provided by Dr. Yow-Pin Lim of Prothera Biologics. To determine an adequate amount of MAB 69.26, various volumes are added to 1.2µm nitrocellulose (Whatman® G3514143). MAB 69.26 are added in 0.6µL increments for a total of three volumes (0.6µL, 1.2µL, and 1.80µL). Between each addition, the antibody is allowed to dry for 3-5 minutes on a hotplate at 37°C. Following the addition of capture antibody, the nitrocellulose and main channel is blocked with the 5wt.% Milk in PBST solution (made by mixing 2.2g non-fat milk powder in 22 g of PBST) and allowed to dry at 37°C. The top tape layers are then added completing the assembly of the chip.

Various concentrations of Biotinylated-Inter Alpha Inhibitor Protein (B-IαIP) are prepared by a dilution in 1wt.% Milk-PBST solution. These dilutions include: 1:10, 1:100, 1:1,000, 1:10,000, & 1:100,000 (B-IαIP:1wt.% Milk-PBST). Each concentration is added to the chips at a volume of 60µL, followed by a 30µL PBS after the sample has been pulled through the device. Then the HRP in 1wt.% Milk-PBST is added at a volume of 30µL, followed by 30µL PBS after the HRP has been pulled through the device. At this point DAB substrate is added at a volume of 30µL to create the color reaction. The chips are scanned at 1200 DPI and the images are processed using the ImageJ software.

3.10.2 Optimization of B-IαIP Concentration

After determining the appropriate volume for the capture antibody, the next step is to determine the optimal concentration for the competitor protein, Biotinylated IαIP (B-IαIP).

The simplified chip was again assembled under the same protocol as in the previous experiment. The volume for the capture antibody is increased to a total of 2µL and is applied in 5µL increments to the 1.2µm nitrocellulose. After the chips are fully assembled, B-IαIP is diluted in 1wt.% Milk-PBST solution at ratios of 1:10, 1:100, 1:200, 1:400, & 1:800. For example, a 1:10 concentration would be 10µL of B-IαIP in 90µL of 1wt.% Milk PBST solution. In a subsequent experiment, the dilution ranges are increased to 1:10, 1:100, 1:500, 1:1000 and 1:10,100. Under the same procedures, HRP-Strep and DAB are added to create the color change.

The idea behind this experiment is to determine the corresponding concentration for the saturation point of the capture antibodies. When looking at the signal to concentration curve, this value lays just before the plateau. The concentration correlated with this value would offer an adequate competition within the competitive ELISA scheme.

3.10.3 Development of Standardized Curve

It was determined that a concentration of B-IαIP between 1:100 and 1:400 is sufficient for the competition of un-labeled IαIP. In an effort to develop a standardized curve for a competitive ELISA on paper, the test chips were assembled, treated again with 2µL of detection antibody (MAB 69.26), then blocked with 5wt.% milk in PBST.

An unlabeled IαIP was provided by Dr. Yow-Pin Lim to serve as the competitor to the labeled IαIP (B-IαIP). This is diluted with a solution of 1:200 B-IαIP in 1wt.% Milk-PBST to create concentrations of: 40, 20, 10, 5, 2.5, 2.0, 1.0, 0.5, 0.25, 0.2, and 0.02 mg/ml. These are then added to the chips at volumes of 60µL, followed by HRP-Strep (30µL), PBS (30µL), and then DAB (30µL).

This experiment is then repeated with a 1:400 concentration of B-IαIP in order to determine if the standardized curve shifts to change the limit of detection. As the concentration of IαIP in B- IαIP increases there should be a lower signal as more detection antibody is occupied by the un-labeled proteins.

3.10.4 Reduction of one fluid

Under the previous experiments by Hanno Teiwes and under the protocol from the previously described experiments, HRP-Strep is added as a separate fluid after the main sample, followed by the DAB substrate. Since it was determined that a wash step can drastically increase the signal/noise, there are 4 total fluids needed (sample fluid, HRP-Strep, PBS Wash, and DAB). In a fully autonomous set up, this would complicate the device.

Ideally, the test would include 3 or fewer fluids. To achieve a lower level of complexity, the HRP-Strep is added to sample fluid to incubate with B-IαIP. This is done for the 1:400 B-IαIP in 1wt.% Milk-PBST at the same ratio as previous experiments. For the 60µL sample, 30µL of HRP are added and mixed. This is then added to a prepared chip to determine if an adequate signal may be generated.

3.10.5 Complete Competitive Test

With the generation of a standardized curve and reduction of fluids, the next step is to develop the Competitive ELISA on a fully autonomous device. The 3-Fluid design may be used to develop a signal curve.

The 3-Fluid chip is used. To prepare the chip, each layer must be blocked with 5 wt.% Milk-PBS solution. It is important to note that PBS is used rather than PBST, in order to prevent premature opening of valves. Since Tween 20 serves as the surfactant, PBS must be used instead. This should be repeated for the dilutions of B-IαIP and furthermore for the dilution of unlabeled IαIP. Surfactant areas are treated

with 3µL of a 5 wt.% solution. The hydrophobic areas for the wash and subsequent DAB substrate are treated with higher volumes of hydrophobic solution 4µL and 8µL to ensure that valves held. The full chip is then run with 1000, 200, 80, 40, 20, 8, 4, 2, 0.4, 0.02 mg/ml concentrations of IαIP diluted in 1:400 B-IαIP 1wt.% MILK-PBS solution. The added solution volumes are as follows:

- 100µL of Sample Volume (dilutions of IαIP) incubated with 50µL of HRP-Strep prior to testing
- 60µL PBS as the secondary fluid
- 60µL of DAB as the tertiary fluid

The chips are then scanned at 1200 DPI and the results are processed with ImageJ.

4 CHAPTER 4 – FINDINGS

4.1 Sequential Flow of Fluids

4.1.1 Gerberss and Föllscher's Design

Using a previous design by Roman Gerbers & Wilke Föllscher and assembly techniques introduced by Hong Cheng a chip was fabricated to test the sequential loading of three fluids. Hong Cheng's assembly methods use small disks between each layer; these disks were treated with surfactant and hydrophobic solution to create the fluidic valve. This methodology can be referenced in his PHD dissertation [48].

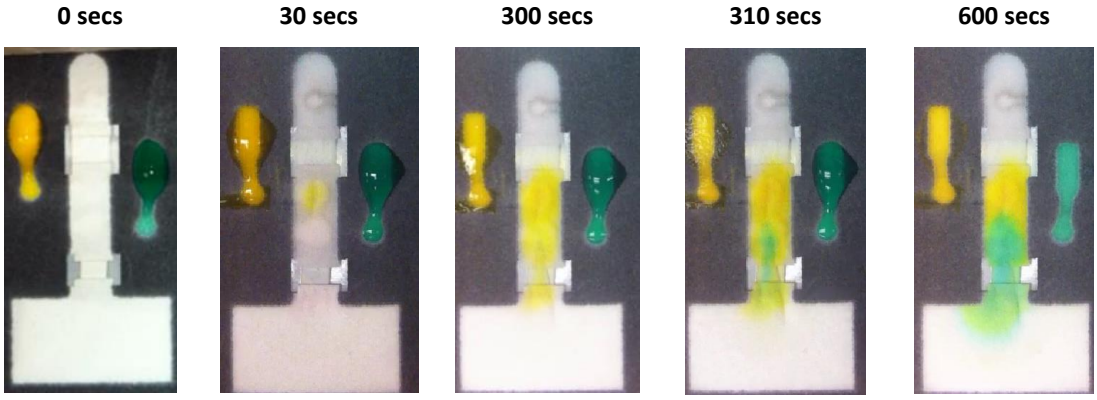


Figure 4-1: Results of sequential Loading of a two fluid circuit

As can be seen from the above images, three fluids were used, two of which included food coloring to demonstrate when fluids came through to the top layer of the device. As previously discussed, each fluidic valve holds the colored fluids until they are triggered by fluid flow in the 3rd layer. The first fluid (yellow) begins to come through at 30 seconds and continues to flow until about 310 seconds when the second fluid (green) comes through to the top layer. By showing that it is possible to

sequentially flow fluids with the above design, developments can be made to add biology and trigger fluids in the same sequence.

4.1.2 Protocol Development Chip (Double-Sided Tape)

Using the protocol described by Gerbers and Föllscher and the construction described in section 3.5.2 the following result was obtained.

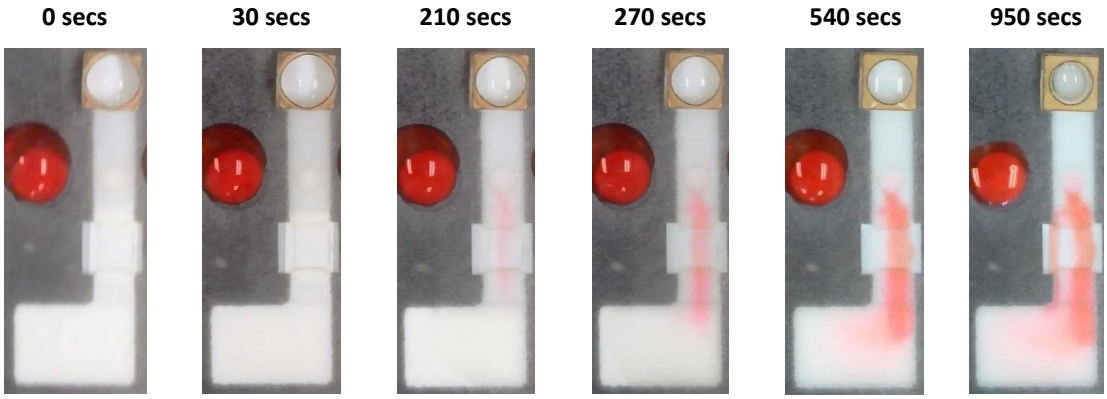


Figure 4-2: Results of protocol development chip

After about 3 minutes and 30 seconds, the secondary fluid can be seen coming through from the reservoir and into the main channel. This simply shows that it is possible to store a fluid and trigger it with an input fluid within the protocol development chip. One observation is the amount of time needed for the secondary fluid to be fully absorbed, at least 15 minutes. This may be a result of the resistance between the various layers of paste and paper. As can be seen best in the last photo, the fluid seems to be coming from the bottom of the paste area located underneath the top layer. Comparisons are later made to the flow within the spray adhesive assembly.

4.2 Re-optimization of Components in Spray Adhesive

4.2.1 Optimization of Surfactant

Three batches of chips were made with 4 tests on each batch dedicated to the variations of surfactant solution (30, 25, 20, 15, 10, 5 wt.%). For each chip, first the reservoir fluid was added (red fluid from figure 4-2) before any clear fluid was added to the sample input. This was done to make sure that the hydrophobic areas would hold back the red fluid until the sample fluid is added. The table below lists the results of the surfactant optimization.

Table 4-1: Results of Surfactant Optimization

30 wt.%	25 wt.%	20 wt.%	15 wt.%	10 wt.%	5 wt.%
Failed in < 30secs	Failed in < 30secs	Failed in < 30secs	Failed in < 30secs. **One test held**	**All held**	**All held**

In the above tests, the 30-15 wt.% of surfactant failed because there was too much surfactant solution. As a result the surfactant, a soap like substance, did not fully dry and rendered the hydrophobic solution inactive prior to adding any sample fluids to the chip. Also, the surfactant solution was in such a high concentration that it actually allowed fluid to pass directly through the bottom most wax layer and directly into the absorption pad without traveling back up to the main channel and though the waste area.

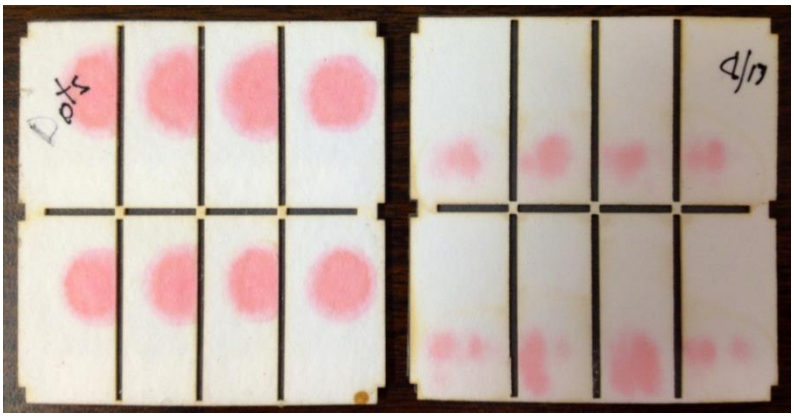


Figure 4-3: Surfactant dots bridging the wax layer (5th layer)

The chip on the left shows how surfactant has bridged the wax layer and gone directly down to the absorption area. On the right is a case there a chip has functioned accordingly by flowing through the waste area. It should be noted that this phenomenon may also occur if pressure is applied to the reservoir area containing fluid.

Initially, speculations were made blaming the interactions between spray adhesive and hydrophobic/surfactant material for the failure of the fluidic valves, but as the experiments moved into the 10 & 5 wt.%s all tests successfully held.

4.2.2 Optimization of Hydrophobic Solution (5 Layers)

Following this test, optimization procedures were developed for the hydrophobic areas. According to the methodology of Gerbers and Föllscher [53], hydrophobic areas were treated with 8µl. With the optimal concentration of surfactant solution (5-10 wt.%), the amounts of hydrophobic solution were decreased in the range of 8, 6, 4, and 2µl. Three batches were assembled for each concentration for a total of 12 batches each with 8 individual chips or tests. Each

concentration of hydrophobic volume was also combined with 10 and 5 wt.% of surfactant to measure the full efficacy of each. During the procedure, the red fluid was added first, and allowed to sit for at least 5 minutes before adding the main sample. This was done to ensure that the hydrophobic valves held. The following chart shows the average opening times for each volume.

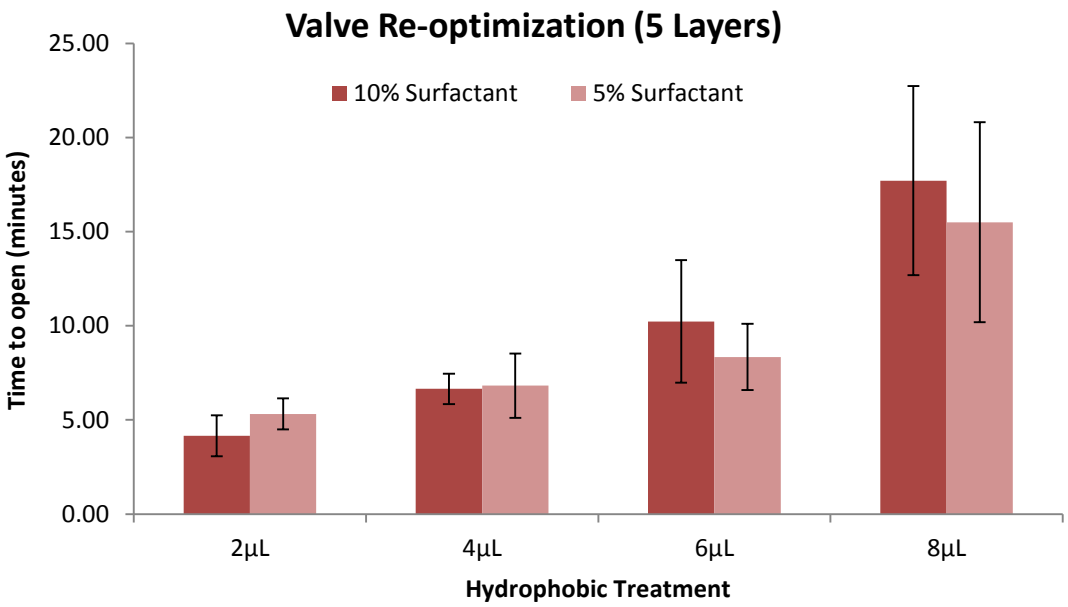


Figure 4-4: Valve Re-optimization for Surfactant and Hydrophobic Treatment in an Assembly with Spray Adhesive (5 Layers)

The data from this experiment is shown in the appendix. As can be seen in the chart above, there is no significant difference between 10 wt.% and a 5 wt.% surfactant solution on the opening times of the valves. This is especially apparent for the 6 and 8µL volumes, where one might expect the 5 wt.% to take longer to open. Furthermore, as the amount of hydrophobic treatment solution is increases so does the time needed for the valves to open. This data shows that it is possible to integrate a fluidic valve into a chip which has been built with a spray adhesive.

4.2.3 Optimization of Hydrophobic Solution (4 Layers)

Under the same methodology as the 5 Layer assembly, Hydrophobic treatments were tested in the range for 8, 6, 4, and 2µL. Surfactant solution was added directly to the third layer in 2 and 1 µL volumes at a 5 wt.% solution.

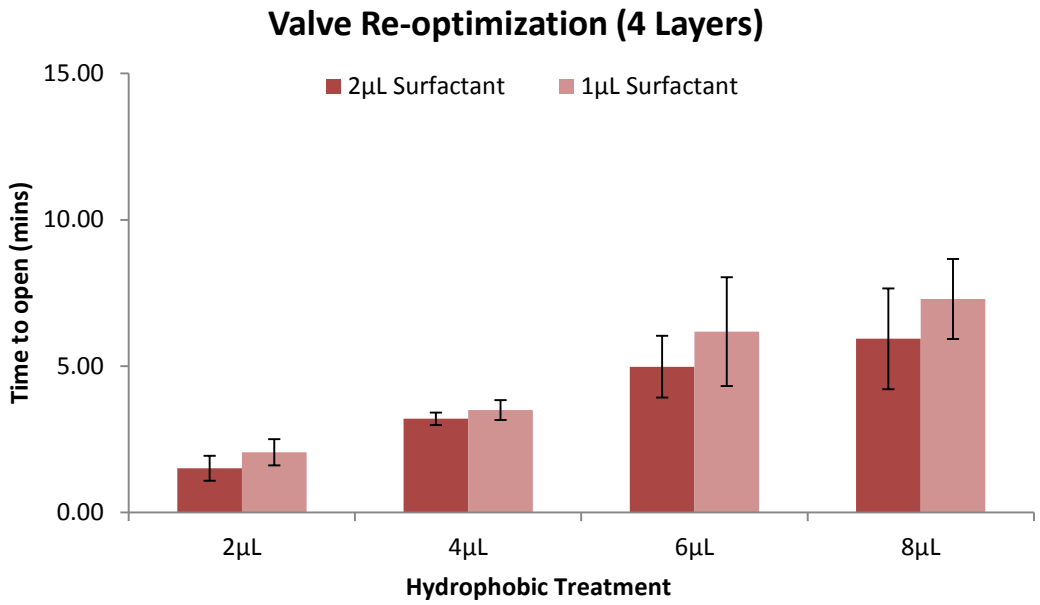


Figure 4-5: Valve Re-optimization for Surfactant and Hydrophobic Treatment in an Assembly with Spray Adhesive (4 Layers)

In comparison to the 5 layer assembly, the opening times for the 4 layer assembly decreased by a factor of about 2. This likely due to the fact that the fluid travels through 3 layers as compared to 4 layers. Within this setup, there was only 1 of the 24 tests that failed as a result of valve prematurely breaking. The data for this experiment is listed in the appendix.

The various combinations of surfactant and more importantly hydrophobic solution may be taken advantage of during fluidic circuit design. For example, higher or lower combinations of surfactant may be combined with timing channels to help

ensure the sequential flow. This could be particularly advantageous for multi fluidic circuits when it must be ensured that one fluid is sequentially released after another fluid. This may also be an important factor when chips are miniaturized, which would not leave as much space for timing channels. In this particular case, different volumes of hydrophobic solution may be used to ensure a later triggering of a secondary fluid. Within this experiment no particular fluid is optimal, however the results may serve as a reference for designing future circuits which may be require more complex geometries.

4.3 Comparison of Time to Assemble Adhesive vs. Double-Sided Tape

When preparing all materials for chip assembly, the major components for each assembly method remain the same. However, the assembly times for each chip differ. The following charts describe the differences in assembly along with a breakdown of the time needed for each step in the assembly process.

Table 4-2: Component Comparison of Double Sided Tape and Spray Adhesive (listed in the order of assembly from bottom up)

Double Sided Tape Components	Spray Adhesive Components

• **Absorption Layer**
• **3rd Tape Layer**
  ○ **Absorption Pad**
• **3rd Wax Layer**
• **2nd Tape Layer**
  ○ **Absorption Pad**
  ○ **Surfactant & Hydrophilic Paste**
• **2nd Wax Layer (Hydrophobic Layer)**
• **1st Tape Layer**
	• Absorption Layer
	• 4rd Wax Layer
	• 3rd Wax Layer (Surfactant)
	• 2nd Wax Layer (Hydrophobic)
	  ○ Nitrocellulose & Conjugate Pad
	• 1st Wax Layer
	• Top Tape Layer

- Absorption Pad
- Nitrocellulose & Conjugate Pad
- Hydrophilic Paste

- **1st Wax Layer**
- **Tope Tape Layer**

- **TOTAL = 16 Components**
- TOTAL = **8 Components**

Moving from Double-Sided Tape to a Spray Adhesive, there is a major drop in number of components; as shone shown by Table 4-2, there is a 50% reduction in number of parts. The most significant implication of this the reduced amount of time needed for fabrication. This is especially apparent when comparing the times for each step in the fabrication methods. The following table shows the times for each step in the preparation and assembly processes.

Table 4-3: Fabrication and assembly times for double sided tape and spray adhesive

Double Sided Tape		Spray Adhesive	
Process	Time (mins)	**Process**	Time (mins)
Cutting and Preparation			
Absorption Area Cut	4.00	Absorption Area Cut	4.00
Nitrocellulose Cut	10.00	Nitrocellulose Cut	10.00
Conjugate Pad Cut	2.00	Conjugate Pad Cut	2.00
3 Layers of Wax Cut	4.00	4 Layers of Wax Cut	4.00
3 Layers of Tape			
Prepare Tape	5.00		
Cut & Clean Up	11.00		
Absorption Squares	2.00		
Hydrophobic and Surfactant			
Hydrophobic Treatment	5.00	Hydrophobic Treatment	5.00
Surfactant Paste	7.00	Surfactant Treatment	5.00
Hydrophilic Paste	5.00		
Assembly			
Absorption Area	1.00	Spray Absorption Area	0.17
+ 3rd Tape	2.00	Spray bottom of 4th Wax Layer	0.17
+ Absorption Pad	1.00	Adhere	0.17

+ 3rd Wax Layer	1.00		
Bottom Assembly		Spray Top of Bottom Assembly	0.17
+2nd Tape Layer	2.00	Spray bottom of 3rd Wax Layer	0.17
+ Absorption Pad	4.00	Adhere	0.17
+ Surfactant Paste Application	1.00		
+ Hydrophilic Paste Application	1.00		
+ Dry	10.00		
+ 2nd Wax Layer	1.00		
1st Tape Layer	2.00	1st Wax Layer	
+ Nitrocellulose	4.00	+ Clear Top Tape	0.17
+ Conjugate Pad	2.00	Spray Bottom of 1st Wax Layer	0.17
+ 1st Wax Layer	0.17	+ Conjugate Pad	3.00
+ Top Tape Layer	1.00	+ Nitrocellulose	4.00
+ Hydrophilic Past	2.00	Adhere to 2nd Wax Layer	0.17
+ Dry	10.00		
Bottom Assembly	1.00	Spray Bottom Assembly	0.17
+ 2nd Wax Layer		Adhere to Top Assembly	0.17
+ Top Assembly			
TOTAL STEPS	**28**	**TOTAL STEPS**	**17**
TOTAL TIME	**101.17**	**TOTAL TIME**	**38.17**

Table 4-3 summarizes the total number of steps and time needed to assemble 1 batch of 8 chips. The steps are explained in chronological order with the chips are generally built from a bottom up approach. With both methods, the chips are built from the absorbent layer up to but not including the 2nd wax layer (hydrophobic layer). The top half of the chip is built from the top down, starting with the top tape layer and assembling till the 2nd wax layer. The final step should be the addition of the top and bottom assemblies to the hydrophobic layer. It should also be noted that no adhesive is applied to the hydrophobic layer, but rather two applications of adhesive are made to the layers above and below.

As can be seen above, there are a few steps which are shared by both assembly methods. However, the overall number of steps needed with the double

sided tape method is 50% greater than the assembly methods of spray adhesive. Also, the time needed is 150% more than with the spray adhesive. Again this is largely due to the number of materials, steps in assembly, and the drying times necessary for the double sided tape method.

Additionally, the double-sided tape tends to be cumbersome to handle and needs more time compared to simply spraying the components with adhesive. Previously the double-sided tape restricted the dimensions of the overall chip. This was partly because the tape is 36mm wide. This would end up defining the overall length or width of a chip. If chips were designed any longer or wider than this width, then there would be the potential for fluid to travel along the seams of parallel tape strips which lay between the various layers of wax. With the spray adhesive, there are a greater range of dimensions and geometries that may be employed which do not have to conform to the size of the double sided tape.

The double sided tape cannot be overlapped, as it has a backing that needs to be removed prior to application. If the tape strips are overlapped, the removal of this backing will be obstructed.

Although multiple spraying steps are described in the chart above, these can be further reduced by spraying all the layers at once. For example, the images below provide some intuition for the spraying of multiple layers and their subsequent assembly.

Spraying of Layers **Assembly of Layers**

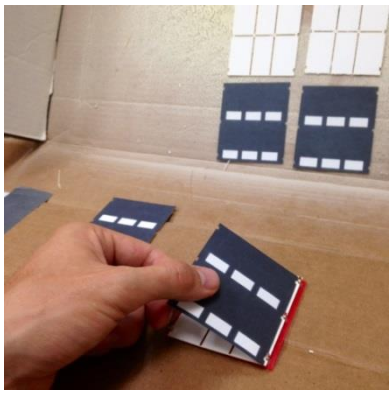


Figure 4-6: Spraying and Subsequent Assemble

Comparatively, the spray adhesive method is a preferred due to the reduced time of and ease of assembly. Especially when prototyping new designs, or testing out new components, the faster time for assembly and easier handling make the spray adhesive a superior method.

4.4 Three fluid device assembled with spray adhesive

A chip capable of handling the sequential flow of three fluids is an integral component of this research. In order to prove the complete efficacy of the spray adhesive, a 3 fluid chip was designed based on the findings of the protocol development chip. When comparing the two (Protocol Development Chip & 3-Fluid Chip) one can see distinct similarities. One major difference is the size at which this chip was developed. Traditionally, a batch of six chips was designed to be 69mm in height. This was primarily done due to the width of the double-sided tape. Since the new assembly is not restricted by this width, a larger design was developed to be 80mm in height for a batch of 6 chips. However, if these chips were to move into a scaled up production, appropriate sizes of double-sided tape could be made in order

that they don't become the limiting factor of the design. Still, within a mass fabrication setting double sided tape may pose problems in assembly.

4.4.1 Confirmation of Sequential Flow

The following images show a sequence of flow along with the times that were recorded when fluids arrived into the 1st wax channel. Two separate chips are shown with sequential flow of a three fluids chip.

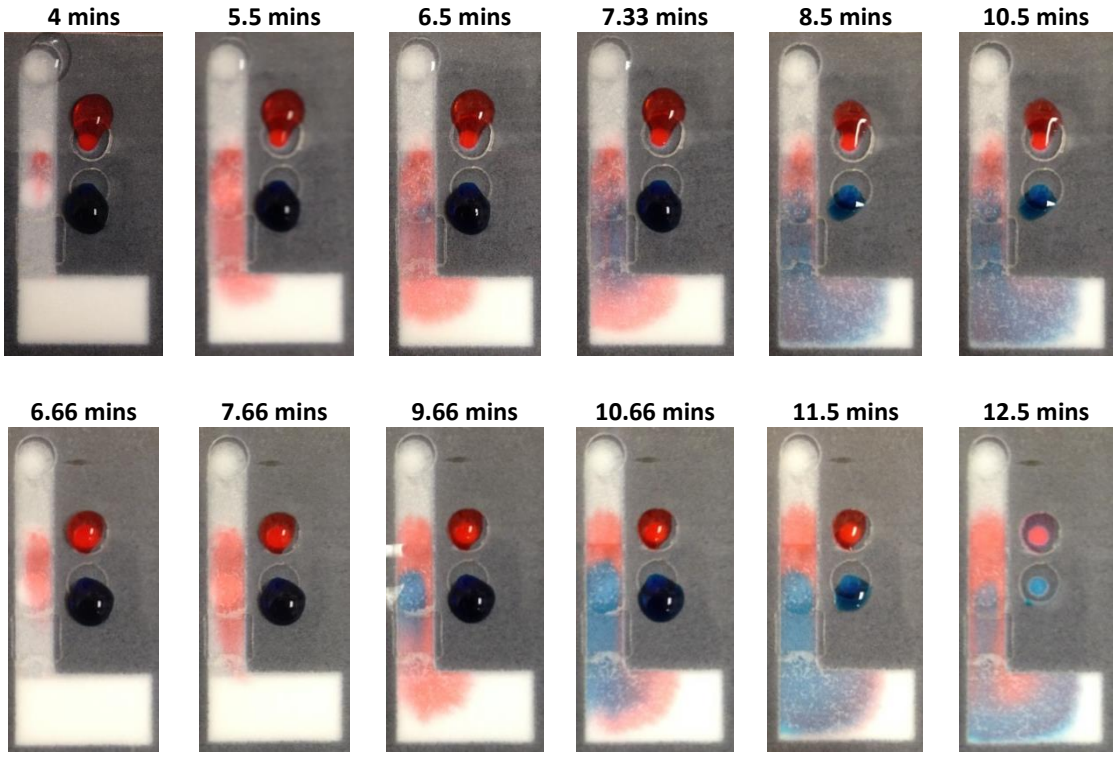


Figure 4-7 : In-Chip Sequential Flow of Three Fluids

These were two chips out of 36 which were tested under the parameters described in section 3.7.2. Of the 36 chips tested, 22 chips exhibited the expected sequential flow of first the red fluid then the blue fluid, leaving a total of 61%. The remaining 14 chips failed likely as a result of faulty hydrophobic treatment. As mentioned in the methodology section, hydrophobic areas were only treated with 2

µL. If this was increased to 4µL, the failure rate of the valves could decrease.

4.4.2 Controlling Backward flow

In an effort to reduce the amount of backward flow within the 3rd wax layer the narrower sections or 'kinks' were added to the channels to restrict flow. Seen below, are the effects of adding these narrowed sections.

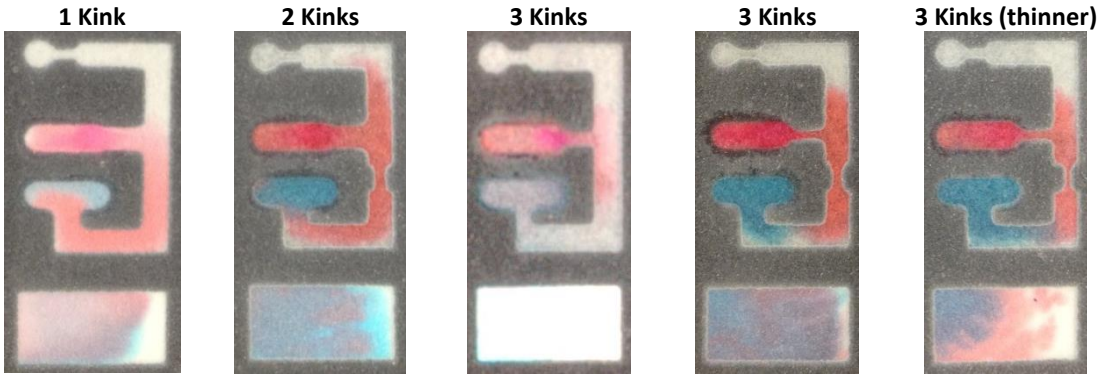


Figure 4-8: Results of kinked areas within the 3rd wax layer

As the designs incorporate more 'kinks' within the second layer, it is clear that the fluids are more restricted. This is most clearly seen by the occupied by the red fluid. With the addition of the kinks the red fluid progressively travels a shorter distance from its origin. For the blue fluid, a kink was added to the middle picture above. There is a narrower channel below the pill shaped area that connects the blue fluid to the main channel. Although the photo is not as clear, the blue fluid manages to travel to the area in the channel just before the contraction. Again, it is favorable to lose less fluid, especially biologicals, which could be detrimental to running a diagnostic test.

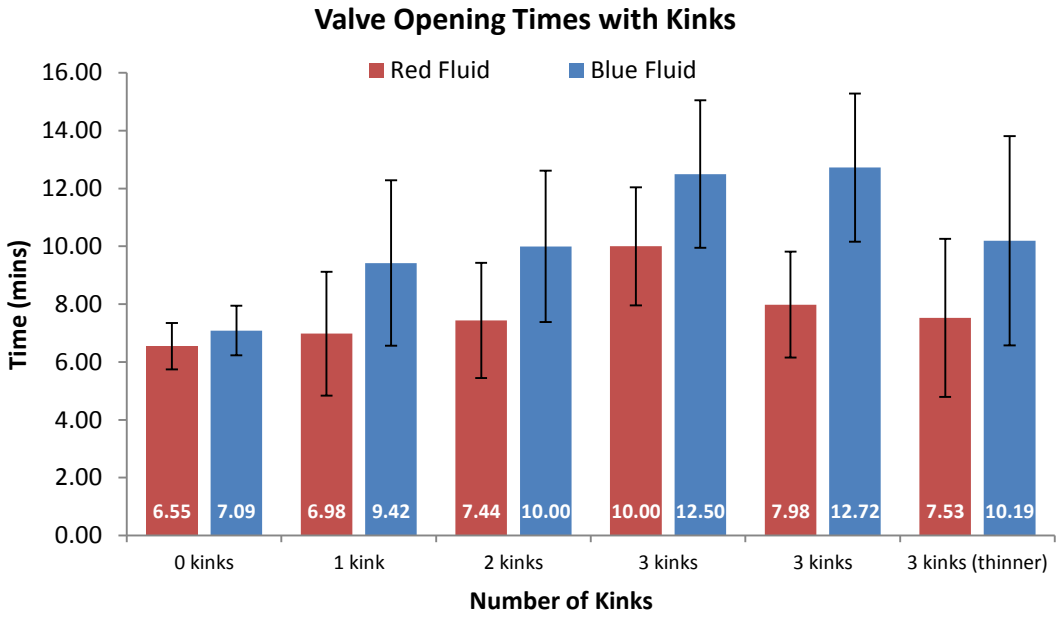


Figure 4-9: Effects of 'Kinks' on Valve Opening Times

When one kink is added to the second wax layer, the average time needed for the valve to open increases by about 2 minutes. This phenomenon is seen again when a third kink is added to the design of the third layer and the secondary fluid needed yet two more minutes to open.

By using this design element, a higher level of control over the fluids is achieved. This element can be used effectively to control backward flow and additionally can be used as a delay for secondary and tertiary fluids. This design element may be combined with the ability to add different volumes of hydrophobic treatments to achieve even a higher level of control with in a multi-fluidic device.

4.5 Enzymatic Activity Test

4.5.1 Alkaline Phosphatase Activity

As a model for the eventual enzymatic test of IαIP, the following results were obtained from the ALP enzymatic activity test.

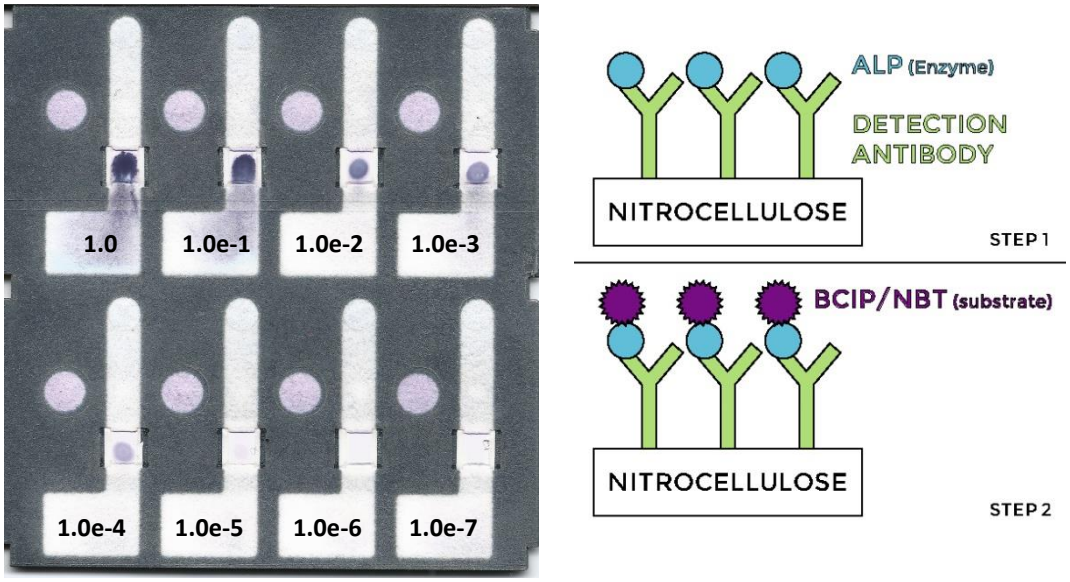


Figure 4-10: Image of the results obtained from the Enzymatic activity test. Various concentrations of detection antibody (Mouse Monoclonal antibody labeled with ALP) were placed on the nitrocellulose. The concentrations of applied detection antibody are shown at the bottom of each chip and are in the units of 'mg/ml.' After the detection antibody was placed on the nitrocellulose it was allowed to dry. The substrate BCIP/NBT was then added to the top of each chip. As it flowed down the main channel, the above color reactions developed as a result of the ALP-BCIP/NBT reaction. This has been graphically displayed as well.

When processing the dot intensities with Imagej, a value between 0 and 255 is obtained. This is the RGB scale with white registering at 255 and black at 0. With these in mind, the chip above would produce a downward sloping response curve. In order to produce and upward sloping curve, the data is corrected by subtracting from 255. This results in a white value of 0 and a black value at 255. Under these calculations the following chart was obtained.

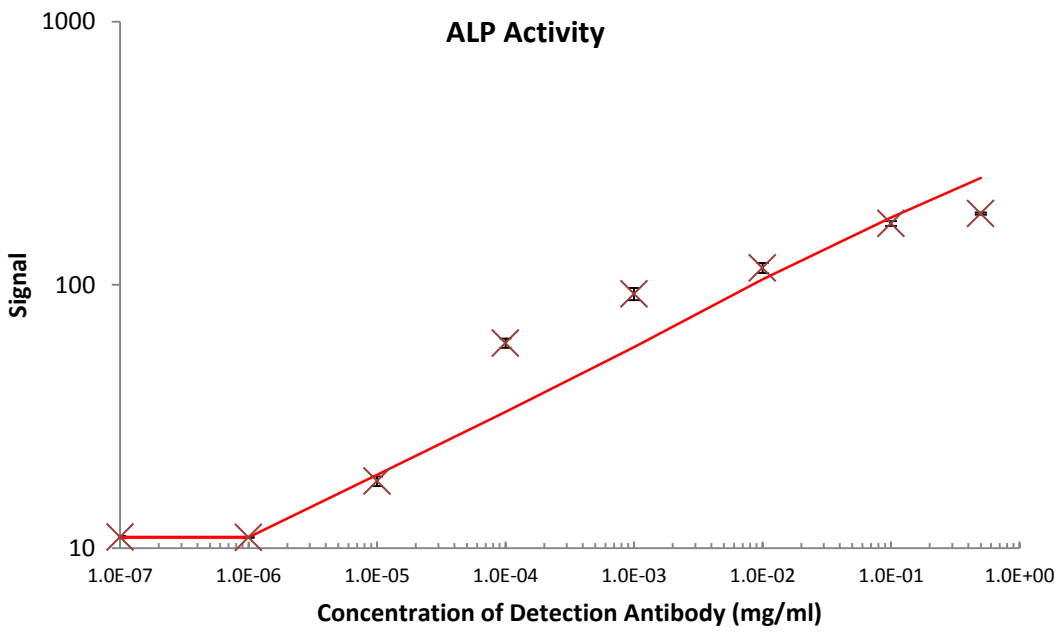


Figure 4-11: Results obtained from the Enzymatic activity test. The crosses represent the values collected corresponding to each concentration along with error bars. A line of best fit has further been included (red line). Various concentrations of detection antibody (Mouse Monoclonal antibody labeled with ALP) were placed on the nitrocellulose. The signal returned is indicative of the interaction between ALP and the BCIP/NBT substrate. Methodology can be found in section 3.8.

The activity response of ALP is plotted on a log-log scale in accordance with Macarron and Herztberg [58]. As can be seen in the above chart, the signal is a maximum with the concentrations of 0.5mg/ml and 0.1mg/ml. The lowest response is with 0.1e-6 and 0.1e-5 mg/ml with values at approximately 10 on the RGB scale. The above chart follows $\log(y) = k * \log(x)$ with k being the slope. For the points between 10^{-7} and 10^{-6} k=1, and for the sloping area between 10^{-6} and 1 k is approximately 0.24 or $\sim{}^{1}/_{4}$. This indicates that there is route of 4 in the sloped region, suggesting some level of signal saturation at higher concentrations of ALP. The values obtained further show a low level of variance, as can be seen by the standard deviation bars.

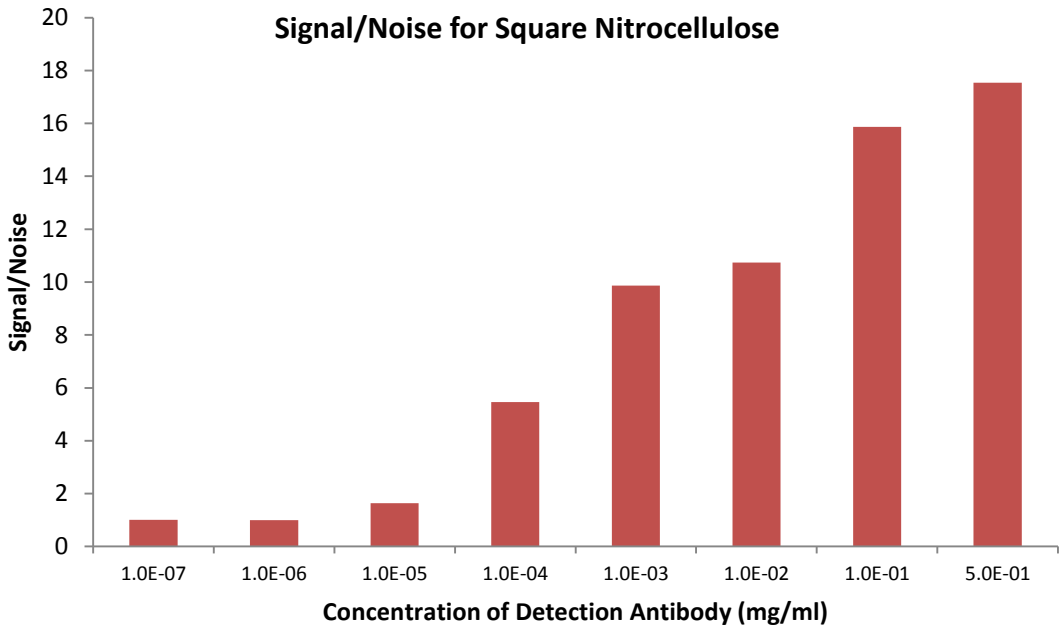


Figure 4-12: Signal /Noise Results obtained from the Enzymatic activity test. Various concentrations of detection antibody (Mouse Monoclonal antibody labeled with ALP) were placed on the nitrocellulose. The signal returned is indicative of the interaction between ALP and the BCIP/NBT substrate. Methodology can be found in section 3.8.

The signal to noise ratio is calculated by dividing the signal obtained from the purple spot by the signal from the clearest point on the nitrocellulose. The signal on the nitrocellulose was around 245 on the RGB scale and when it was inverted it came out to about 10. Again, the data is presented on a log-log scale in accordance with [58].

As mentioned previously, ALP and BCIP/NBT are one of the most widely used enzyme-substrate reactions within ELISA. One of the major reasons for this is that are stable and produce a high signal to noise of nearly 20, as seen by the above chart.

4.5.2 Nitrocellulose Varying Widths

When moving to a narrower width for the functionalized area of the

nitrocellulose, there was not much deviation between each width and the signal returned from the addition of BCIP/NBT. Although there is no differentiation between each width, the below data further confirms the possibility of performing an enzymatic activity test on a lateral flow device. The signal returned matches the original data and shows the reproducibility of this test.

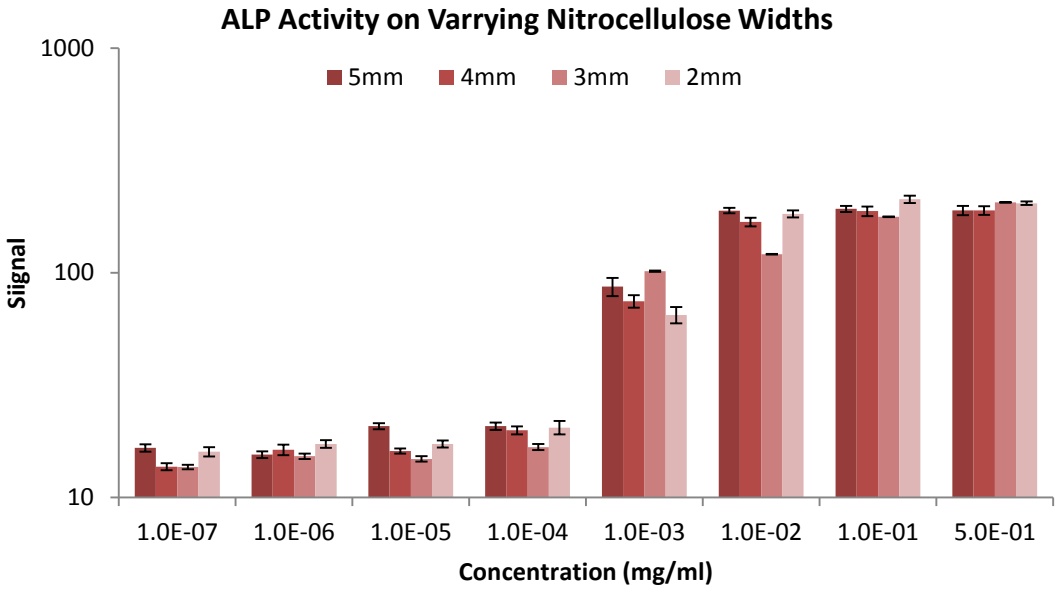


Figure 4-13: ALP Activity after isolating an ALP labeled detection antibody on Nitrocellulose of the on Nitrocellulose of Various Width (5, 4, 3, & 2mm). The various widths of nitrocellulose are displayed in the legend. Images of the nitrocellulose may be found in 3.8.2.

The following data shows the signal to noise ratio observed after a signal was returned from BCIP/NBT-ALP activity. When comparing this data to the previously obtained data there is a slight decrease in the Signal/Noise ratio. This value will change depending on if the nitrocellulose has dried completely or not. After examining the assembly, one may notice that nitrocellulose sits directly upon a black wax area from the 2nd wax layer. When the nitrocellulose is wetted from a sample it

becomes slightly transparent, therefore the intensity of the white color is not as strong. If the nitrocellulose is allowed to dry for 30-60mins prior to scanning and processing, the signal to noise ratio will increase as the contrast between the purple signal and the white background increases.

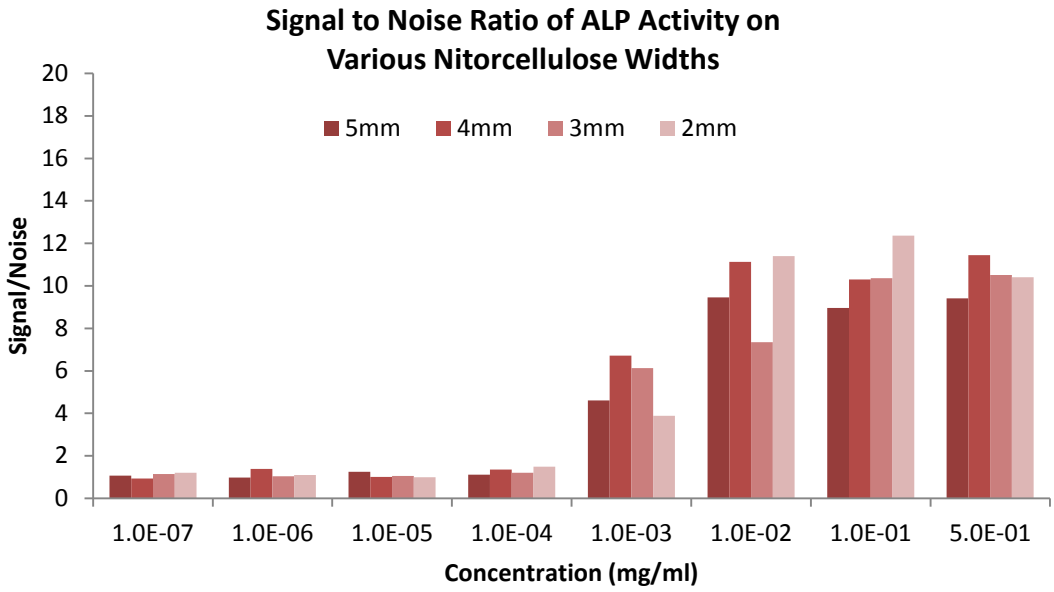


Figure 4-14: Signal to Noise of the ALP-BCIP/NBT interactions after isolating an ALP labeled detection antibody on Nitrocellulose of the Varying Widths. The various widths of nitrocellulose are displayed in the legend. Images of the nitrocellulose may be found in 3.8.2.

Although the signal created by the substrate is not altered to a significant degree, there may be cases in which the narrowing of nitrocellulose plays an effect. For example during the step when detection complexes flow over the nitrocellulose, more of these complexes may be captured within a thinner nitrocellulose which would have capture antibodies on 80-95% of the width.

The laser cutter provides some restrictions in this method. When cutting a nitrocellulose sheet, high speed and low power settings must be used so that the fibers are not damaged. With these settings, intricate cuts do not come out as

cleanly. For example diagonal lines end up being perforations and require much time to separate from a nitrocellulose sheet after cutting.

In summary, this test reinforces the fact that it is possible to run biological experiments on a chip that has been assembled with spray adhesive. The favorable assembly times and ability to run biology within this platform present a convincing case for spray adhesive as a method for assembly.

4.6 Reducing Background Signal

After performing the various washes of HRP-Strep, the following signal/noise ratios were calculated with ImageJ.

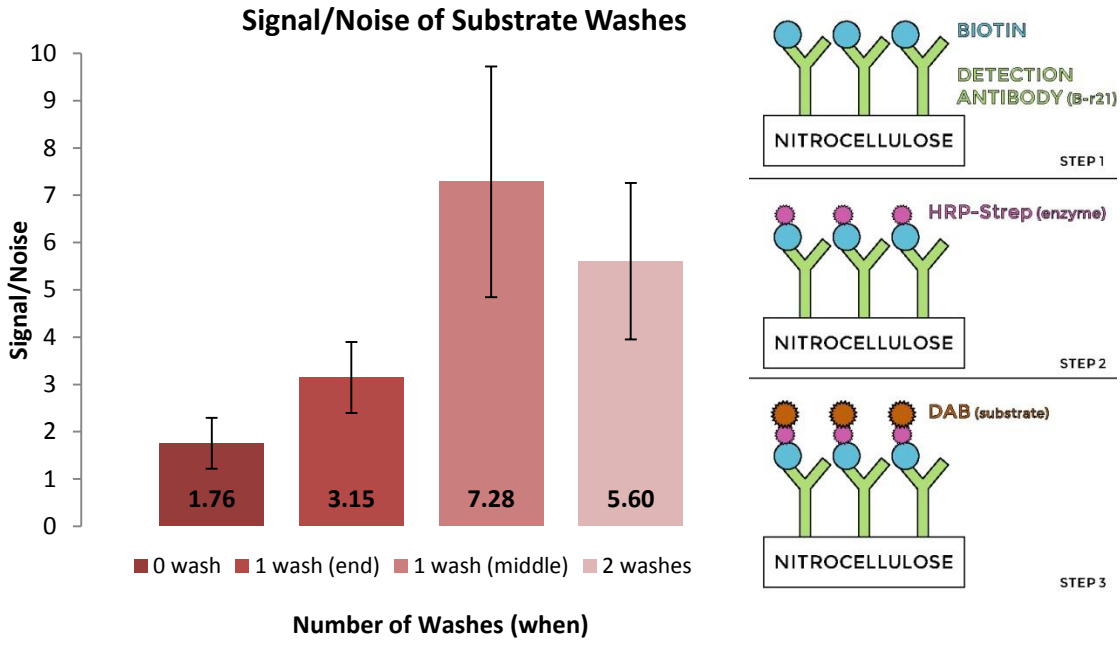


Figure 4-15: Signal/Noise calculated during the washing steps of HRP-Strep (enzyme) and DAB (substrate). The biological reaction is shown in the illustration on the right hand side. Biotinylated Antibodies are placed onto nitrocellulose, then the HRP-Strep is added and binds to Biotin, lastly, the DAB substrate is added to create the color reaction. Wash steps were added intermittently after step 1 or/and step 2 in order to investigate the chance to clean up the background signal. Methodology can be found in 3.9.2.

It can be seen from the above chart that as more washes are used during the addition of HRP and DAB, a higher signal/noise ratio is achieved. When adding a wash step at the end, after both HRP and DAB are added, the signal to noise ratio is doubled. A higher ratio may not have developed here due to the staining of the nitrocellulose that occurs when DAB is added to the chip. In preliminary tests, there did not seem to be a significant removal of the background noise when only a wash at the end was added. However, when adding a wash step between the addition of HRP and DAB, the signal/noise is increased 4&5 fold. There is not a significant difference between the '1 wash (middle)' and '2 washes', so it seems that only a single wash between HRP and DAB is enough to significantly reduce the background.

The results from the 0 wash (1.76) are slightly different from results obtained from Hanno Teiwes when he obtained a maximum of 1.2 for the signal/noise. This indicates that there may be other techniques to decrease the value of the background noise. After discussing this with Dr. Yow-Pin Lim, a suggestion was made that there may be excess biotin within the stock solution of the R21 detection antibody. As this excess biotin lingers on the nitrocellulose, it develops a signal as the substrate is added, resulting in a higher background signal.

4.7 Competitive ELISA Development

The following section focuses on the conduction of experiments working toward a functional and autonomous lateral flow device for the detection of IαIP through competitive ELISA. As mentioned in the methodology, many of the

optimization steps were conducted by pipetting fluids onto the main channel and allowing them to flow toward and through the nitrocellulose. An autonomous test was used only toward the end of the research.

4.7.1 Optimization of Detection Antibody (MAB 69.26)

The first major goal in developing a competitive ELISA was to maximize the signal that could be generated by optimizing the amount of capture antibody (MAB 69.26). As mentioned in the methodology, the antibody was added in separate 0.6µL volumes. This was done because a 7x4mm nitrocellulose could not handle higher volumes without overwhelming the nitrocellulose. By adding only 0.6µL the effective detection zone could be controlled by making it a clear dot that fit within the confines of the nitrocellulose geometry.

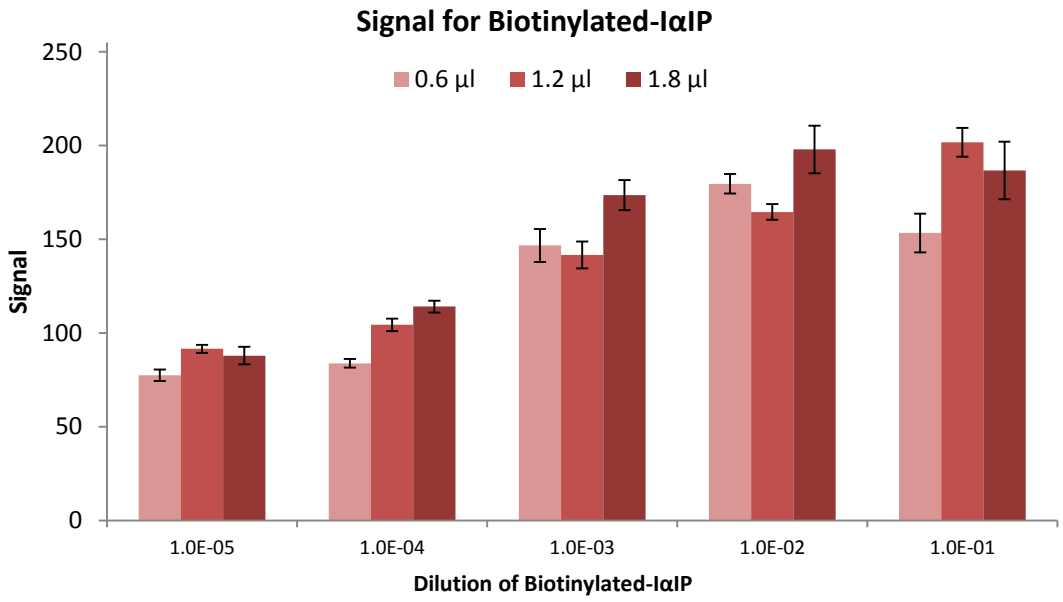


Figure 4-16: Signal returned the capture of Biotinylated-IαIP by Monoclonal Antibody 69.26. Various volumes (0.6, 1.2, 1.8µl) of capture antibody were placed onto nitrocellulose. Afterward HRP-Strep and the substrate (DAB) were added.

After scanning the chips and calculating the returned signal from the capturing of B-IαIP, the 1.8µL volume showed to generally capture more protein and therefore produce a higher signal. With the 1.8µL volume a signal of nearly 200 can be generated, which is in line with the results from ALP capture.

On the following chart the signal/noise is plotted. When comparing the wash tests, a similar signal to noise ratio of 2.5 is achieved only at the higher volumes of 1.8µL. A signal/noise of 7 was not reached mainly because of the increased background noise that occurs as a result of staining from the HRP-Strep/DAB substrate. However, when comparing the experiments of the 'Sandwich' ELISA, this is a 2-fold increase in signal/noise. For the reason of capturing the most amount of protein, the highest volume of capture antibody is needed.

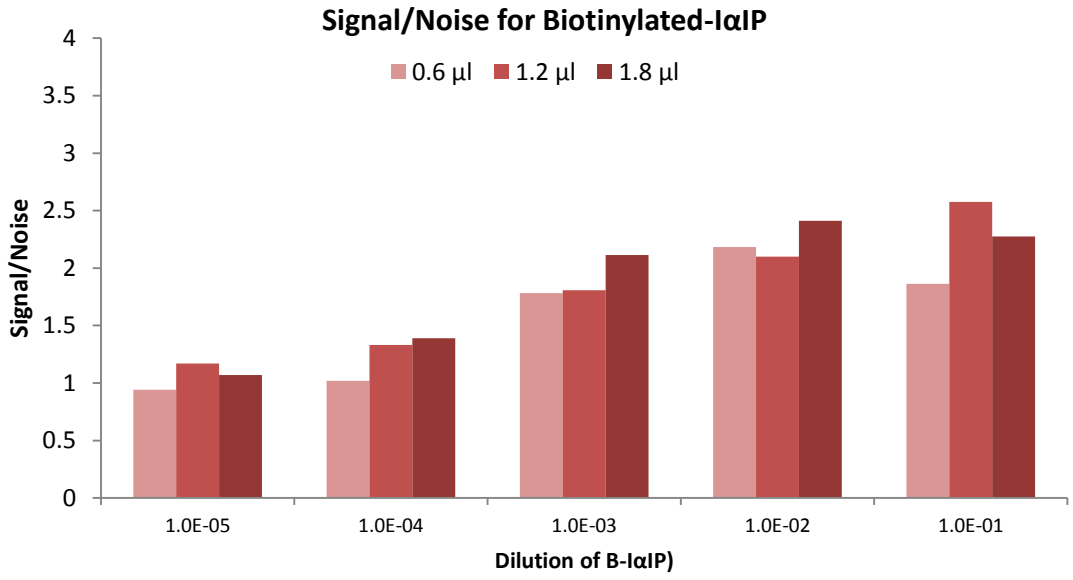


Figure 4-17: Signal/Noise returned Biotinylated-IαIP by Monoclonal Antibody 69.26. Various volumes (0.6, 1.2, 1.8µl) of capture antibody were placed onto nitrocellulose. Afterward HRP-Strep and the substrate (DAB) were added. Based on this, future tests would include a volume slightly higher, at 2µL, which would be added in 0.5µL increments. It offers the chance to capture the most protein and furthermore shows a general higher signal/noise over 0.6µL and 1.2µL.

4.7.2 Optimizing Biotinylated-IαIP Concentration

The next step involved determining the concentration of B-IαIP, which would serve as a competitor for the un-labeled IαIP. The goal was to determine the appropriate saturation point for B-IαIP (the point where signal does not increase with increased concentrations) with which to dilute the un-labeled protein with. This point can be found corresponding to the values just before the plateau or saturation region on the signal vs concentration curve. As shown, by the following graphs, the point at which this occurs seems to be between 1:100 and 1:400.

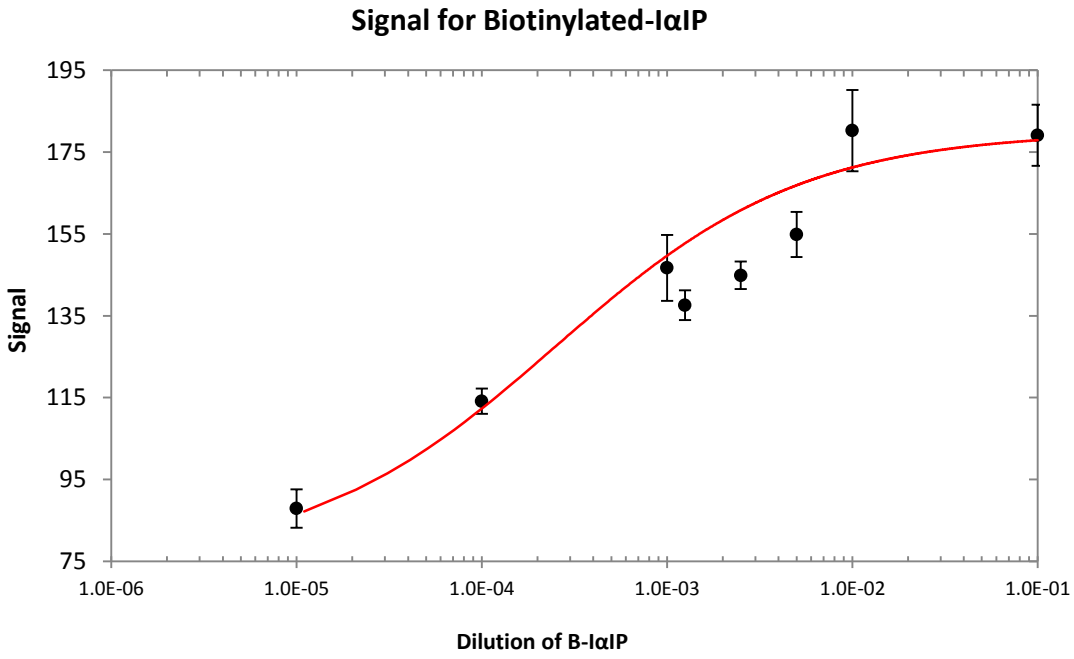


Figure 4-18: Results obtained during experiments conducted to optimize the competitor protein (Biotinylated-IαIP). Chips were prepared by placing the MAB 69.26 on to nitrocellulose then running various dilutions of B-IαIP followed by the HRP-Strep and the substrate, DAB. Methodology can be found in section 3.10.1

From this data the 1:200 and 1:400 dilutions would be used in attempt to generate a standard curve during the competitive set-up. These dilutions offer the

best chance for an un-labeled IαIP to effectively compete with a labeled protein.

4.7.3 Development of Standardized Curve

Since the optimal competitor concentration was determined to be between 1:200 or 1:400, dilutions of un-labeled IαIP were created and run through the chips. As shown by the below figures the first signs of a standardized curve are seen within the 1:200 B-IαIP. The signals returned have a clear plateau region which begins to decrease when the concentration of unlabeled IαIP in B-IαIP increased. There is a decrease in the signal produced as this concentration increases because of the fact that there is a lower number of labeled proteins available for capture relative to the number of labeled proteins.

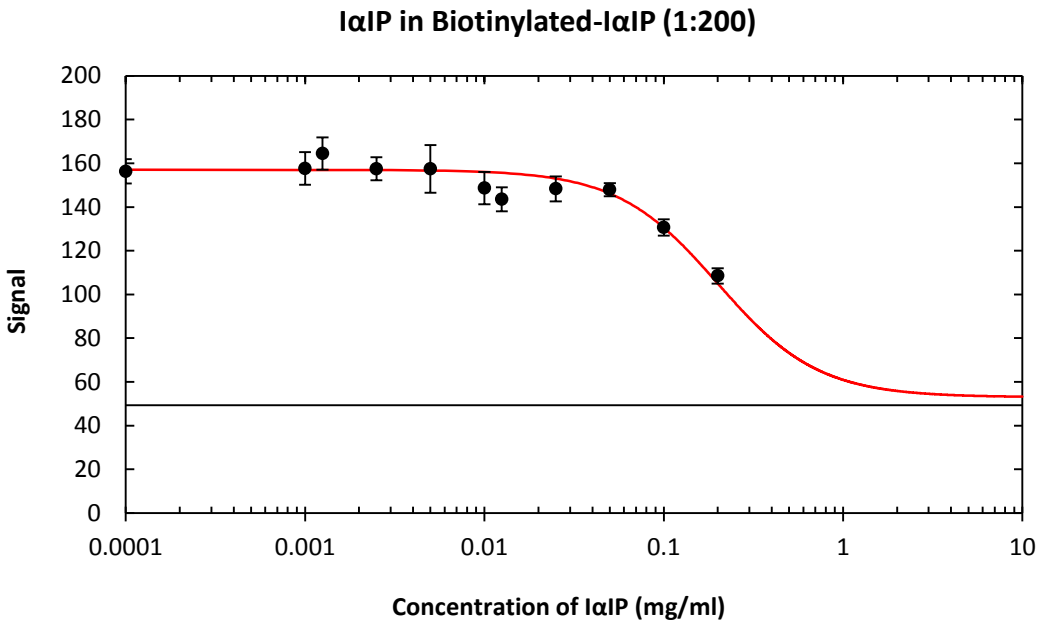


Figure 4-19: Signal returned when competing IαIP with B-IαIP (1:200 in 1wt.% Milk-PBST). After preparing the chips with the capture protein (MAB 69.26), various dilutions of IαIP are added to the chip followed by the enzyme and substrate. The reaction scheme can be visualized in section 1.7.2

On the above chart there is a lower black line at the signal value of about 47, this value represents the value of the background. Essentially it should be where the lower limit of detection should be.

Since the lower limit of detection is not visible, the experiment was repeated with dilution of the un-labeled IαIP in 1:400 concentration of B-IαIP in an effort to shift the standard curve and develop the lower plateau region. The results are shown in the below graph.

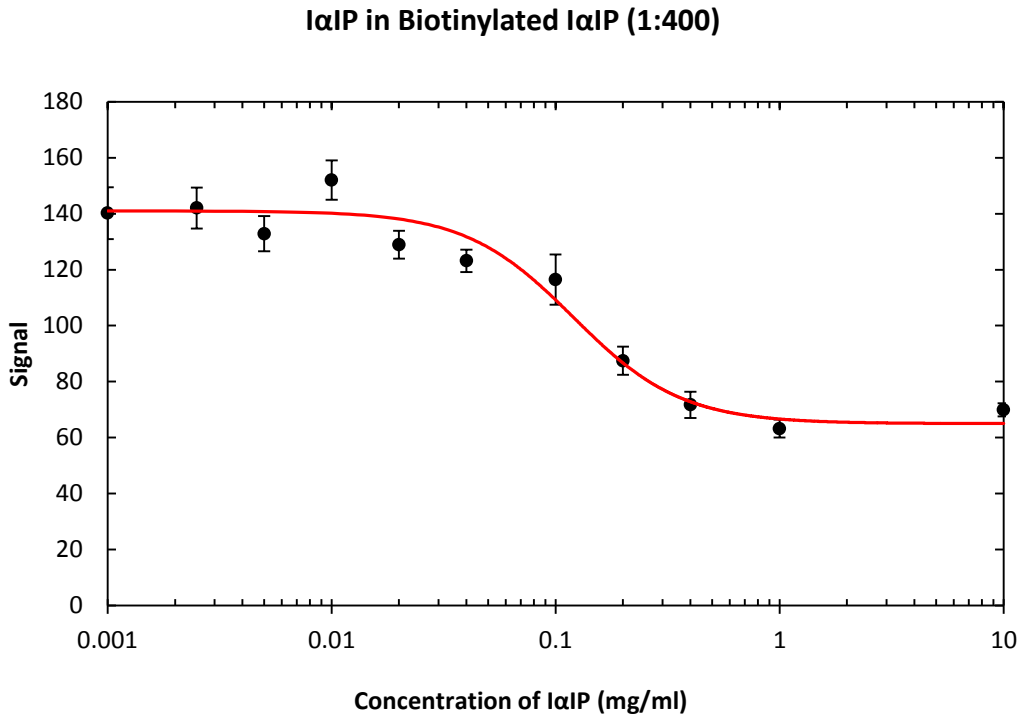


Figure 4-20: Signal returned when competing IαIP with B-IαIP (1:400 in 1wt.% Milk-PBST). After preparing the chips with the capture protein (MAB 69.26), various dilutions of IαIP are added to the chip followed by the enzyme and substrate. The reaction scheme can be visualized in section 1.7.2

When the concentration of B-IαIP is reduced to 1:400, the full standardized curve is developed. The curve shows the first signs of competition at 1:200 and signal continues to reduce until the 1:1 concentration.

Again, there is a steady decrease in signal which is indicative of competition and furthermore the lower plateau is developed. This curve can be used as the standard with which to compare individual tests. For example, if a sample resulted in a signal of 130 or more, this would be an indication that there is less than 1:500 concentration of IαIP within the sample.

From this data, it is clear that it is possible to detect the presence and further to quantify this presence using the competitive ELISA for IαIP on a paper-based platform. The next step is to move this test onto a fully autonomous platform in order to satisfy the requirements of a 'simple test' as developed by FDA (these are described in 1.2).

4.7.4 Removal of One Fluid

When moving toward the autonomous device it is important to minimize complexity where ever possible. When more fluids and thus more valves are introduced, the success rate of the chips falls. The following images show a comparison of two chips designed to capture IαIP. In an effort to try to minimize the number of fluids that need to be entered into this device, an experiment was conducted to rather add the HRP-Strep directly to the sample fluid (fluid containing B-IαIP). Only the 1:400 B-IαIP in 1 wt.% PBST was tested, as it would be the concentration that would produce the strongest signal. This served as a base line to move forward.

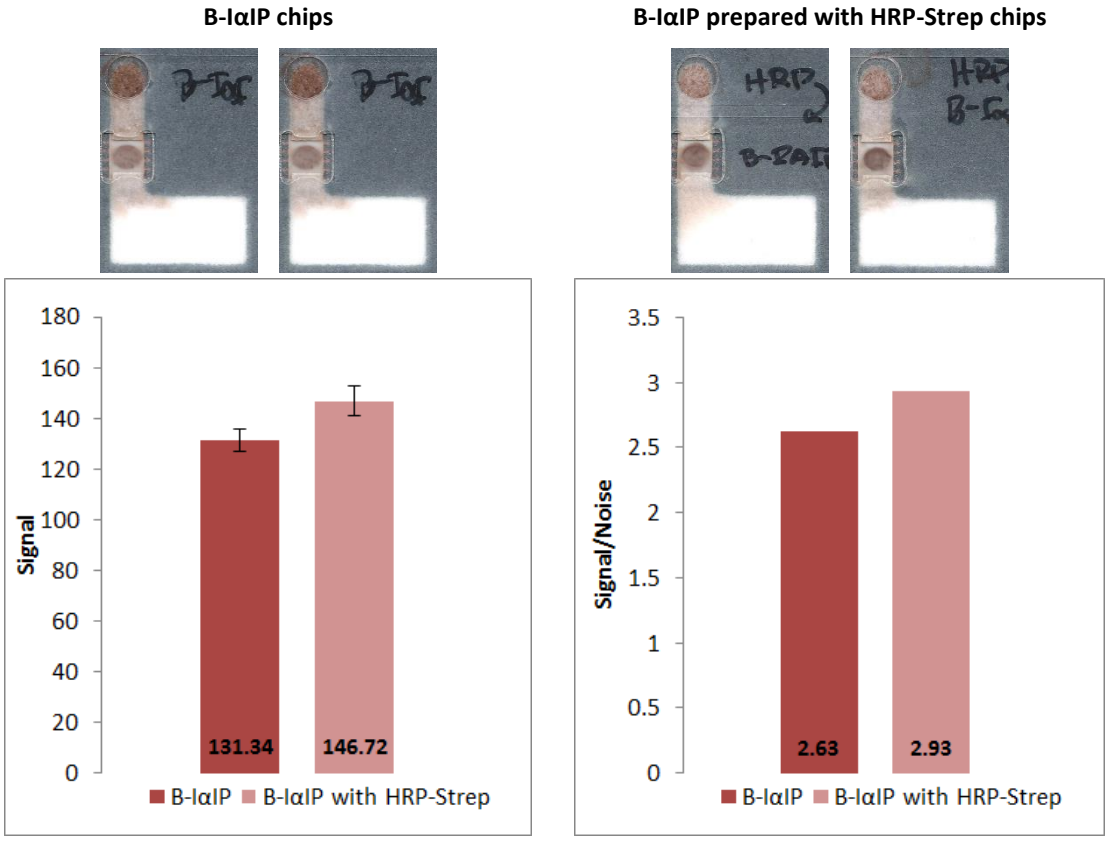


Figure 4-21: In previous experiments, first, the IαIP solution is added, second, the solution of HRP-Strep is added, then the DAB substrate is added to the chip. The above compares the previous method along with a method that incorporates HRP-Strep into the IαIP Solution, in essence reducing the number of fluids that need to be added to a chip. Included above are images of the signal generated on the Chips, along with the Signal and Signal/noise Charts. Methodology can be referenced in section 3.10.4.

The first two chips (in the upper left) tested the ability to capture B-IαIP and second two chips (in the upper right) tested the ability to capture B-IαIP which was prepared with HRP-Strep prior to its addition.

As can been seen by the above images, adding HRP-Strep to the prepared solution rather than to the chip achieves comparable results. Not only is a slightly stronger signal achieved, but also the signal to noise ratio is increased. Although this was calculated by ImageJ the difference can be seen by the naked-eye. This confirms that the HRP-Strep may be integrated into the sample fluid and furthermore that a

PBS wash can take its position in a 3-fluid circuit, when moving toward the development of an autonomous chip,

Another finding of this experiment came as a result of changing the dilution fluid from 1wt.% Milk-PBST to just PBST. As the results were calculated this change yielded a much higher background signal. So it seems that the 1wt.% Milk-PBST is an integral component for reducing the background signal and thus increasing the signal/noise.

4.7.5 Complete Competitive ELISA

After determining that the competitive ELISA could be run with only three fluids, forgoing the necessity to design a more complex chip, the following results were achieved. The below table shows the signal created from each concentration ratio of IαIP to 1:400 B-IαIP in 1wt.% Milk-PBS solution. Again it is important to note here that PBS was used instead of PBST, as Tween 20 is the surfactant that is responsible for opening the valves.

Table 4-4: Complete Competitive ELISA Results for each Concentration of IαIP (µg/mL)

0.2	0.4	0.8	2.0	4.0	8.0	10	80	200	1000
									

From the above images it can be seen that the signal remains until the 1:5 dilutions, at which point it begins to fade due to the higher concentration of un-labeled protein. The following chart was generated from data of the above images which was calculated with ImageJ.

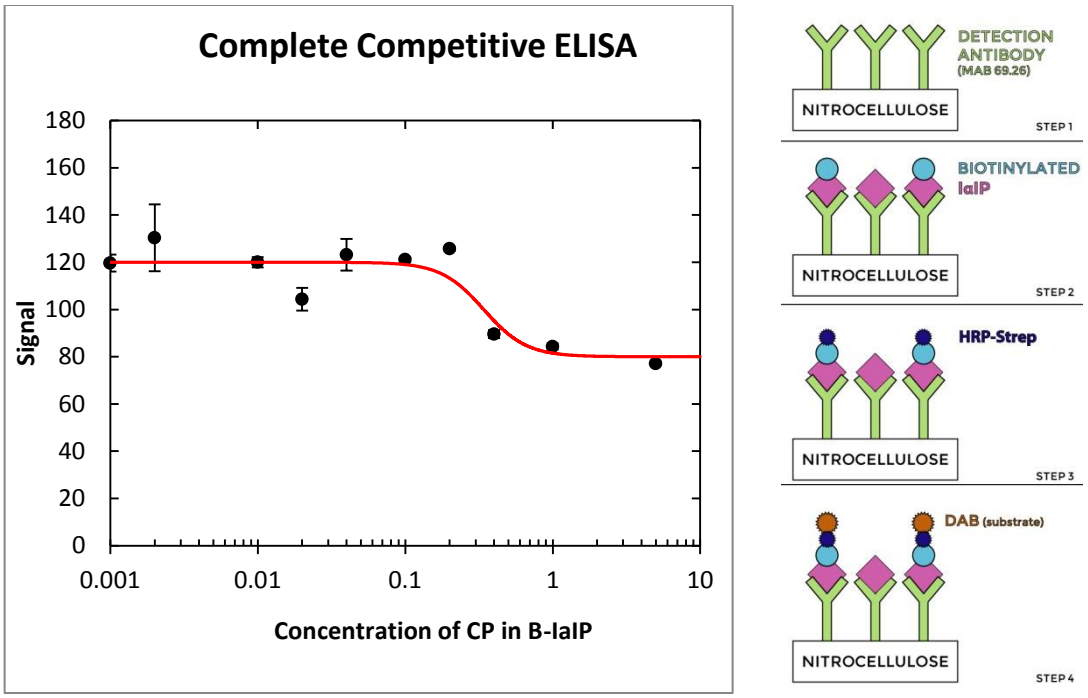


Figure 4-22: The above figure displays the computed results from a fully autonomous test. A three fluid device was fabricated and prepared with MAB 69.26. The sample fluid including HRP-Strep first flows through the nitrocellulose, then a wash step to remove unbound proteins, followed by the DAB substrate. The mechanism at play can be viewed in section 1.7.2. These results are calculated based on the images from Table 4-4. The Biological mechanism at play is also graphical displayed.

The curve again is reproduced and shows a decrease in signal at the same point as previously, at the 0.25-0.1 dilutions. Again this decrease of intensity occurs due to the higher number of un-labeled proteins relative to the single production labeled proteins. Within this test for samples containing a low level of un-labeled protein, signal/noise turned out to be slightly lower averaging around 2.

As can be seen by the above data, this paper based chip has the ability to run a competitive ELISA to quantitatively detect a concentration of IαIP within a sample. Compared to the current bench top test the range for the paper-based set up is lower. The bench top set up by Dr. Yow Pin Lim can detect concentration variations of IαIP as low as 1:320 and as high as to 1:5. The competitive ELISA on the multifluidic

platform is operating at 3-5% of that range. In order to make this a viable product that range needs to be increased.

5 CHAPTER 5 – CONCLUSION

During the course of this research the development of the Paper Based Diagnostic test was progressed in three particular areas. The first being the construction with spray adhesive, which drastically reduces the time for assembly and the number of necessary components. With a spray adhesive, the tests became more reliable and easier to assemble. It also allowed for greater possibilities in geometry as the dimensions were no longer restricted by the widths of double-sided tape.

The second innovation was through addition of 'kinks', which added further control for timing and reducing loss of secondary fluids. This technique show cases the broad spectrum of possibilities that are available for a paper based device.

The third innovation and likely the most impactful came through the development of a competitive ELISA as well as a complete ELISA by Hanno Teiwes. By developing a standard curve for the detection of IαIP through the competitive ELISA scheme, a new method for detection is presented. This method is simpler, easier to use, and of course faster than conventional laboratory tests. An important finding through this study was the addition of HRP-Strep into the sample fluid which allowed for a less complex device. This may also be beneficial to the research of Hanno Teiwes as his device can become less complex as well.

5.1 Recommendations for Future Work

Within this section directions for further research are discussed to further streamline the fabrication, reliability, and application of a PBD.

5.1.1 Increasing detection range

Increasing the detection range capabilities is next major challenge to be tackled in this project. Compared to competitive ELISA conducted in a 96 well plate, the paper based set up is operating at about 5% the detection capacity. Greater ranges may require optimization of competitor protein concentration, adjustment of sample and substrate volumes, and potential increases in detection protein volume on the nitrocellulose. Up to this point this work serves as a proof of concept and a foundation from which to build upon.

Another problem with this methodology lays in the fact that all reagents are pipetted by hand. As a result this causes great variances in the various sample concentrations and especially in the detection antibodies. When adding detection antibodies with a pipette at low volumes (0.5 µL) it is impossible to be accurate. This inaccuracy then carries over into the signal generation which then has variances. When examining the data of Figure 4-19 and of Figure 4-20, the top and bottom plateau regions show the effects of this phenomenon. In those regions, there is not a perfectly level line. For example, if a mechanical instrument, one that has a high level of precision and accuracy, were to place detection antibodies smoother curves and more consistent data could be developed. In many test strips, a robotic needle is

dragged along a strip of nitrocellulose to create a test line by precisely placing a specific amount of detection antibody (examples of these can be seen in Figure 2-14).

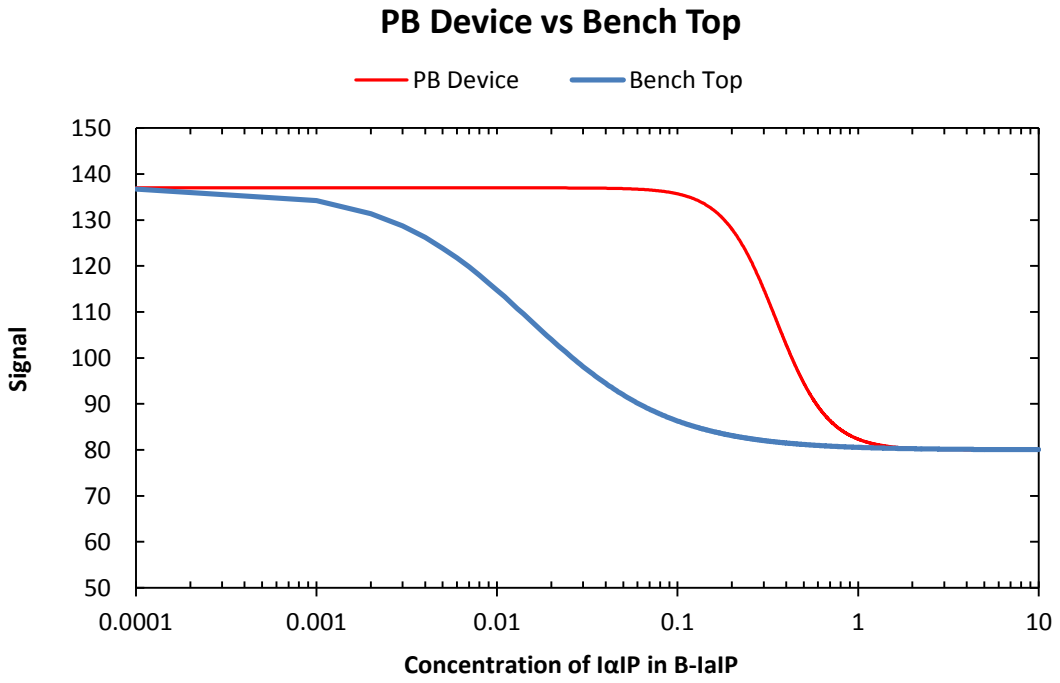


Figure 5-1: The capabilities of a paper based test are compared with the current capabilities of a bench top test for the detection of IαIP.

As a visual to compare the current state of this PB Competitive ELISA, Figure 5-1 shows the detection capabilities for the PB Device vs the current bench top set up used by Dr. Yow-Pin. As mentioned currently this test is can detect only higher concentration of IαIP of 1:5. Perhaps great advancements could be made through the précising that could be created by a robot. Such an instrument could greatly improve the consistency and reproducibility of detection ranges for this research.

5.1.2 Enzymatic Inhibitory Assay

As discussed in the second chapter, the enzyme trypsin is well known to interact with IαIP. Early in this research, experiments were conducted to test the possibility of developing an inhibitory assay for the detection of IαIP. Currently, this can be done in-solution with along with the substrate BAPNA. Preliminary test on paper yielded weak results due to the light yellow color change that is created by the BAPNA substrate. One option may be to investigate the chance to use another substrate such as ALP, which is shown to have a very high signal/noise in a paper based setup. If such a test could be applied to a paper-based device, there may be the chance to have lower limits of detection as compared to current bench-top test.

5.1.3 New Substrates

One of the weaker components of this research was the signal returned as a result of using DAB and HRP-Strep. After repeated experimentation the background signal proved too high, even with multiple wash steps. In order for this platform to be competitive with the current bench top set-ups, a higher signal/noise ratio needs to be determined. Perhaps this could be developed by using other substrates that create higher signal intensities. If biotin was instead conjugated with ALP, a BCIP/NBT substrate could be used, which has been shown to generate signal/noise in the range of 17-20 at the highest concentrations. There may also be the chance to add amplifiers as discussed in chapter 2, which could lead to better signal generation.

5.1.4 Construction

Another area that has a great frontier yet to be explored is in the construction of paper based devices as they pertain to competitive ELISA.

5.1.4.1 Miniaturization

In particular the miniaturization could improve this device in many aspects. The foremost improvement would come by the reduction of cost. As of now, each sheet of filter paper costs nearly $2. On this one sheet of filter paper, there is only enough room to print one batch of 6 chips (with all the necessary layers). A miniaturization would drive down the cost per chip. This would also offer the opportunity to run more optimization tests over a short period of time. Subsequently, smaller geometries may be able to have a higher yield of success.

5.1.4.2 Making Valves more Reliable

A large area of development may come in refining the valve technology. As it stands a 60% yield was achieved within this research, which needs to be improved if such a device were to go into mass production. This is one of the most important components to a multi-fluidic device as it offers the chance to run ELISA and other more complex tests than are currently available for PBDs. As biologicals are introduced into chips integrated with valves, further optimization and reconfiguring may also need to be refined.

5.1.4.3 Nitrocellulose Contact with Main Channel

One of the biggest problems seen in the development of a competitive assay was the connection between the main fluid channel and the nitrocellulose pad. Often there was not a strong enough connection to ensure consistent fluid transfer from the main channel through the nitrocellulose and further into the waste pad. This is the most important functional component in the device and it is imperative to the results to ensure a consistent reagent flow. Usually, a small amount of pressure was applied to the overlapping area of the main channel and the nitrocellulose which would speed along the flow. Perhaps this pressure could come from an enclosure or housing.

6 CHAPTER 6 – Appendices

6.1 IαIP Structure and Physiological Pathways

Inter-α-inhibitor or Inter- α-trypsin inhibitor was isolated in the 1960's, and its function remained a mystery up until the research in the last two decades revealed much about its structure and physiological influences [32].

The IαIP is made from three polypeptides including two heavy chains and one light chain. Another major component is the bikunin end which is responsible for the inhibitory activity of IαIP.

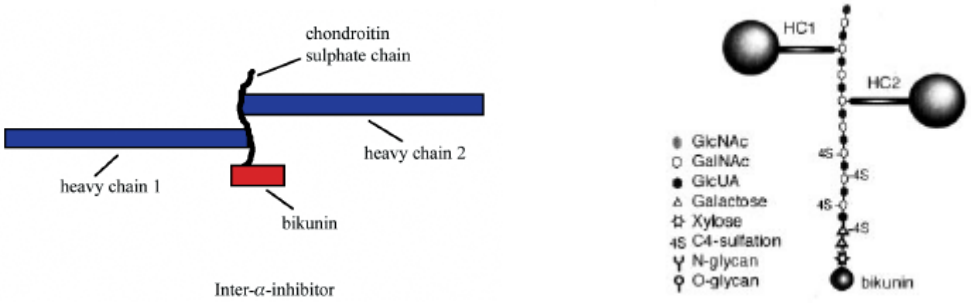


Figure 6-1: Illustration Inter-alpha-Inhibitor protein structure [32] [59]

IαIP is synthesized in the liver by hepatocytes, which are the cells making up 70-80% of the liver's tissue. Hepatocytes are responsible for protein synthesis, storage, transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, and are also responsible for detoxification, modification and excretion of exogenous and endogenous substances.

Initially the protein family was thought be involved with protease inhibition, however, it has been shown that proteases are more efficiently inhibited by their respective physiological inhibitors [60]. Research has shown that IαIP rather acts as a

shuttle that captures and transports protease inhibitors to the physiological inhibitors [61]. The bikunin end has been shown to also inhibit granzyme K, which is found in T-cells and is involved with cell-mediated cytotoxic defenses [62]. Other functions of bikunin included mitogenic activity, inhibitory activity of hyaluronidase, prevention of premature delivery, ischemia stress and renal failure, and suppression of pancreatitis, colitis and arthritis [63]. The research that was involved in these findings was mostly focused on bikunin, but another phenomenon has been shown with relation to this protein. [59]

In other areas of the body, bikunin has been present in forms both with one heavy chain and without any heavy chains. More functions are described with relation to hyaluronan (HA), a glycosaminoglycan which contributes to cell proliferation and migration, and also has shown functions with knee joint lubrication. HA has been found also in complex with serum-derived hyaluronan-associated protein (SHAP). HA is bound to SHAP though an ester bond. [59]

6.1.1 SHAP-HA, IαI, and inflammatory diseases

The SHAP-HA complex has additionally been found more highly expressed in patients with rheumatoid arthritis. The complex has been found within synovial fluid, which is needed for lubricated joints particularly in the knee. It has been suggested to play a role in the inflammatory response, by regulating the adhesive properties of leukocytes, or white blood cells. Leukocytes are also responsible for defending the body against foreign bodies and infectious diseases.

With application to inflammatory bowel diseases, the SHAP-HA complex also shows importance for binding leukocytes to the cells of inflamed tissues. A structure of extracellular HA complexes provide the sites for leukocytes to bind and activate.

6.1.2 A Graphical View of IαI and its pathway

The main pathway and interactions of IαIP are summarized by the below image.

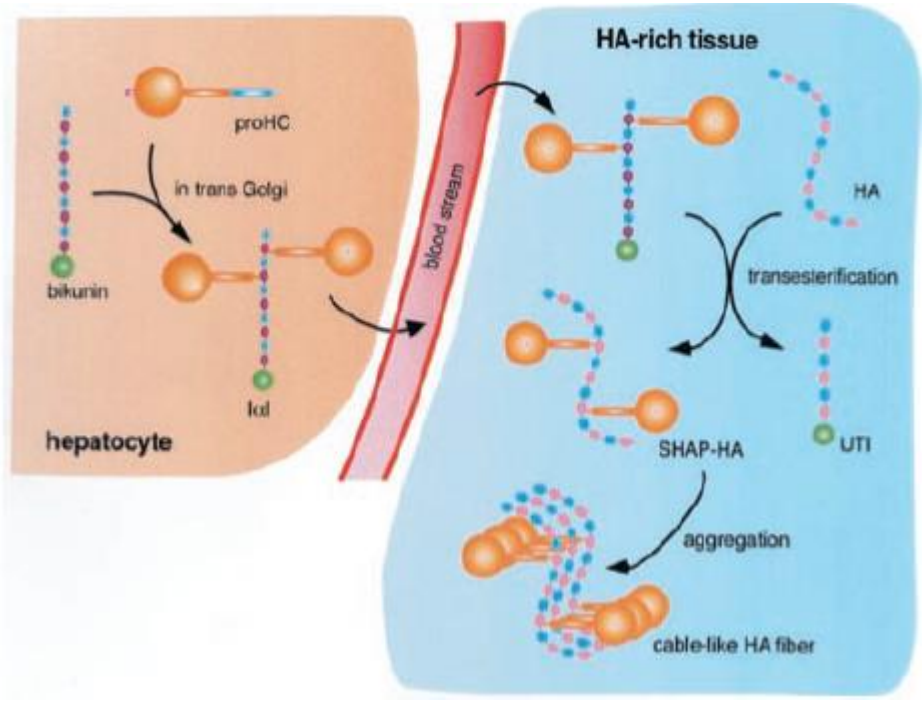


Figure 6-2: Illustration of the formation of Inter-alpha-Inhibitor protein and a depiction of the interaction with HA and SHAP [59]

When IαI is formed in the liver it travels to the blood stream and eventually reaches HA-rich tissue. At that point the heavy chains are transported to locally available HA and form the SHAP-HA complexes. These complexes aggregate into cable like structures and additionally play a role in tissue matrices of inflammatory tissues [59].

6.2 Dimensions of Components

6.2.1 Alignment Tool Dimensions

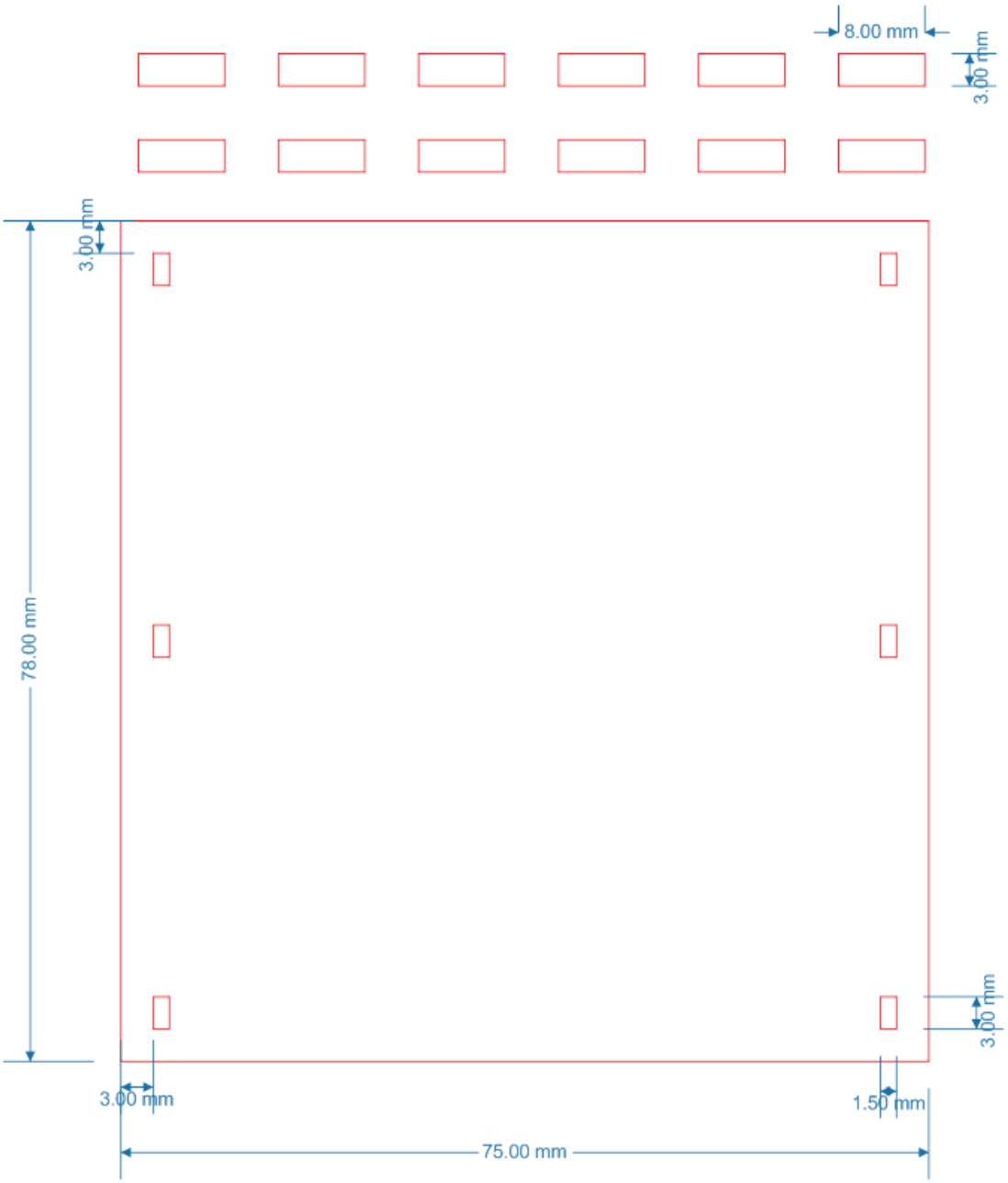


Figure 6-3: Dimensions of Alignment Tool

6.2.2 Modified Design

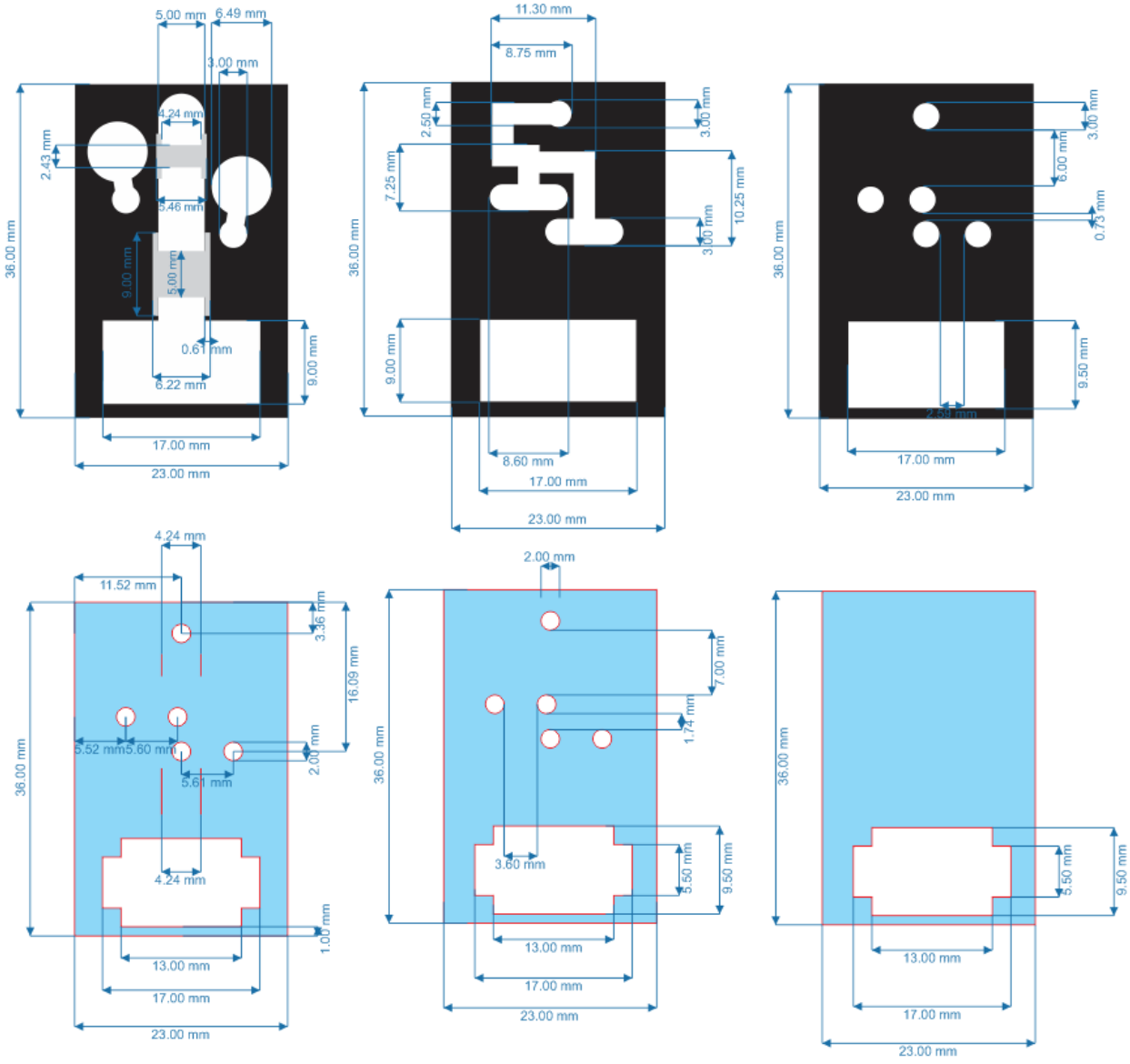


Figure 6-4: Dimensions of the New Design (Printed in Batches of 6)

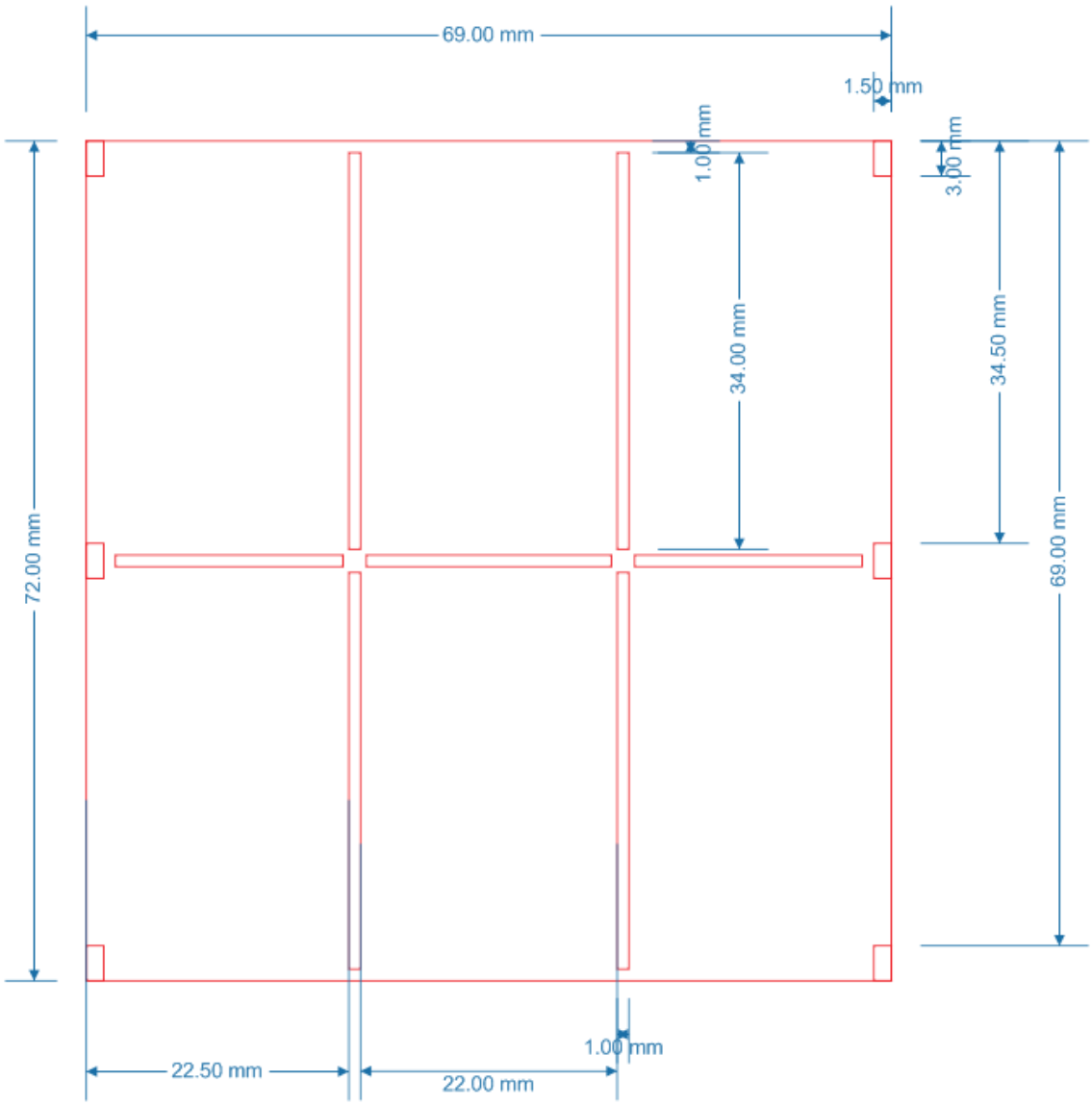


Figure 6-5: : Dimensions of Modified Design Absorption Area

6.2.3 Protocol Development Chip

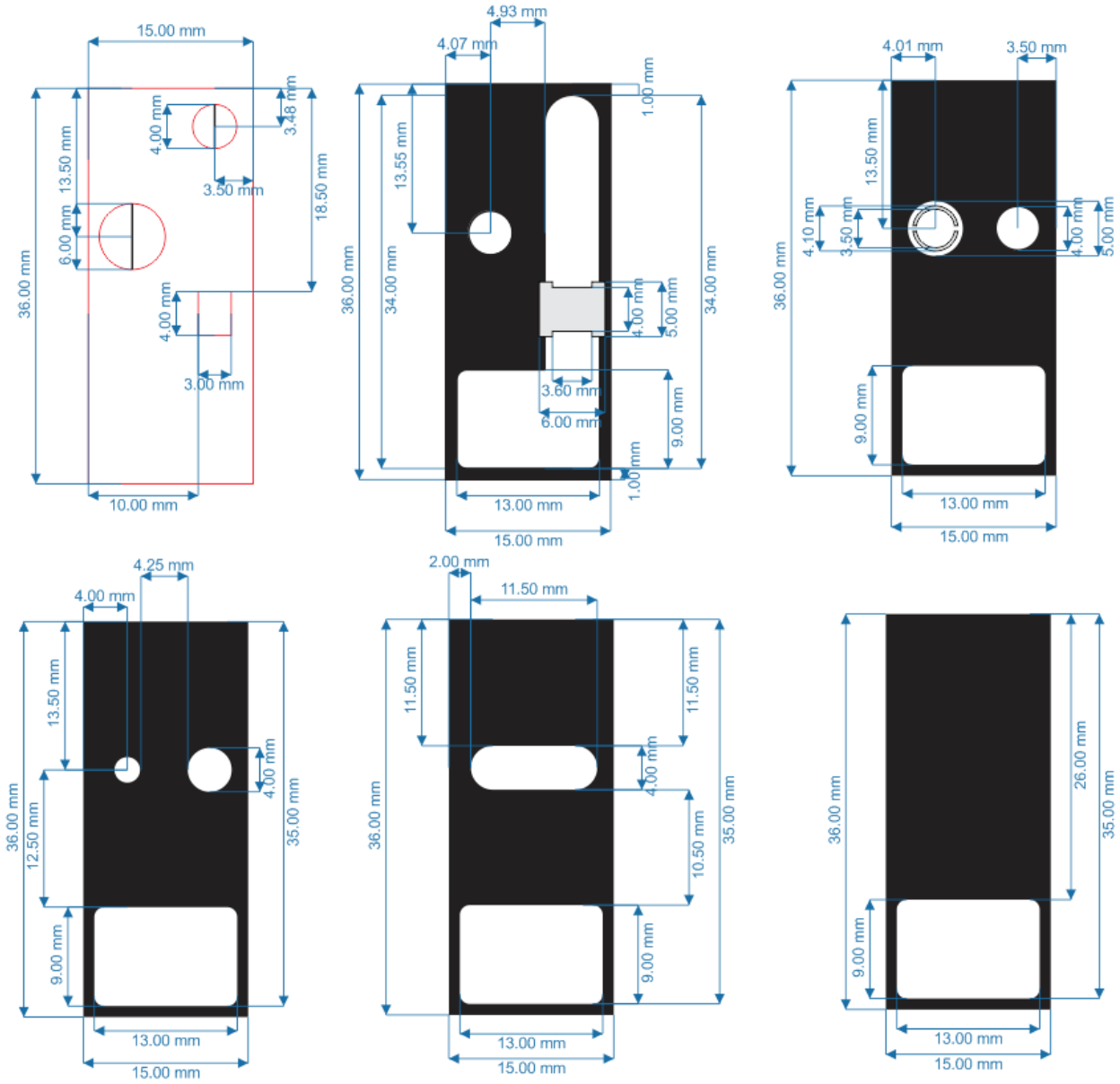


Figure 6-6: Protocol Development Chip Dimensions (Printed in Batches of 8)

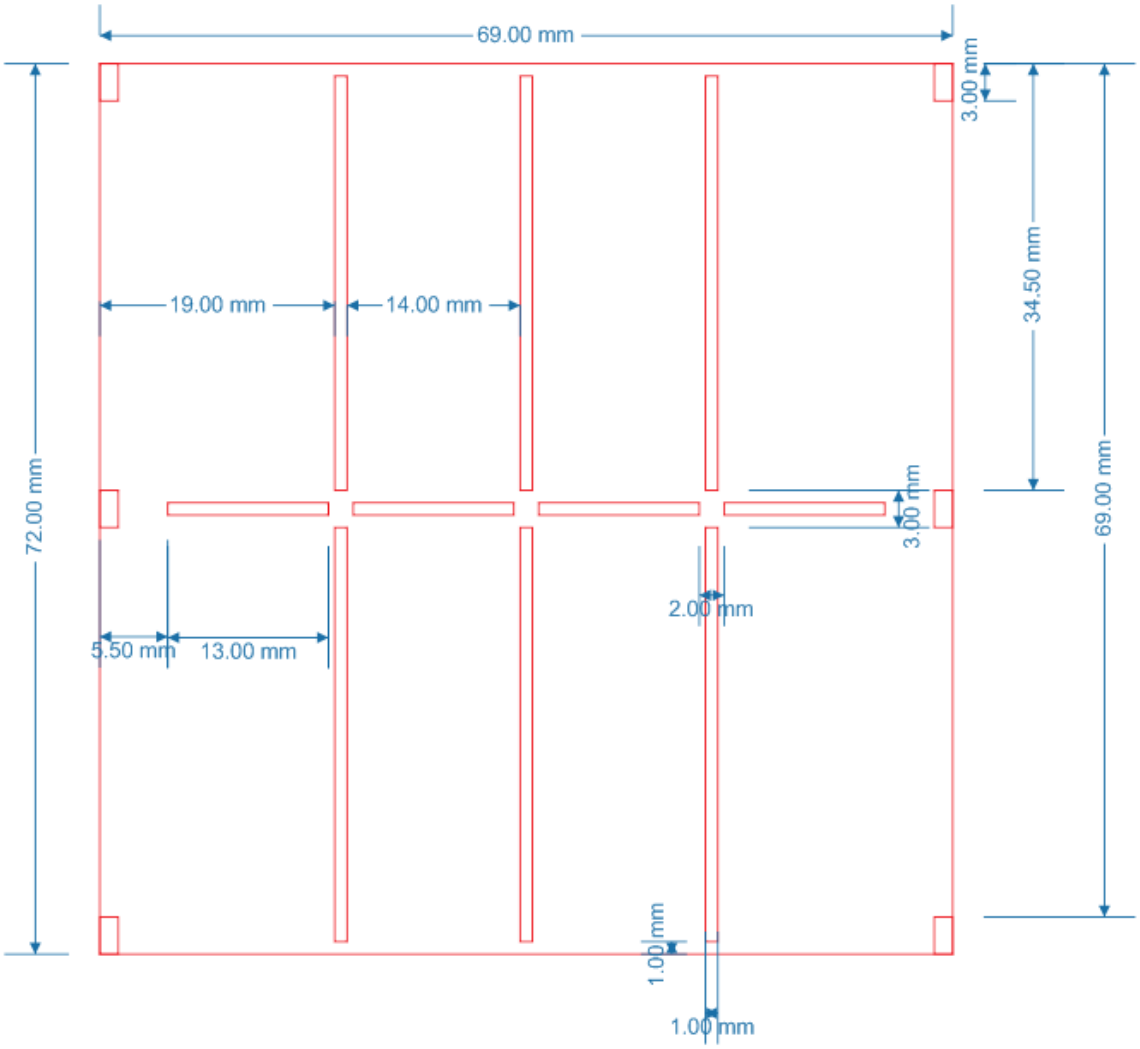
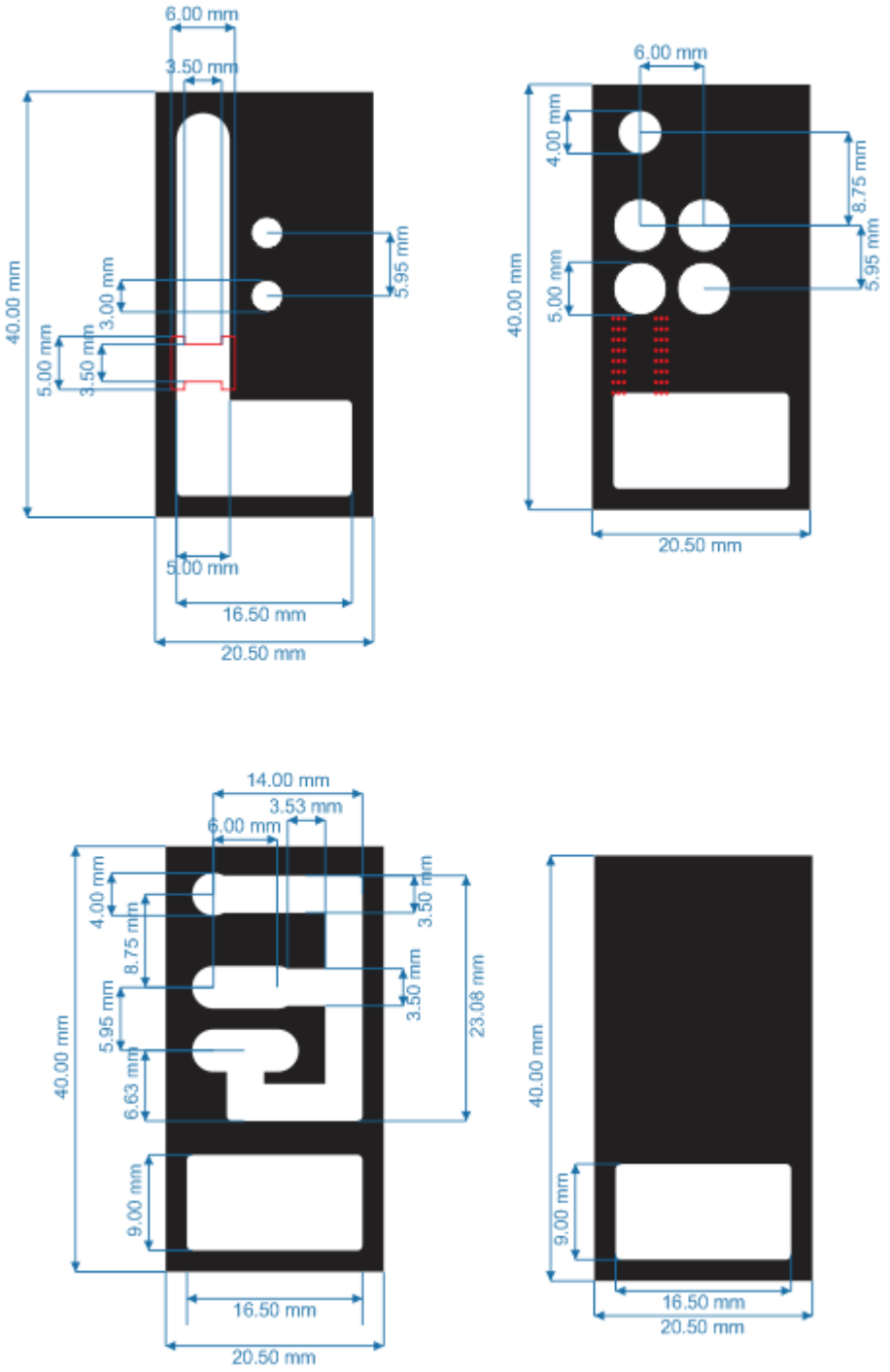


Figure 6-7: Dimensions for Protocol Development Chip and Corresponding Absorption Area

6.2.4 3 Fluid Chip Dimensions



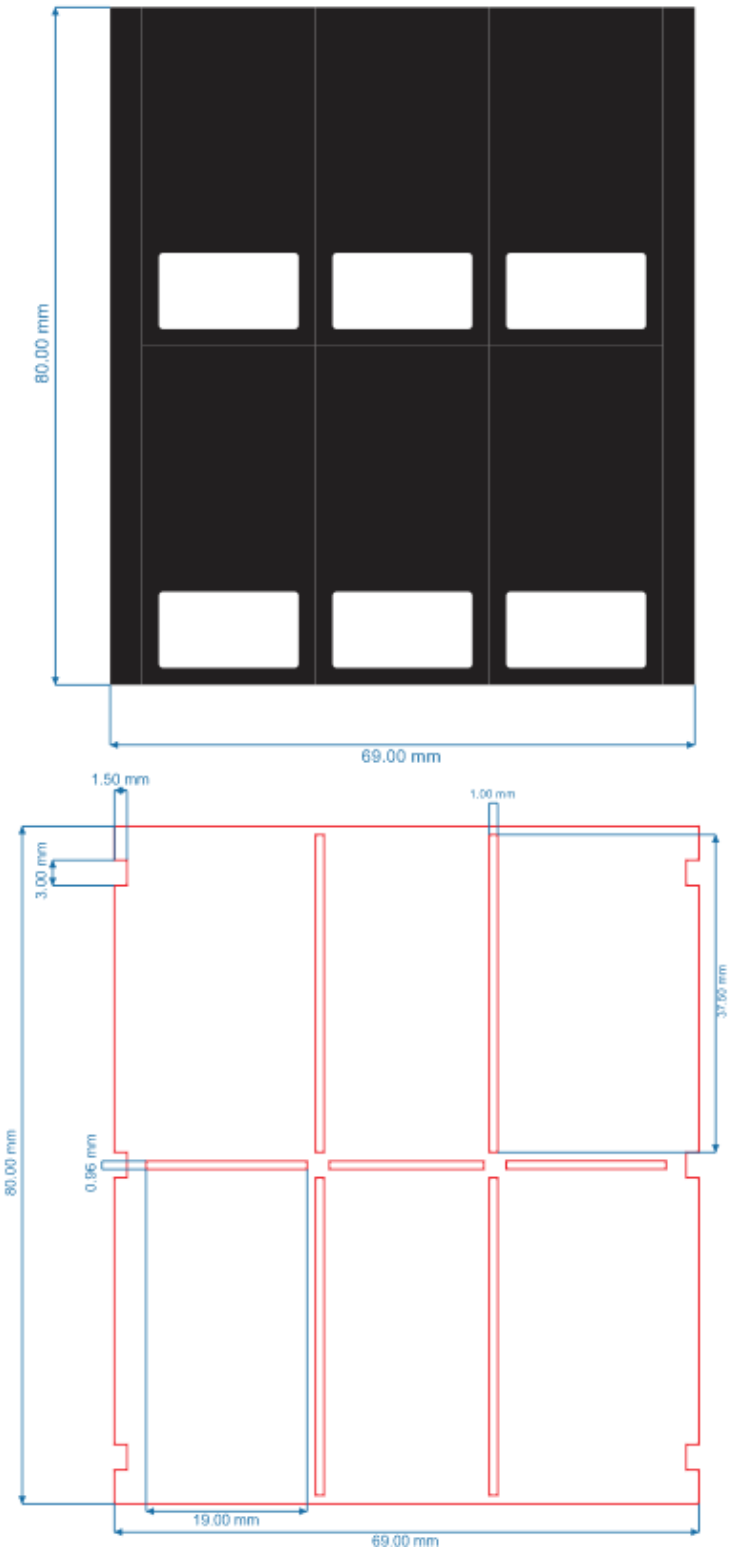


Figure 6-8: 3 Fluid Chip Dimensions

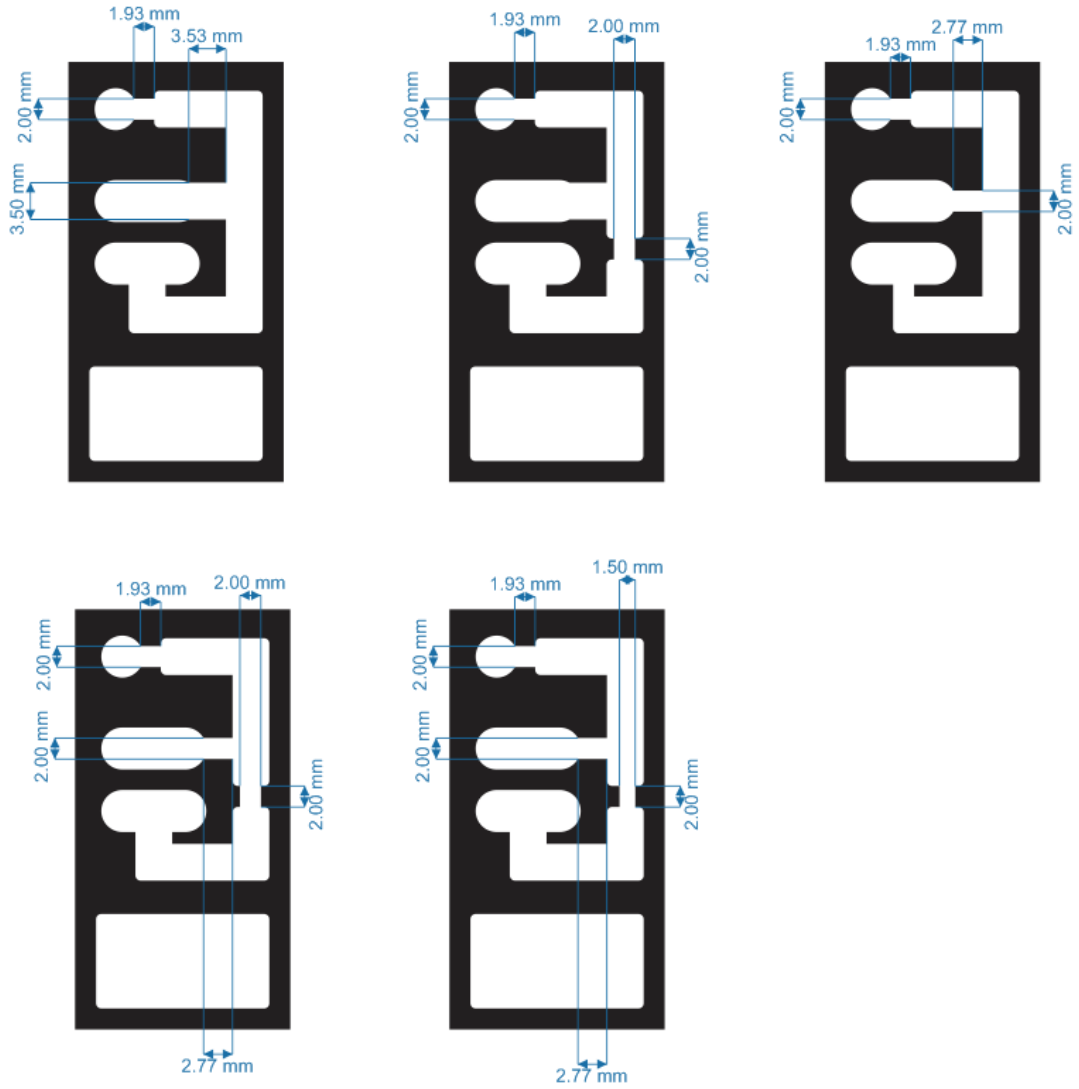


Figure 6-9: 'Kink' Dimensions

	8 µL				6 µL				4 µL				2 µL			
	10%		5%		10%		5%		10%		5%		10%		5%	
	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted
	32.30	**26.60**	12.60	**6.90**			9.50	**4.50**	24.60		9.30	**4.30**	9.85	**4.85**	9.50	**4.50**
	21.00	**15.30**	14.50	**8.80**	10.50	**5.50**	12.00	**7.00**	11.00	**6.00**	11.75	**6.75**	8.75	**3.75**	11.30	**6.30**
	32.60	**26.90**	11.25	**5.55**	10.75	**5.75**	13.60	**8.60**	14.75	**9.75**	9.25	**4.25**	8.00	**3.00**	9.60	**4.60**
	13.30	**7.60**	14.00	**8.30**	10.50	**5.50**	11.60	**6.60**	24.60		8.50	**3.50**	7.50	**2.50**	9.60	**4.60**
	34.60	**28.60**	30.50		14.00	**8.75**	16.00	**10.75**	11.00	**5.75**	11.25	**6.00**	11.15	**5.90**	11.00	**5.75**
	34.70	**28.70**	35.50		11.50	**6.25**	14.50	**9.25**	10.30	**5.05**	13.00	**7.75**	8.50	**3.25**	17.00	
	24.60	**18.60**	35.30		24.00	**18.75**	14.00	**8.75**	10.75	**5.50**	11.30	**6.05**	8.00	**2.75**	8.60	**3.35**
	31.50	**25.50**	31.30		12.00	**6.75**	11.50	**6.25**	12.60	**7.35**	9.75	**4.50**	7.75	**2.50**	9.00	**3.75**
	11.50	**5.25**	26.50	**20.25**	28.00	**22.50**	11.60	**6.10**	12.60	**7.15**	23.85		19.30		14.50	**9.25**
	12.75	**6.50**	35.60	**29.35**	22.30	**16.80**	23.00	**17.50**	24.00		16.00	**10.55**	15.00	**9.55**	11.00	**5.55**
	11.50	**5.25**	35.60	**29.35**	11.25	**5.75**	12.00	**6.50**	24.00		20.00	**14.55**	8.95	**3.50**	11.00	**5.55**
			1.20		11.25	**5.75**	11.50	**6.00**	24.00		19.75	**14.30**	9.25	**3.80**	17.30	
AVG	23.67	**17.71**	25.70	**15.50**	15.48	**10.23**	13.57	**8.35**	16.38	**6.65**	13.09	**6.82**	10.25	**4.16**	11.10	**5.32**
STDV	9.94	**10.04**	10.40	**10.61**	6.63	**6.51**	3.60	**3.51**	6.40	**1.61**	4.92	**3.42**	3.69	**2.18**	2.53	**1.66**

Figure 6-10: Data from Re-optimization (5 Layers)

For each concentration of surfactant there are two columns of data. The column titled 'initial' shows the time that was observed for the opening of the valve. The 'adjusted' column represents the time adjusted to subtract the approximate 5 minutes that was needed to ensure the valves were opening. These values represent the times needed for the valves to open after the addition of the main sample fluid ('clear fluid'). There are some values which have been marked in red. These values represent a failure of the valves to open until a small amount of pressure was applied to better the connection between the first and second layers of the chips. These values have been disregarded as an effect.

Figure 6-11: Data from Re-optimization (5 Layers)

	2µL		4µL		6µL		8µL	
	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted	Initial	Adjusted
2µL	11.00	1.00	13.30	3.14	18.05	7.73	21.50	11.02
2µL	11.60	1.58	13.30	3.12	15.75	5.41	15.55	5.05
2µL	12.00	1.95	14.00	3.79	14.30	3.93	14.25	3.72
2µL	BROKE	BROKE	13.00	2.77	13.25	2.86	14.50	3.95
AVG	11.53	1.51	13.40	3.21	15.34	4.98	16.45	5.94
STDDEV	5.78	0.85	0.42	0.43	2.08	2.11	3.41	3.44
STDDEV/2	2.89	0.43	0.21	0.21	1.04	1.05	1.71	1.72
1µL	13.50	3.41	14.00	3.75	17.25	6.84	14.90	4.33
1µL	11.75	1.64	14.55	4.28	21.60	11.17	19.25	8.66
1µL	11.75	1.61	13.60	3.30	13.50	3.04	21.00	10.38
1µL	11.75	1.59	13.00	2.68	14.15	3.67	16.45	5.81
AVG	12.19	2.06	13.79	3.50	16.63	6.18	17.90	7.29
STDDEV	0.88	0.90	0.65	0.68	3.70	3.72	2.74	2.73
STDDEV/2	0.44	0.45	0.33	0.34	1.85	1.86	1.37	1.37

	2µL	4µL	6µL	8µL
2µL Surfactant	1.51	3.21	4.98	5.94
stddev/2	0.43	0.21	1.05	1.72
1µL Surfactant	2.06	3.50	6.18	7.29
stddev/2	0.45	0.34	1.86	1.37

For each concentration of surfactant there are two columns of data. The column titled 'initial' shows the time that was observed for the opening of the valve. The 'adjusted' column represents the time adjusted to subtract the approximate 10 minutes that was needed to ensure the valves were opening. These values represent the times needed for the valves to open after the addition of the main sample fluid ('clear fluid').

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