2015

Enzymatic and Enzyme Inhibitory Activity on a Paper-Based Lateral Flow Device

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ENZYMATIC AND ENZYME INHIBITORY ACTIVITY ON A PAPER-BASED LATERAL FLOW DEVICE

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MECHANICAL ENGINEERING AND APPLIED MECHANICS

UNIVERSITY OF RHODE ISLAND

2015

MASTER OF SCIENCE THESIS

OF

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UNIVERSITY OF RHODE ISLAND

2015

ABSTRACT

In this work it was demonstrated for the first time that Alkaline Phosphatase (ALP) enzymes spotted and dried onto a nitrocellulose membrane remained active. This was accomplished by passing a fixed volume of the BCIP/NBT substrate solution over the immobilized enzymes and measuring the resulting color density at the spot where the enzymes were spotted. A dose response curve was produced of the concentration of the enzymes spotted on the test area versus the color density measured at the spot after the substrate had flowed past it indicating that the assay produces quantitative as well as qualitative results. This experiment was conducted in paper based lateral flow devices.

In a second experiment, prior to the flowing of the substrate over the test spot where ALP enzymes were immobilized, a solution of Sodium Orthovanadate (Na_3VO_4) was introduced to the system. Sodium Orthovanadate (Na_3VO_4) is an inhibitor to ALP. By varying the concentration of Na_3VO_4 in the solution that flowed past the enzymes, a certain number of the enzymes were deactivated. When subsequently the BCIP/NBT substrate solution was flowed over the enzymes, the intensity of the color produced depended on the concentration of Na_3VO_4. It was then possible to generate again a dose response curve, of the concentration of the Na_3VO_4 inhibitor in the solution versus the color density at the spot where the enzymes were dried. This is a novel result in that it is again the first time where an inhibitory activity assay was run on a paper based lateral flow device. This experiment also demonstrated that the bond between the Na_3VO_4 molecules and the immobilized ALP molecules is

strong enough so that at least not all of the inhibitor molecules were washed away by the substrate solution.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Mohammad Faghri, who supported me from when I started work with his microfluidics group as an undergraduate research assistant, through my studies abroad, to the completion of my graduate studies. His enthusiasm for microfluidics inspired innovations and brought an atmosphere of learning and discipline to the group. I am also very grateful to Dr. Constantine Anagnostopoulos, who always had fresh ideas when mine ran dry and was always able to explain the more difficult concepts in ways I could easily understand. I would also like to thank Dr. Sigrid Berka, who encouraged me to study abroad during my graduate studies and supported me both here at URI and at TU-Braunschweig. I also want to thank Dr. David Heskett, who is a great physics professor and an even better neighbor. A big thanks also goes to my laboratory colleagues, Dr. Peng Li, Dr. Hong Chen, Dr. Toru Yamada, Dr. John D. Jones, Michael Franzblau, Nick DeFilippo, Alex Pytka, Alexander Sherry, Michael Godfrin, and Jeremy Cogswell, who frequently aided me in their respective academic fields. This work is a continuation of the work done by the aforementioned people and would only be possible with the solid foundation they built. I would also like to thank Dr. Yow-Pin Lim, who taught me about some of the complexities of diagnostic biology and provided many of the reagents needed to make this work possible. A big thank you also goes to Kevin, Tunde, Aaron, and Sogand, for helping me to run experiments, fabricate chips, and keep me sane on long days. I am also very grateful to the faculty and staff of the Mechanical Engineering Department without whom this work would not have been

possible. Finally, I want to thank my family for their love and support for the many years that I have been a student.

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LIST OF ABBREVIATIONS

AKD	Alkyl ketene dimer
AcdP	Acid phosphatase
ALP	Alkaline Phosphatase
ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable
AuNP	Gold Nanoparticles
B- IαIP	Biotinylated Inter-Alpha Inhibitor Protein
CMC	Critical micelle concentration
DNase I	Deoxyribonuclease I
DPI	Dots per Inch
DAB	Diaminobenzidine
ELISA	Enzyme-linked immunosorbent assay
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
IU/L	International units per liter
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)
mg/mL	Milligrams per milliliter
MGV	Mean Gray Value
mIU/mL	Million International Units per Milliliter
NBT/BCIP	Nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate
PDMS	Polydimethylsilaxane
PBD	Paper Based Device
POC	Point of Care
RGB	Red Green Blue
TBPB	Tetrabromophenol blue
TEA	Triethanolamine
TRIS	Tris (hydroxymethyl) aminomethane
U	Units of enzymatic activity (U = 1 µmol/min)
vol.%	Volume Percentage
wt.%	Weight Percentage

1 Introduction

Hospitalizations for sepsis in the USA surpassed 1,000,000 people in 2008 [1]. Sepsis is fatal for up to half of the people who get it [2] [3]. The Center for Disease Control and Prevention (CDC) defines sepsis as "the body's overwhelming and life-threatening response to infection which can lead to tissue damage, organ failure, and death [4]." From pneumonia to a scraped knee, sepsis can stem from any infection in the human body and is characterized by inflammation as a response to a foreign microbial infection. The most common symptoms for sepsis are fever, chills, rapid breathing, elevated heart rate, confusion, disorientation, muscle and joint pains, a sense of impending doom, rash, and poor feeding (infants and children) [5]. Because these symptoms are unspecific, a diagnosis of sepsis is often delayed [6]. The progression of sepsis in some patients seems to be very slow and they will deteriorate in the late stages of their illness, yet in others, it progresses much more quickly and can be fatal within a few hours [5]. Diagnosis usually relies on blood or tissue cultures that take 6-48 hours. Cultures give false negative results in 30 % of the cases due to antibiotics taken before the test or because sepsis can also be related to toxins produced by the pathogen rather than the pathogen itself [2]. For these reasons, a rapid detection of sepsis is critical to the effort in increasing patient outcomes.

1.1 Inter-alpha inhibitor protein and its link to sepsis

Until recent years, the search for biomarkers for sepsis has been largely unsuccessful [2]. In 2000, changes in Inter-alpha Inhibitor Protein (IαIP) levels were

linked to inflammation and septic shock, and an IαIP specific enzyme-linked immunosorbent assay (ELISA) was developed [7]. IαIP are plasma-associated serine protease inhibitors that are mainly produced by the liver. Plasma levels in adult sepsis patients are decreased by 20 – 90 % and are inversely correlated with unfavorable patient outcomes. In 2009, a decrease in IαIP levels in neonates has also been discovered to indicate bacterial sepsis [6], which can be seen in figure 1. The administration of IαIP to neonatal mice with sepsis has showed improved survival rates [8], reinforcing the link between IαIP and sepsis.

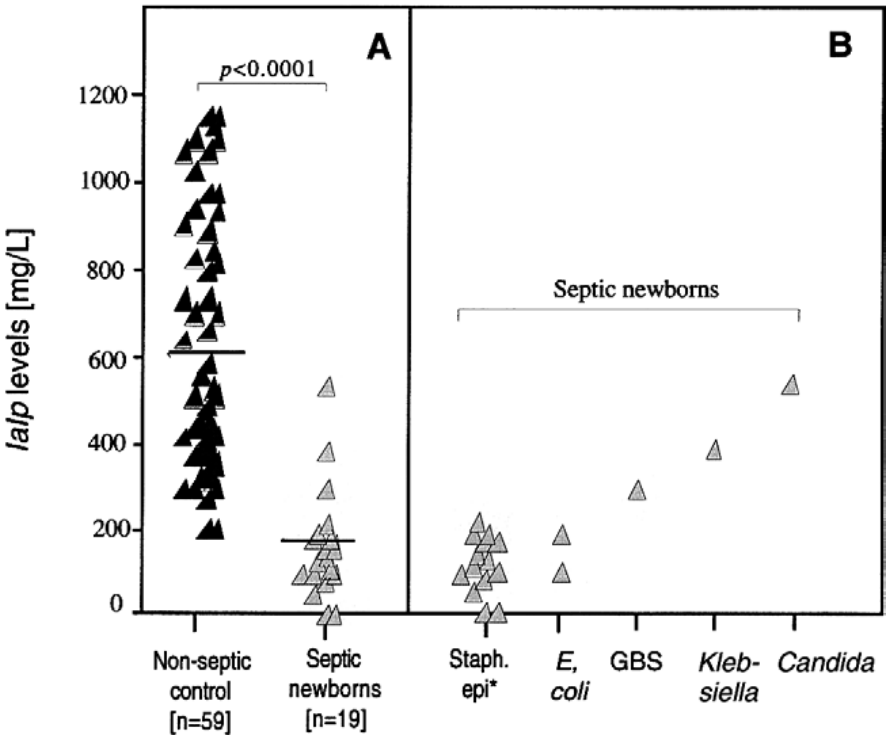


Figure 1: A: Plasma IaIP levels in newborn sepsis. Blood from newborns with clinically proven sepsis were collected at the time of diagnosis (positive blood culture) and blood from age-matched newborns without evidence of sepsis, were collected as control. B: Septic newborns grouped based on pathogens found in blood culture. [6]

1.2 Sandwich ELISA

A sandwich ELISA, specific to each of the heavy chains of IαIP, can quantitatively measure the level of IαIP [7]. A sandwich assay is typically built up on a 96-well micro-titer plate. A ‘capture’ antibody is immobilized (anchored) on the well surface. Next a sample solution is added to the wells that contains the analyte. The immobilized capture antibodies will bind to the analyte to form the base of the ‘assay sandwich.’ Next, a buffer solution is used to wash away any unbound proteins, after which a labeled antibody solution is introduced to the assay, completing the ‘sandwich’ as shown in figure 2. These antibodies are tagged with an enzyme that in the presence of a matched substrate will produce a detectable color change [9].

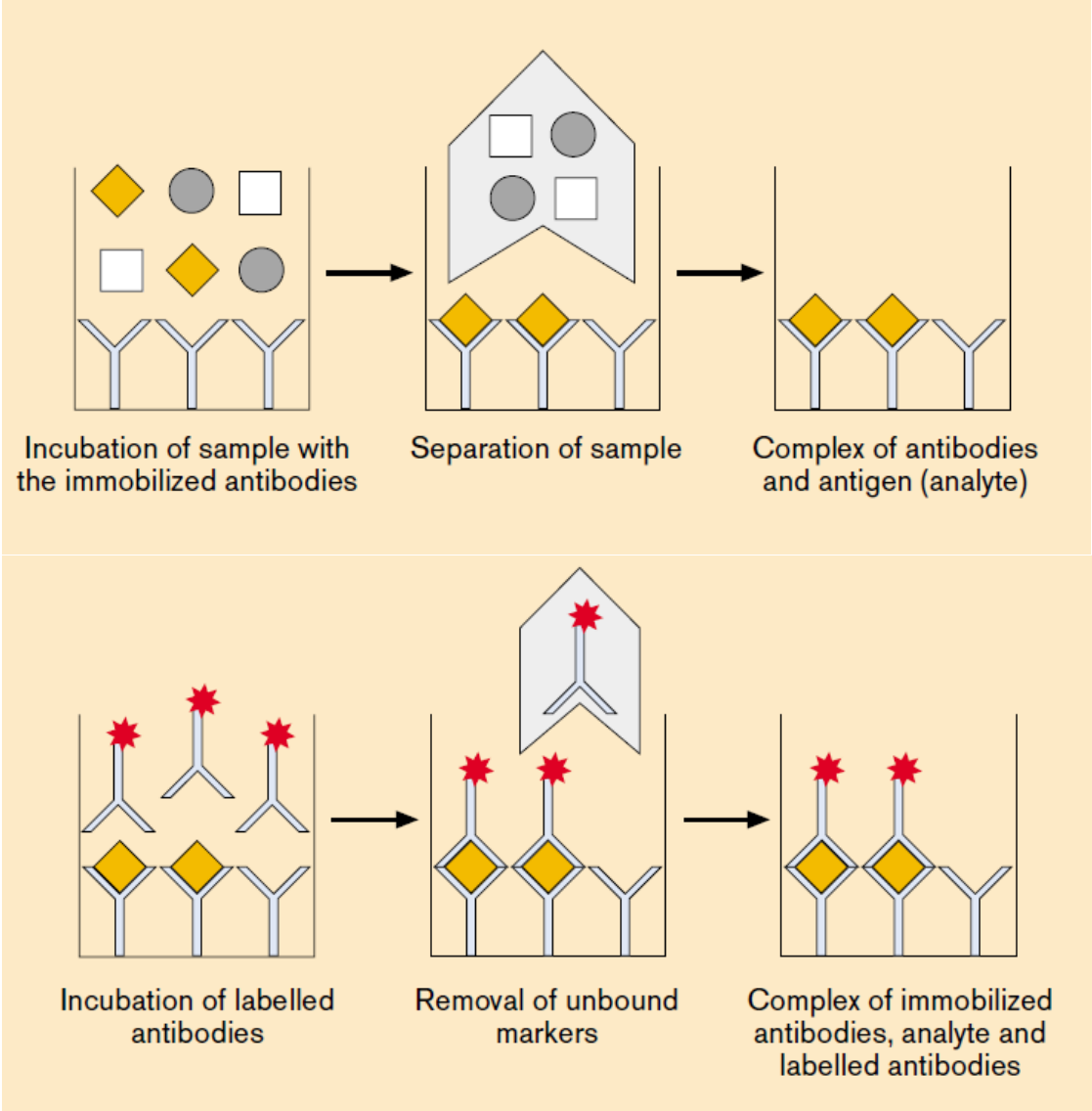


Figure 2: Steps in building of sandwich assay. [9]

1.3 Competitive ELISA

A sandwich assay is more applicable to high molecular weight analytes such as proteins or allergens. For the detection of low molecular weight analytes, a competitive assay is used. The principle behind this assay is that analytes from the sample must compete with added labelled analytes for immobilized binding sites. The capture antibody specific to the analyte in question is immobilized on the well surface

just like in the sandwich assay. Next, a prepared sample solution with the added labeled analytes mixed with the latent sample analytes is introduced. If a high concentration of sample analyte is present, fewer labeled analytes will find binding sites, decreasing signal and vice versa. Figure 3 shows a representation of this competitive assay principle [9].

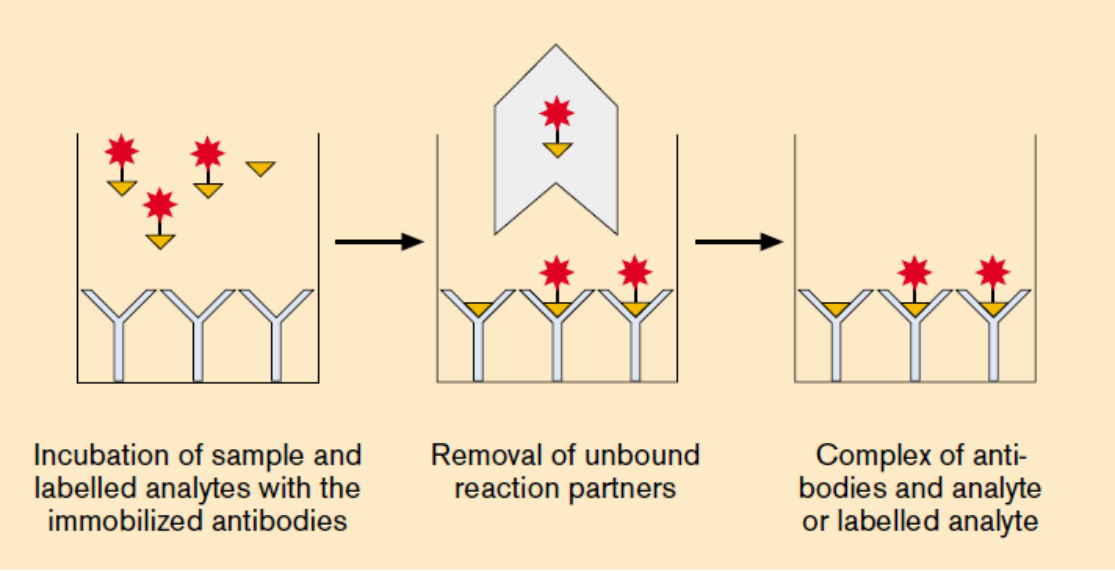


Figure 3: Steps in competitive assay. [9]

1.4 Point of care devices

Not only for sepsis, but many other diseases progress quickly or can progress unnoticed for a long period of time, thus requiring a quick, cheap, and simple means of diagnosis [10]. Point of Care (POC) devices are an exciting development in medical diagnostics that can be used outside of hospital/laboratory settings and can be used from range of detection applications from diseases and pathogens to explosives and toxins. POC devices are usually one of three main categories: permanent integrated

instruments, permanent instruments with disposable components, or pure disposables. Permanent integrated instruments require the instrument to purge itself of one sample before analyzing another to prevent contamination, and require calibration by a trained technician, which may not be useful in a remote setting [11]. These devices are not typically affordable by individuals and will therefore only useful in labs and hospitals. Permanent instruments using disposable components have a permanent but portable analyzer that uses disposable cartridges. The analyzer controls the pumping, thermal control, timing, and detection. The disposable cartridges are typically made of glass, silicon, or polydimethylsiloxane (PDMS) and are patterned to contain a microfluidic circuit with reaction and detection chambers where the chemical or biological reactions take place [12] [13]. This prevents the cross contamination from one sample to the next but cartridges can still be rather expensive to manufacture. Pure disposables radically reduce the material and reagent costs of these tests. They are typically run on a paper or other fibrous substrate that can wick fluid through capillary action, removing the need for external pumps. These lateral flow devices (LFD) also reduce the volumes of the reagents needed reducing cost even more. The most noticeable drawback of pure disposable devices is their decreased sensitivity and most cannot produce quantitative results [11].

1.5 Paper-based lateral flow devices

Guidelines from the World Health Organization suggest that for the developing world, POC diagnostic devices should follow the acronym **ASSURED**: **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid, and robust, **E**quipment-free, and **D**eliverable

to the end-users [12] [14]. Paper-based lateral flow devices (PBD) easily cover the majority of these guidelines, though their sensitivity and specificity are often lacking. The simplest of such tests are dipsticks such as pH test strips that are treated with a range of concentrations of acid-alkali solutions and will change to certain color depending on the pH of the sample solution being tested [12].

1.5.1 Lateral flow immunosorbent assay

A more complex PBD, a lateral flow immunoassay (LFIA), matches biological reagents to the material properties of the substrate (usually a filter paper or nitrocellulose) to detect various biomarkers in blood, urine, or saliva. As can be seen in figure 4, a LFIA typically has a sample inlet, a conjugate pad, a detection membrane that includes a test line and a control line, and an absorption pad to drive the flow. The main strip of fluid channel is usually made from hydrophilic filter paper to allow the capillary wicking of water-based fluids, while the detection membrane is typically made of porous nitrocellulose or nylon that are tailored to be used with biological reagents.

LFIA commonly use the ‘sandwich assay’ model for their biological scheme. After the sample fluid is introduced at the sample inlet, it flows through the conjugate pad, where labeled antibodies conjugate (combine) with the analytes in the sample. The conjugate then flows farther down the strip passed the test and control lines, where ‘capture antibodies’ are immobilized and ready to bond with them. The accumulated tagged sandwich complexes can then be detected in many different ways

depending on how it was labeled, such as colloidal gold, colored dyes, fluorescent dyes, and magnetic components [15].

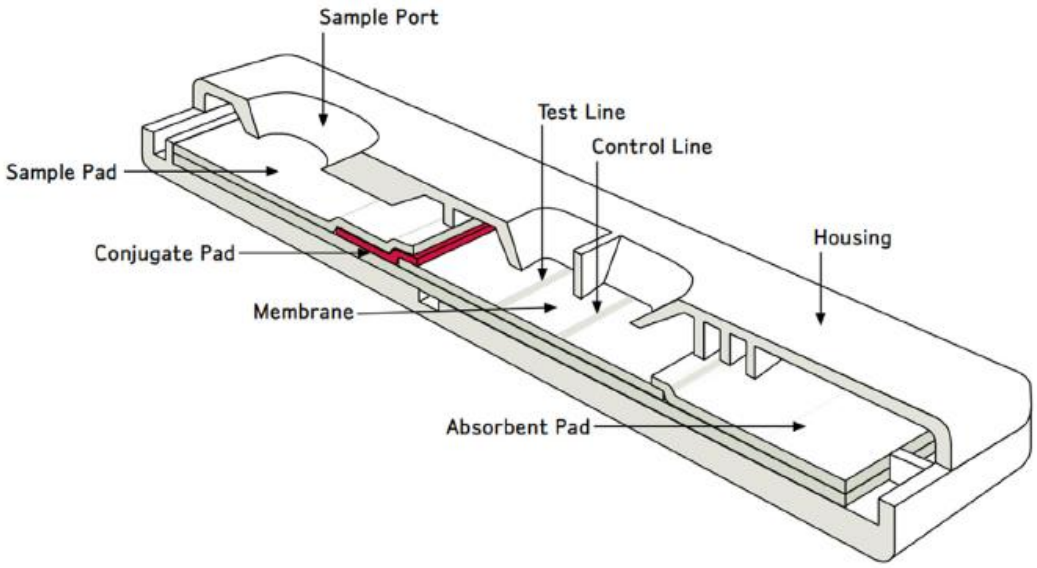


Figure 4: Lateral flow immunosorbent assay test and its components. [12]

1.5.2 LFIA with multiple fluids

For more complex chemistries, more reagents (i.e. more types of fluids) need to be introduced to the LFIA. For enzyme linked immunosorbent assays (ELISA), the labeled antibody is tagged with an enzyme that on its own does not give off any kind of signal. An additional substrate solution needs to be added to the system and when the substrate comes into contact with the enzyme, the enzyme will cleave the substrate. One of the products after conversion will be measurable through a colorimetric or electrochemical signal [13]. The difference between a conventional ELISA test on a micro-titer plate and an ELISA being run on an LFIA can be seen in figure 5.

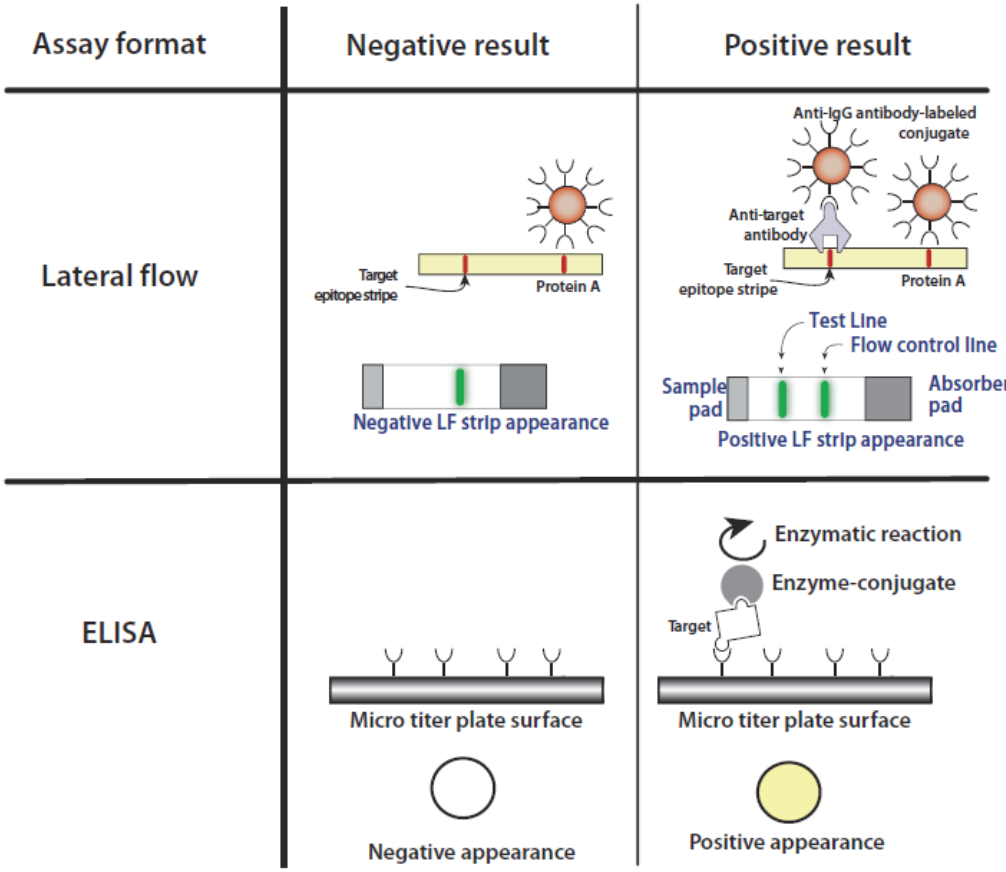


Figure 5: Lateral flow immunosorbent assay(LFIA) vs a typical ELISA [13]

Until recent years, LFIA were only able to handle one fluid at a time. Martinez developed a way to handle multiple fluids by inventing a mechanical fluidic switch, where the user can push a button to trigger fluid reservoirs. Figure 6 shows the various paper layers that are need to fabricate the mechanical switch [16]. The requirement for the user to trigger fluids at appropriate times adds the possibility of user error, which is very important to avoid for a diagnostic device. Through the invention of a paper-based fluidic valve [17], complex fluid circuits can now be designed to automatically sequentially load multiple fluid reagents. A representative schematic of a paper-based fluidic valve can be seen in figure 7.

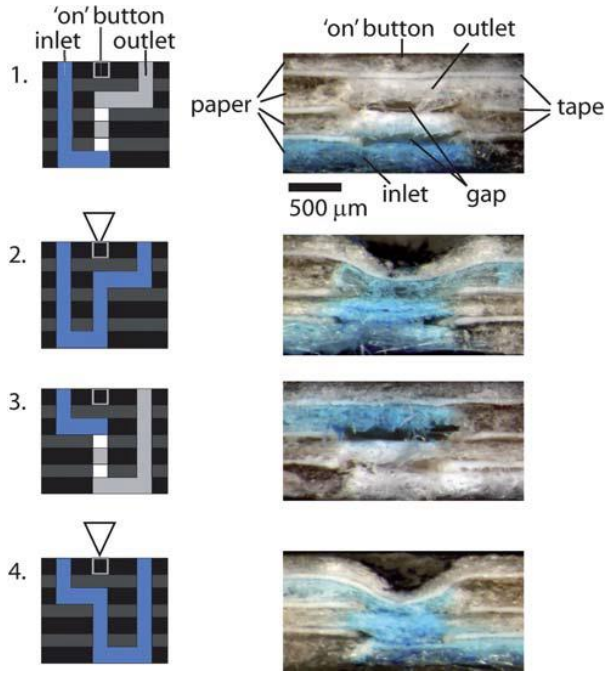


Figure 6: Schematic representation and photographs of the cross sections of four buttons. Buttons 2 and 4 were switched on by compression with a ball point pen. [16]

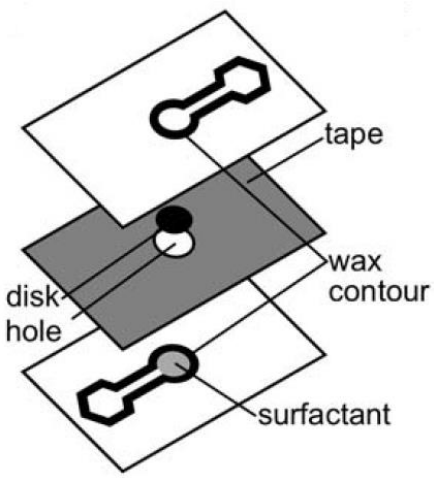


Figure 7: Schematic of the layers of a basic paper-based fluidic valve. [17]

1.5.3 IαIP ELISA on PBD

Once multi-fluid PBDs were possible, Giannakos was able to rapidly measure IαIP levels using the competitive ELISA method. He used a similar version of the three-fluid PBD designed by Gerbers and Foellscher [18] shown in figure 8, containing a sample inlet and two fluid reservoirs for the two additional fluid reagents required. The sample volume containing the unknown concentration of IαIP is incubated with an HRP-Strep solution prior to testing to form a conjugate. The sample fluid flows by capillary action through the device and over the nitrocellulose detection spot, where immobilized capture antibodies bind to the conjugates flowing by, anchoring them. Meanwhile, the sample fluid is following another fluid timing channel that then triggers the first reservoir containing a PBS wash. This new fluid introduced to the system flows through the detection spot, washing any unbound conjugates. The next fluid to be triggered from the second reservoir contains the substrate, DAB. When the DAB substrate molecules flow passed the immobilized HRP enzymes, they become converted into a colored precipitate that can then be measured colorimetrically. The amount of color change in the detection area produces a quantitative measurement of the IαIP concentration in the sample solution [19].

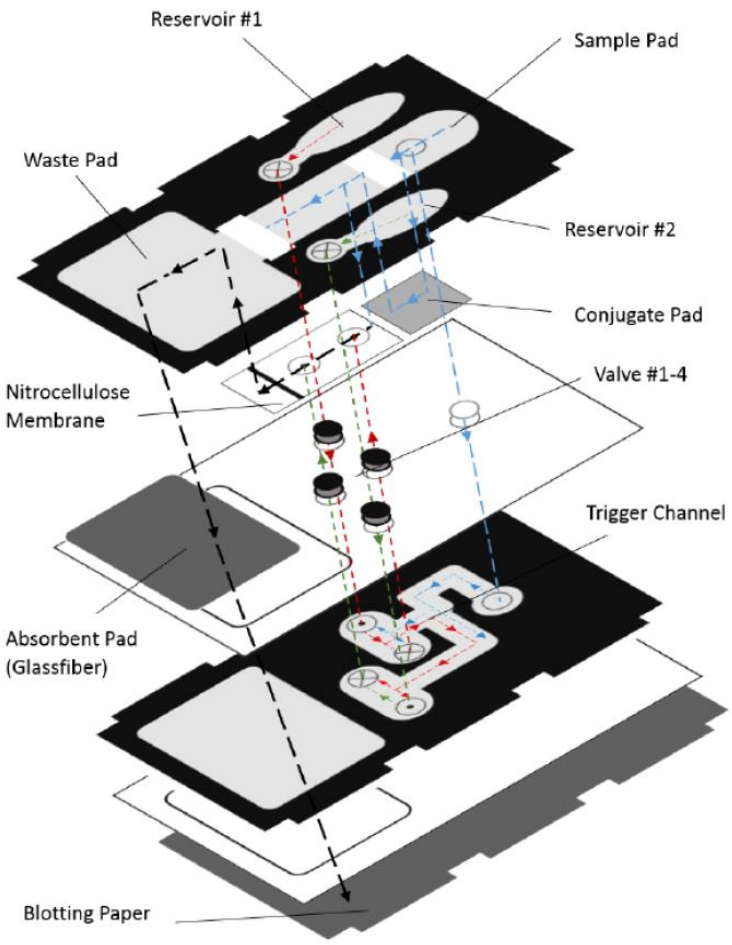


Figure 8: Schematic of the layers of a three-fluid basic paper-based device. [18]

1.6 Objective and Motivation

Each additional fluid reagent needed for bio-tests on a PBD adds to its complexity. To detect an enzyme inhibitor concentration quantitatively in a fluid sample, it may be possible to reduce the number of assay fluids to two by utilizing the enzyme inhibitory behavior instead of treating it as a neutral analyte like in the ELISA method. Inter-alpha inhibitory protein (IαIP) blocks the enzymatic activity of trypsin. Lim uses a 95-well micro-titer plate method to build this assay. First, the trypsin enzyme is added to the unknown sample concentration of IαIP and set to incubate for

at least 5 minutes at 37 °C to allow for the trypsin to IαP interaction. Next, the substrate solution, BAPNA, is added and is allowed to develop for precisely 30 minutes at 37 °C. The color of the resulting fluid is then read at 405 nm to measure the change in color [20].

A two-fluid PBD could be designed for this test with a sample inlet and one fluid reagent reservoir. In the nitrocellulose detection spot, a known quantity of the trypsin enzyme is immobilized. The substrate, BAPNA, will start in the reservoir and an unknown concentration of the inhibitor, IαIP, will be in the sample solution.

The enzyme, alkaline phosphatase (ALP), has been used by this lab to label detection antibodies for ELISAs done on a PBD and quantitative results could be obtained. Because ALP's enzymatic activity has already been seen working in a porous nitrocellulose detection area, it was used to model an enzymatic activity test. Sodium orthovanadate, an inhibitor for ALP, was used to model enzyme inhibitory activity in a two-fluid PBD.

2 Review of Literature

2.1 Enzymatic assay for glucose and protein concentration quantification

A group from Harvard University used a multiplexed paper-based lateral flow device, which can be seen in schematic form in figure 9, for a both glucose and protein detection in urine [21]. SU-8 2010 photoresist was used on chromatography paper to pattern the hydrophobic fluid barriers and the hydrophilic paper channels for the devices. The glucose assay they used is based on the oxidation of glucose to gluconic acid and hydrogen peroxide, which is catalyzed by glucose oxidase (notatin). That is immediately followed by the reduction of the hydrogen peroxide and oxidation of iodide to iodine, which is catalyzed by horseradish peroxidase (HRP) [22]. The protein assay they used is based on the nonspecific binding of tetrabromophenol blue (TBPB) to proteins through electrostatic and hydrophobic interactions. The phenol in TBPB deprotonates and changes color from yellow to blue. The brown color of iodine and the blue color from the TBPB were then quantified colorimetrically by the use of a phone, digital camera, or scanner. These images could be sent electronically to an offsite lab to be analyzed or potentially be analyzed by smartphone application.

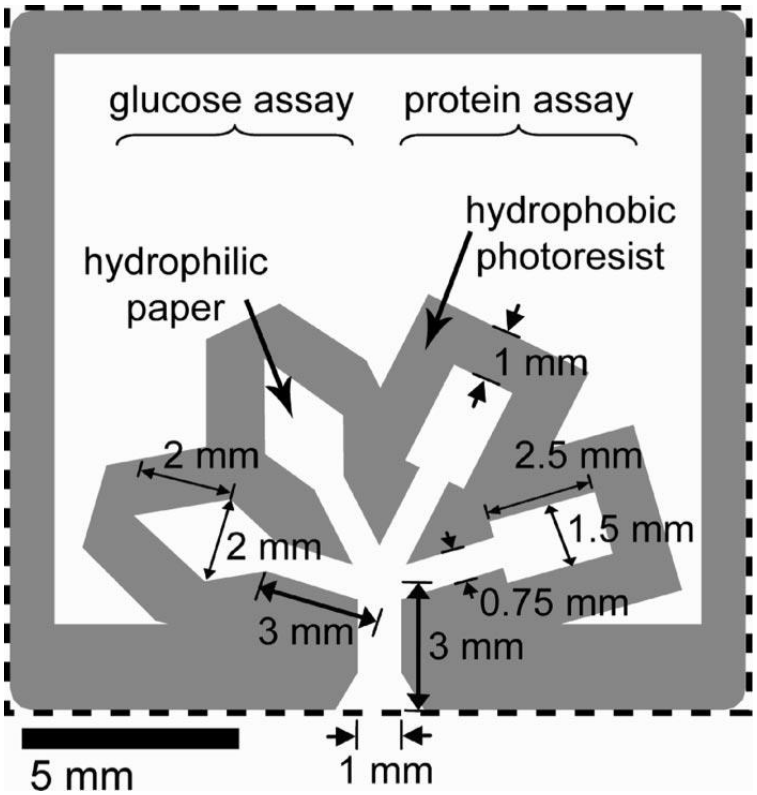


Figure 9: Schematic for a multiplexed paper-based later flow device for the colorimetric quantification of glucose and protein in urine.

Zhao [23] based a paper-based enzymatic assay on blue colored gold nanoparticle (AuNP) aggregates, modified with S1 and S2 that are cross-linked via DNA hybridization. The cross-linked DNA is cleaved by the enzyme Deoxyribonuclease I (DNase I), which breaks the aggregates. Well dispersed AuNPs appear as a red color. As can be seen in figure 10, the assays were carried out on both hydrophilic and hydrophobic filter paper spots at varying concentrations of DNase I, and photos were taken every 10 seconds.

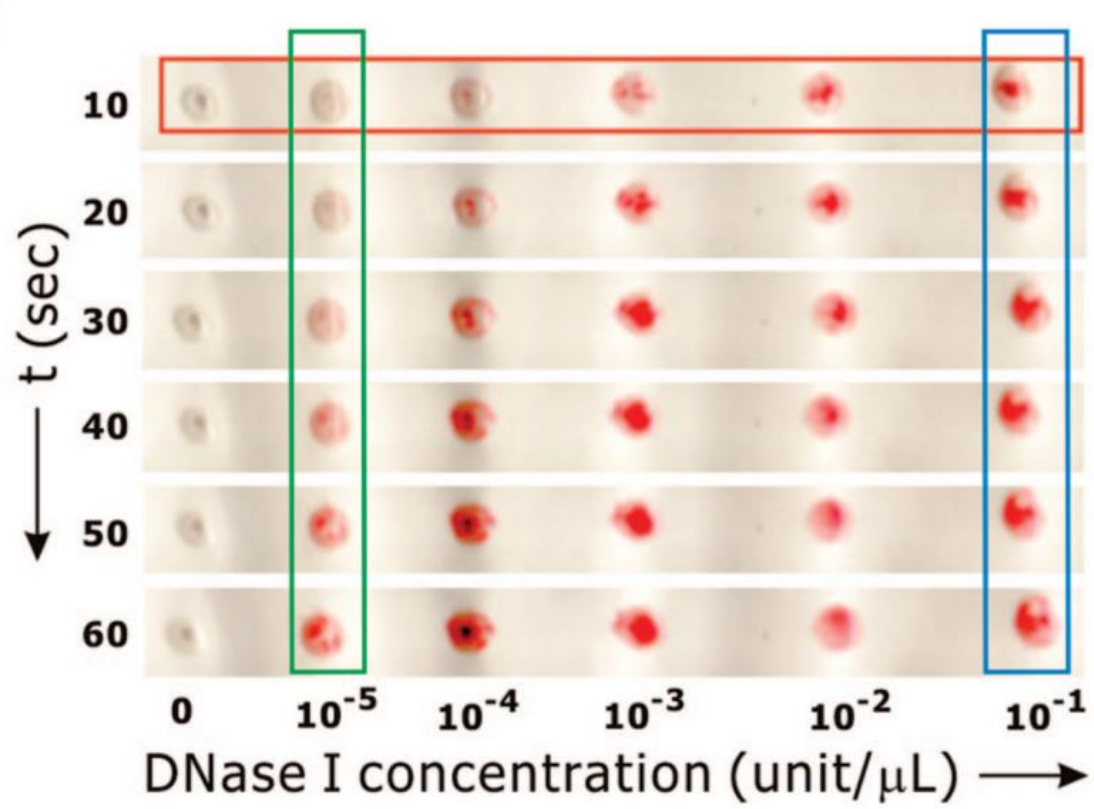


Figure 10: DNase I assay on hydrophobic paper as functions of both assay time and enzyme concentration [23].

2.2 Alkaline phosphatase

Tests for alkaline phosphatase (ALP) may be used to diagnose liver or bone disease. Increased ALP levels can also be seen in children undergoing growth spurts and in pregnant women. Normal ALP levels in blood from adults are 44 to 147 IU/L [24]. ALP is also found in neutral or alkaline soils, while acid phosphatase (AcdP) is found in acidic soils. An appropriate pH for crop growth can then be defined as a soil with a proper AcdP/ALP activity ratio [25].

The substrate for ALP to be used in the enzyme activity tests needs to convert on the same time scale as the fluid flowing passed the detection spot, which typically takes less than 10 minutes depending on the fluid volumes and channel geometries.

NBT/BCIP was chosen as color development happens in ~5 minutes [26]. Another advantage of this substrate is that the product after conversion is a dark purple precipitate that can be easily seen on a white nitrocellulose background. The fact that it precipitates allows the color to accumulate in the porous nitrocellulose, because the particles are too large for unhindered flow [27].

Peng [28] used a spot test on filter paper to test ALP activity with the NBT/BCIP substrate. ALP was immobilized on filter paper using a vacuum method. Three microliters of various dilutions of BCIP/NBT substrate solution were added to the spot and left to convert for 30 minutes. It was scanned at 600 dpi and converted from RGB brightness values to a mean gray brightness value (V) using the formula (V=0.299R+0.587G+0.114B), which are the default weighting factors used to convert RGB to YUV, the color encoding system used for analog television [29]. Ideally, weighting factors to match the dark purple color produced by this substrate conversion should be used. Peng's data can be visualized in figure 11.

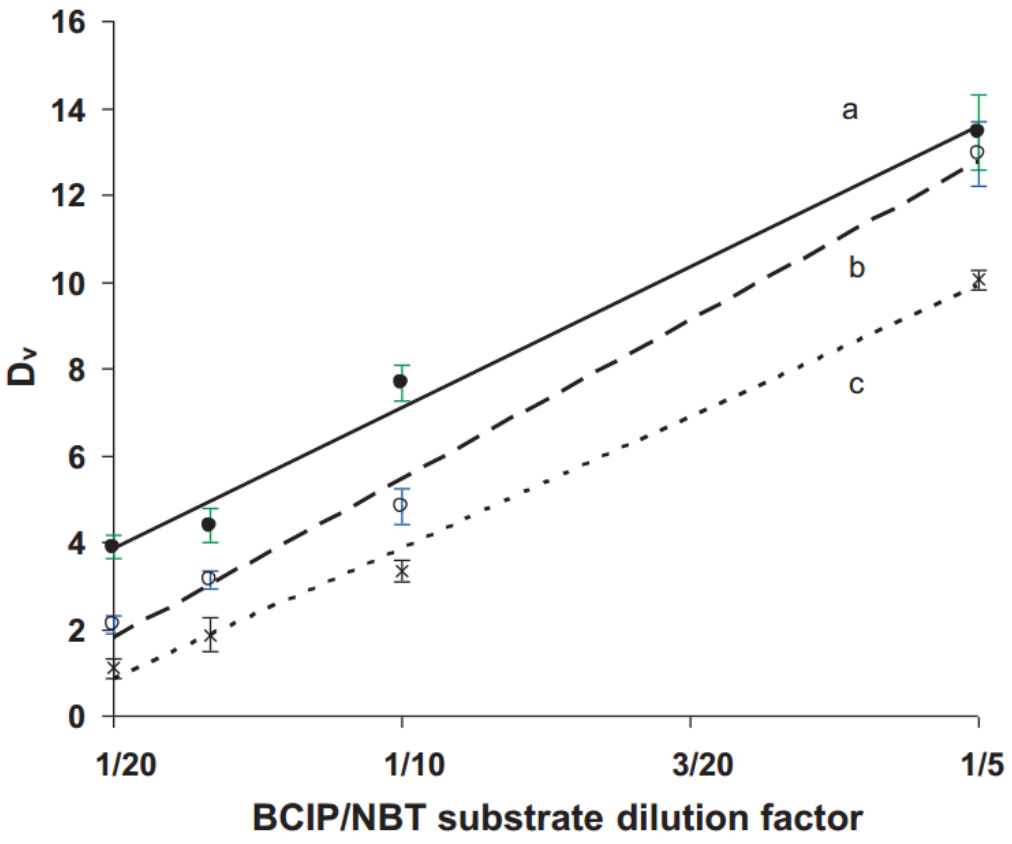


Figure 11: Activity (Dv) of paper with immobilized ALP probed by BCIP/NBT substrate dilutions under various ALP immobilization conditions by filtration: **a)** Cacium Carbonate (CC) flocs, **b)** CC colloids, and **c)** S. Added ALP concentration: 0.0125 mg/mL. [28]

A group from Monash University, in Austrailia, patterned a multiplexed PBD using alkyl ketene dimer (AKD) to hydrophobize filter paper and a plasma treatment through a metal patterning mask to rehydrophilize the regions designated to be fluid channels [30]. The ALP was divided into two parts; one was heated for 10 minutes at >70 °C to deactivate the enzyme. They spotted the active and deactivated ALP into designated filter paper tests spots at the ends of fluid channels. As can be seen in figure 12, after the substrate, BCIP/NBT, was added at the intersection of the six

channels and allowed to wick for a short time, test spots with active ALP enzymes changed to a purple color, whereas test spots with the deactivated ALP did not.

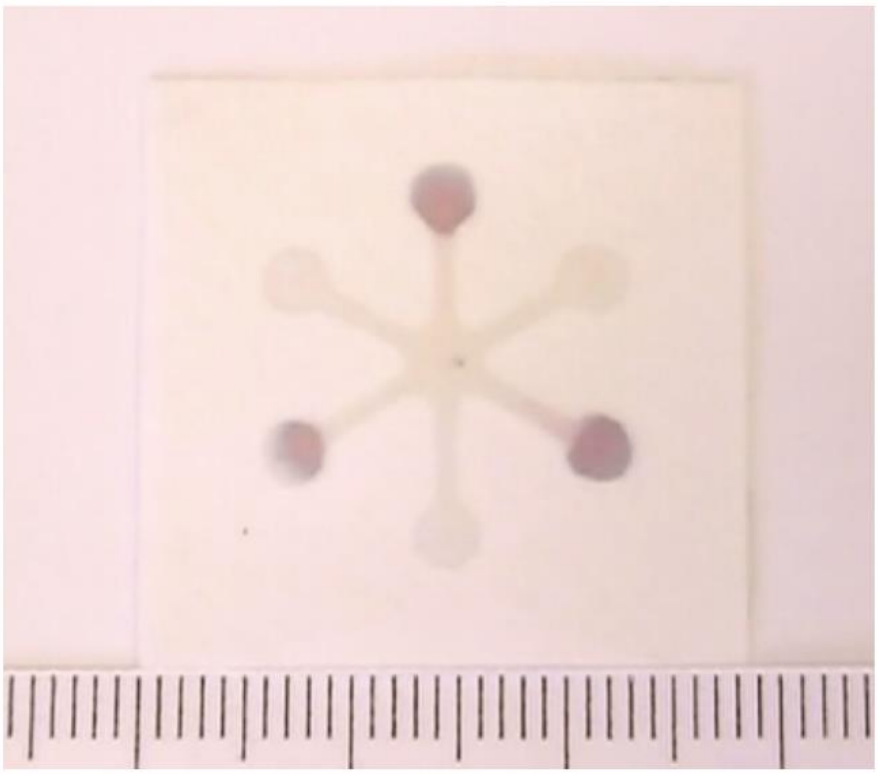


Figure 12: Three active and three deactivated ALP enzyme sample reacting with the liquid substrate BCIP/NBT [30].

Giannakos [19] immobilized various concentrations of mouse monoclonal and polyclonal antibodies tagged with ALP onto a nitrocellulose detection area in a PBD. He also used the BCIP/NBT substrate but in a lateral flow format rather than the direct spotting and incubation. The chips were scanned and analyzed the same way as Peng's in method [28], though only single data points were gathered for each concentration, leaving a lot of uncertainty. Figure 13 shows the data gathered with error bars equal to plus and minus two standard deviations of the individual pixel brightness values that

were averaged together to form each data point. These error bars were calculated incorrectly, as they should be two times the standard deviation of multiple RGB mean gray value data points, should the same 95 % confidence level scheme be followed.

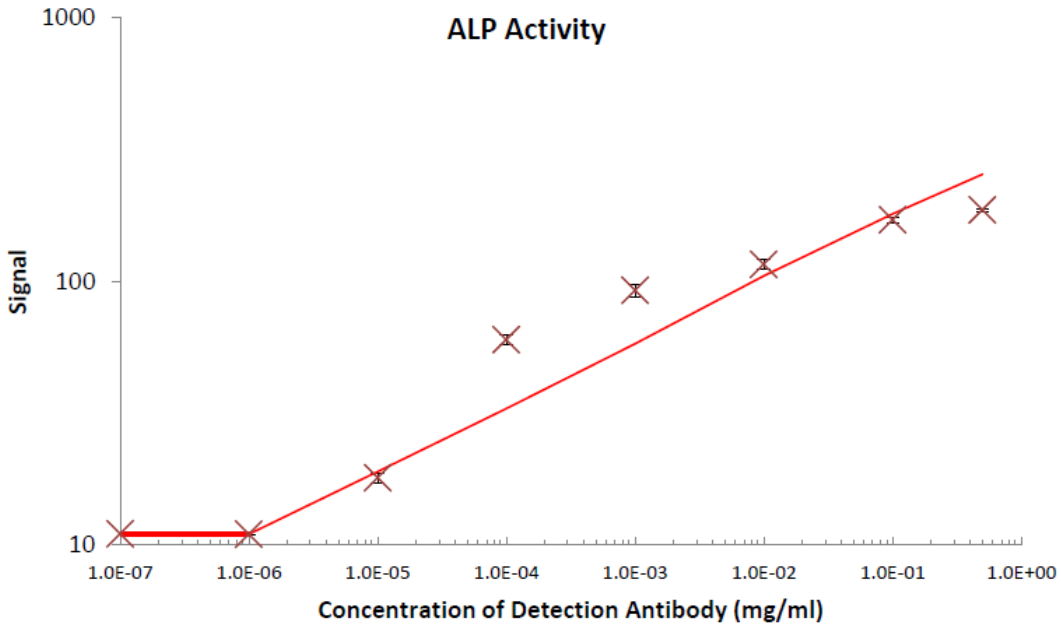


Figure 13: ALP activity test showing signal intensity at various concentrations of mouse monoclonal antibodies tagged with ALP. [19]

2.3 Inhibitory activity of sodium orthovanadate on ALP

Seargeant found that sodium orthovanadate can be used to inhibit the enzymatic activity of ALP produced by a human liver, small intestine, or kidney. The inhibition of ALP activity by orthovanadate can be seen in figure 14. The specific activity of the enzyme from liver was 1300 U/mg [31]. One unit of enzymatic activity, U, is equal to the amount of enzyme that decomposes 1 µmole of substrate per minute at room temperature [32].

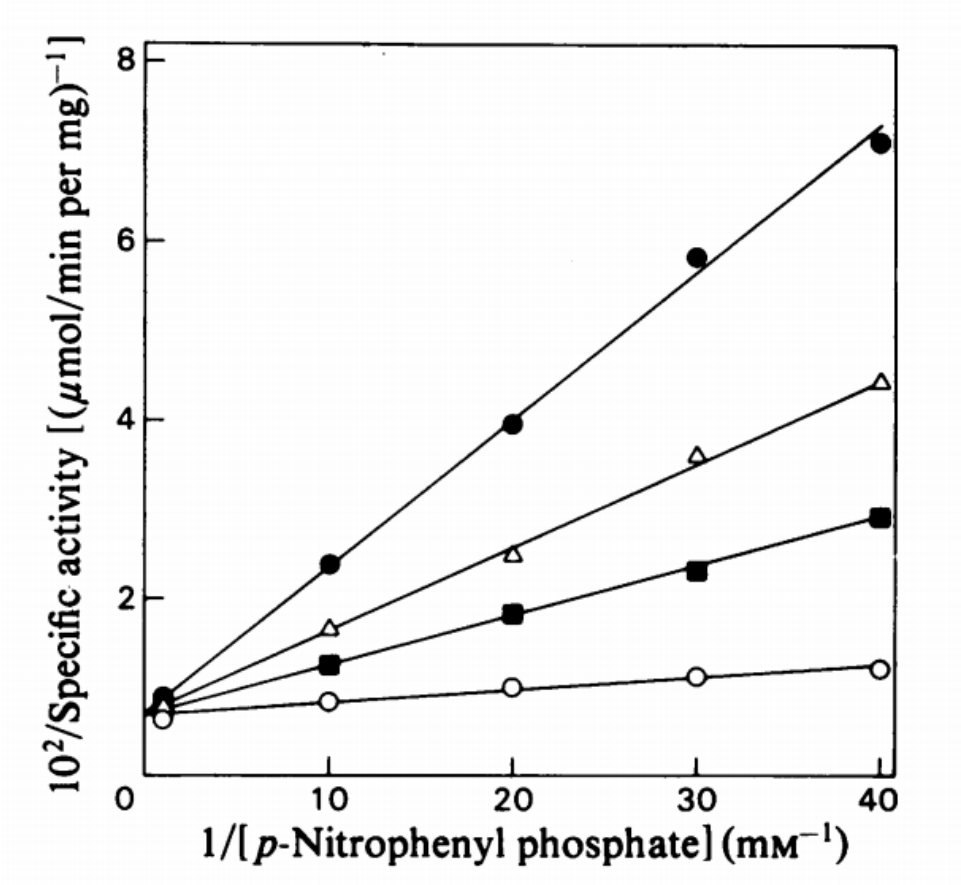


Figure 14: Inhibition of ALP activity by orthovanadate. Double-reciprocal plot for human liver alkaline phosphatase (40 ng/mL) at pH 9.0. Orthovanadate concentrations were: ○, none; ■, 2.5 μM; Δ, 5.0 μM; ●, 10 μM. [31]

2.4 Paper-based fluidic valve

What makes multi-fluid PBDs possible is a single-use paper-based fluidic valve developed by Dr. Hong Chen. He used the chemistry of surfactants to allow water based fluids to wick through hydrophobic paper. Surfactants, like the tween20 used in these valves, is a molecule that has both a hydrophilic head and a hydrophobic tail. Surfactants are surface active molecules that, when mixed with water, will poke their hydrophobic tails through the water-air interface, as seen in figure 15. Once the water-air interface is saturated a so-called critical micelle concentration (CMC) is reached,

the surfactant molecules begin to form micelles, clusters of surfactant molecules that form the lowest energy state of the system. The CMC is important because, below the CMC, changes in concentration have large effect on the surface properties. Above the CMC an increase in surfactant concentration only has a small effect on surface properties, while there is a large increase in fluid viscosity [33]. Surfactant molecules will also form monolayers on hydrophobic surfaces (like channel boundaries or suspended particles), making them hydrophilic for however long the surfactant layers remain [34] [35].

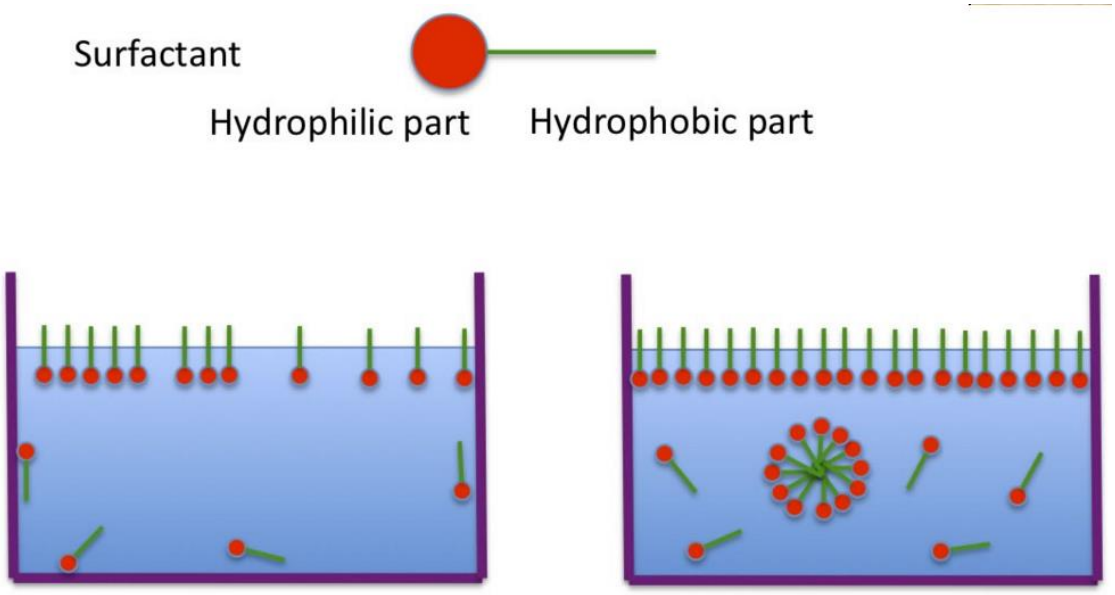


Figure 15: A surfactant molecule and its parts (top). Surfactant molecules in water poking their hydrophobic tails through the water-air interface (left). Saturation of water-air interface and formation of surfactant micelles (right). [36]

Chen developed the 2-d fluidic valve by first patterning fluid channels in filter paper, as seen in figure 16a, by chemically treating the channel boundaries to be hydrophobic. He left a hydrophobic gap between a circular channel and a surrounding

hydrophilic channel. In the circle he spotted the surfactant tween20 and allowed it to dry. Water-based fluid wicking from the non-surfactant side of the ‘one-way fluidic valve’ would stop flowing upon reaching the hydrophobic gap. Were the fluid to flow from the other side, it would mix with the surfactant, allowing it to bridge the small hydrophobic gap. As can be seen in figure 16b, the tails of the surfactant molecules adsorb to the hydrophobic filter paper fibers and exposing only their hydrophilic heads to the water, essentially building a hydrophilic bridge across the hydrophobic gap. In figure 16c, green colored water can be seen at various time intervals flowing through paper channels and interacting with two identical one-way fluidic valves but from opposite sides. The left valve opened after the water mixed with the surfactant that was spotted in the center. The right valve did not open as no surfactant was present on that side of the valve.

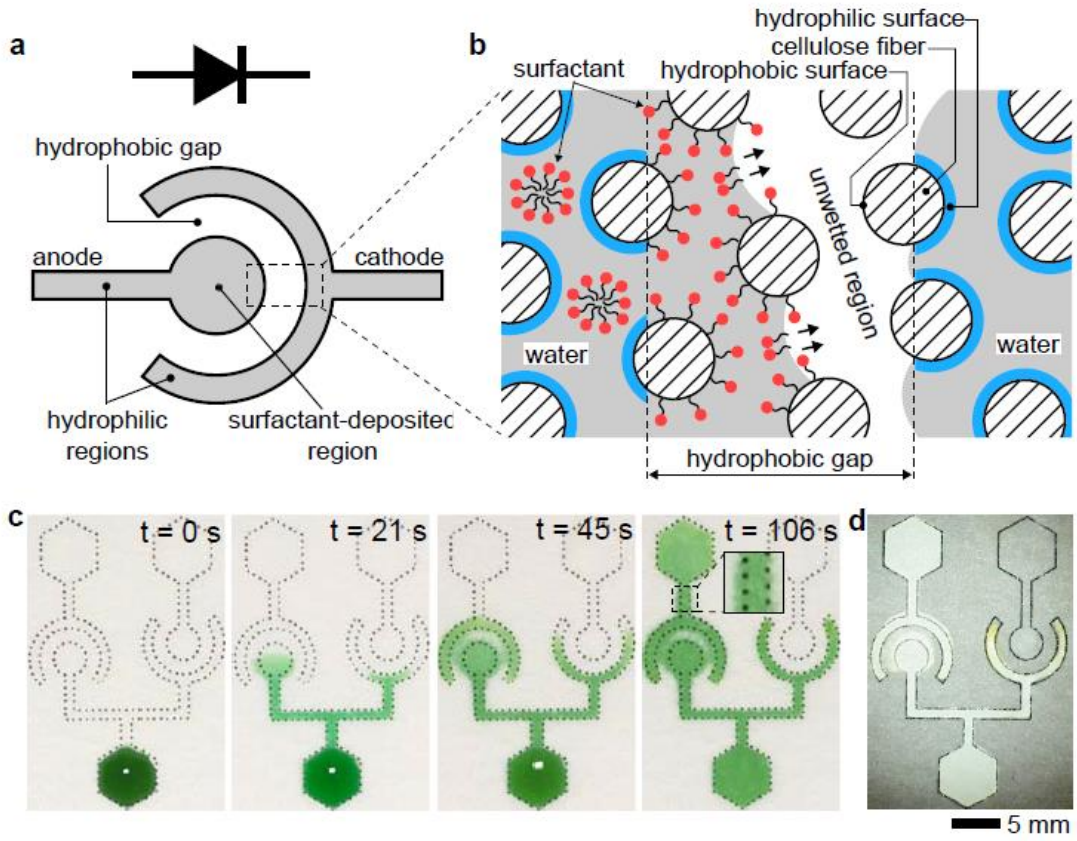


Figure 16: (a) Symbolic and schematic of one-way fluidic valve. (b) Illustration of working mechanism of surfactant bridging the hydrophobic gap. (c) Time-sequential photographs showing (green) water wicking toward two identical one-way fluidic valves positioned in opposite directions. [17]

Chen was also able to develop an improved version of the one-way fluidic valve that is built from multiple layers of filter paper and double sided tape. As can be seen in figure 17, hydrophilic filter paper channels are lined up to intersect over a hole in water impermeable double sided tape. A hydrophobic filter paper disc is set in the hole to block fluid flow and maintain contact between the two filter paper layers through the hole. Surfactant is spotted on one of the filter paper sides to allow fluid flow to pass the hydrophobic filter paper disc only from that side. This basic 3-d one-way fluid valve became a major building block in the development of complex paper-

based fluidic circuits that could then be used for biomarker detection using a variety of multi-fluid assays.

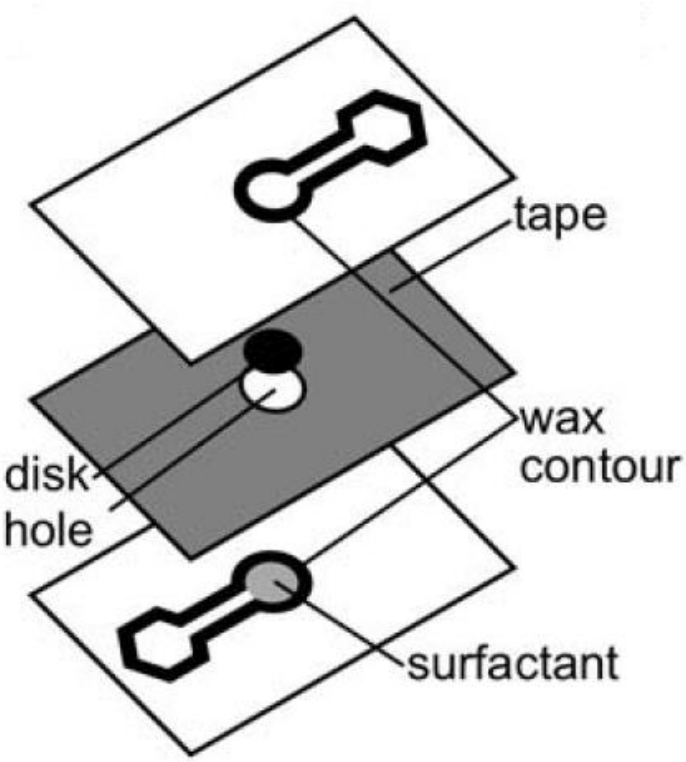


Figure 17: Schematic of the layers of a basic 3-d paper-based fluidic valve. [17]

3 Methodology

In this chapter, the methodology for the fabrication of the paper-based lateral flow devices will be explained in detail. This includes a list of the materials needed, the required tools and equipment, the fabrication of individual parts, the process to assemble the parts, and how to prepare the reagents and experiments for enzyme activity and enzyme inhibitor activity tests. Small tweaks to the conventional fabrication process are also discussed.

3.1 Chip Fabrication Introduction

What makes the paper-based lateral flow devices designed by this research group unique is the ability to automatically handle multiple fluid reagents and sequentially introduce them to the assay. Wax is printed onto filter paper and then melted to produce hydrophobic walls impenetrable by water based fluids. The patterning of these walls produces fluid channels and other fluid manipulation structures like paper level changes and 3d fluidic valves. Important parameters to consider when designing such a chip are channel geometries (length and width), valve sizes, filter paper porosity and dimensions, and wax melting times.

A typical chip design used by this research group is one designed by Gerbers and Föllscher [18]. Despite the fact that in the meantime there have been improvements to this design, it is still a good representation of chips currently being used. The top layer contains a sample inlet, two reservoirs, a potential conjugate pad, a detection zone made of nitrocellulose, and a large connection to the waste pad. The

second layer is a connection layer that separates the top layer from the timing channels. In this design it is made from double sided tape, filter paper discs treated to by hydrophobic, filter paper discs that contain the surfactant tween20, and some native filter paper discs, as well as a large piece of glass fiber paper to help absorb and transfer waste fluid to the absorption pad. This layer is improved in the work by Alex Giannakos [19]. He replaces the double sided tape with 3M® Super 77 Multipurpose Adhesive and consolidates the hydrophobic and surfactant discs into the filter paper layers. This improvement will be seen in the fabrication of the protocol development chip described in section 3.1.2. The second filter paper layer contains the fluid timing channels that connect the sample inlet to the reservoirs and back to the main straight fluid channel containing the detection zone. The geometry of these the timing channels determine the incubation times between the sequential loading of the fluid reagents. Below the timing channel, another tape layer is used to separate the timing channel from the waste/absorption pad made of blotting paper. Once all of the fluid channels in the device are wetted, the blotting paper layer is the primary source of capillary flow and causes the fluid reagents to continue flowing throughout the entire chip. The dashed lines with the arrows show the desired paths for the various fluids to flow through the chip and all ending in the waste/absorption pad.

3.1.1 Paper-based chip fabrication

Materials, software, and equipment needed for paper chip device fabrication:

- Corel Draw® Software
- Xerox® ColorQube® 8570 solid ink printer
- Xerox® Genuine Solid Ink Black
- Epilog® Mini 24 CO_2 laser cutter
- Isotemp® Model 280 vacuum oven
- Whatman® Grade 41 (9572190) filter paper (8x10 in)
- Whatman® Grade GB003 Blotting paper (20x20 cm by 0.8 mm thick)
- Whatman® 12 µm Nitrocellulose 47 mm Diameter (G3514143)
- Sterlitech® GA-55 Absorbent paper
- Ace Hardware® double-sided carpet tape (No. 50106)
- 3M® Super 77 Multipurpose Adhesive
- Reynolds® parchment paper
- Acros Organics® Allytrichlorosilane
- Sigma Aldrich® Tween 20

3.1.1.1 Filter paper patterning

Whatman's® Grade 41 (9572190) filter paper was chosen to carry the fluids in the paper-based lateral flow devices. Corel Draw®, or another vector based drawing software like AutoCAD, is used to design the patterns that are then printed onto the filter paper. The ColorQube® 8570 solid ink printer from Xerox® was used to print

Xerox® Genuine Solid Ink Black onto the filter paper to pattern what will be the hydrophobic walls for the fluid channels, reservoirs, fluid inlets, and 3d fluidic valves.

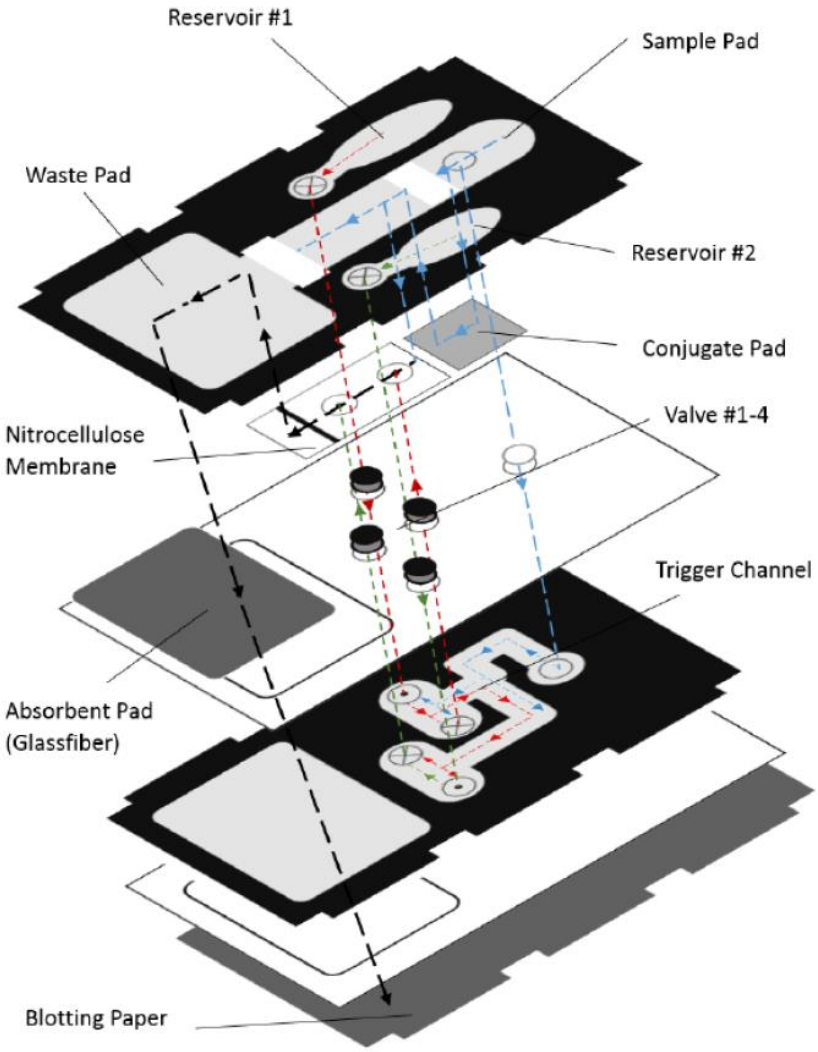


Figure 18: Chip layer and fluid flow paths [18]

3.1.1.2 CO2 laser cutter settings

Using the same drawing software, cut-files are designed to be used with the Epilog® Mini 24 CO_2 laser cutter. This allows for multiple chips to be cut from larger sheets of filter paper, scaling up the fabrication speed. For the design shown in figure

18, six paper-based later flow devices are printed per chip and six chips can be printed on a single 8 x 10 inch (203 x 254 mm) piece of filter paper. Along with any holes that need to be cut into the filter paper layers or double sided tape, the edges of the chips need to be precisely cut to dimension so that they can be fit into alignment fixtures used during assembly. Not only the filter paper layers but the double sided tape, glass fiber paper, nitrocellulose pieces, conjugate pads, and blotting paper layer need to be laser cut. As can be seen in table 1, each material is cut using different power, speed, and frequency setting of the CO_2 laser cutter.

Material	Vector Speed	Vector Power	Frequency
Blotting Paper (waste/absorption)	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

Table 1: CO_2 laser cutter setting for various materials [19]

Cut files are designed as vector files in such a way that the laser can follow a path to cut. To align the designed cut file to the wax printed structures on the filter paper, alignment guide markers are used. For the initial rough placement of the filter paper, two orthogonal black wax lines (can be seen in figure 19) that are printed along with the wax patterns is aligned with the edge rulers of the laser cutter. Using the preprogrammed filter paper cut align laser cutter settings, hairline width crosses are cut over alignment guides also seen in figure 19. The main purpose of the alignment lines is to place the wax printed filter paper parallel to the cut file. The alignment

guides can then be used to shift the cut file horizontally, in the x-direction, and vertically, in the y-direction, to precisely match the placed wax printed filter paper. There are two alignment guides per sheet. Should both alignment guides show differing offsets, it means the wax printed filter paper is not placed with the wax alignment lines parallel with the ruler edges of the laser cutter and needs to be adjusted.

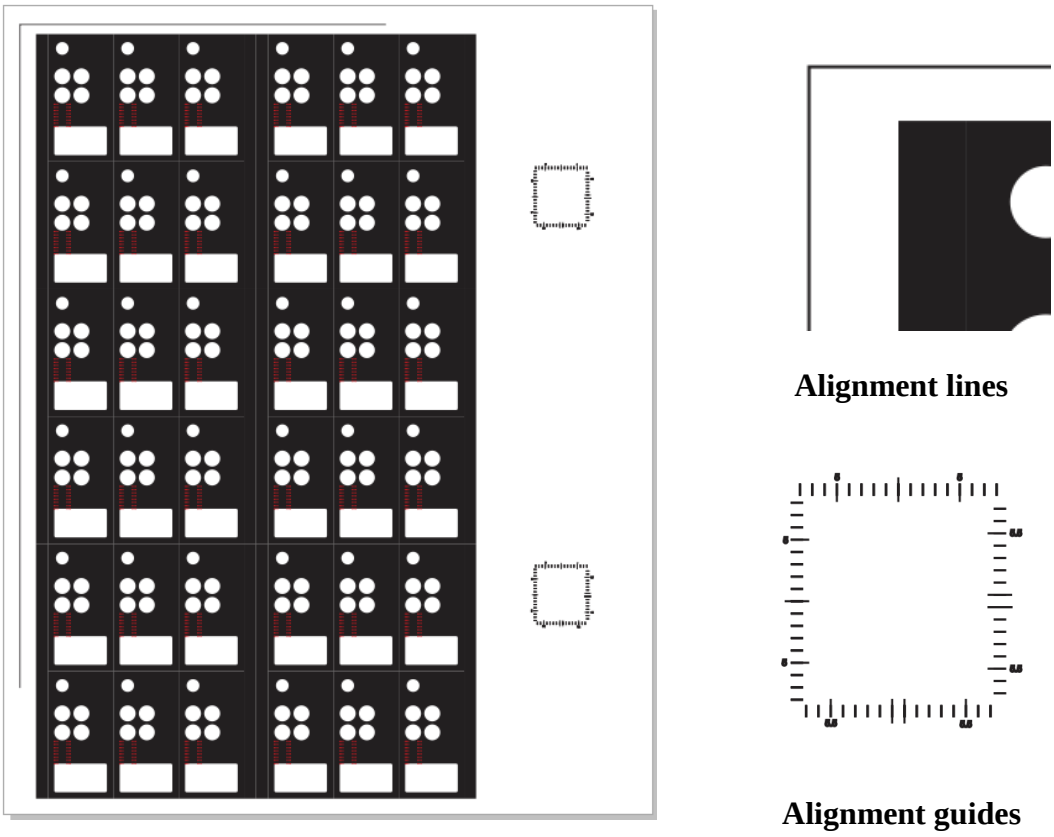


Figure 19: Cutting guides [19]

To cut the waste/absorption pad pieces, the blotting paper cut file is used. The Whatman Grade GB003 blotting paper is simply aligned along the laser cutter ruler edges and do not need additional alignment steps as no wax structures are printed on it. As blotting paper is thicker, it needs additional power to be cut. The power, frequency, and speed settings from table 2 are used.

The nitrocellulose detection areas are cut from circular pieces 47 mm in diameter. A fixture, seen in figure 20, is used to secure the nitrocellulose and keep it flat during cutting. The cut file is pre-aligned to the fixture and alignment tolerances are taken into account so that any additional alignment steps are not necessary. During most cuts, a ventilation fan is used to suck out smoke from the burning materials. For the nitrocellulose cut, the fan is left turned off as it can suck the small cut pieces of nitrocellulose out of the fixture and render them useless. It is important to use gloves when handling all of these materials as the oils from your skin can affect the fluid properties of the paper. With nitrocellulose it is of paramount importance as contaminants from the skin can have an adverse effect on the surface chemistry, which then interferes with the binding chemistry of proteins and other biological reagents on the nitrocellulose detection site.

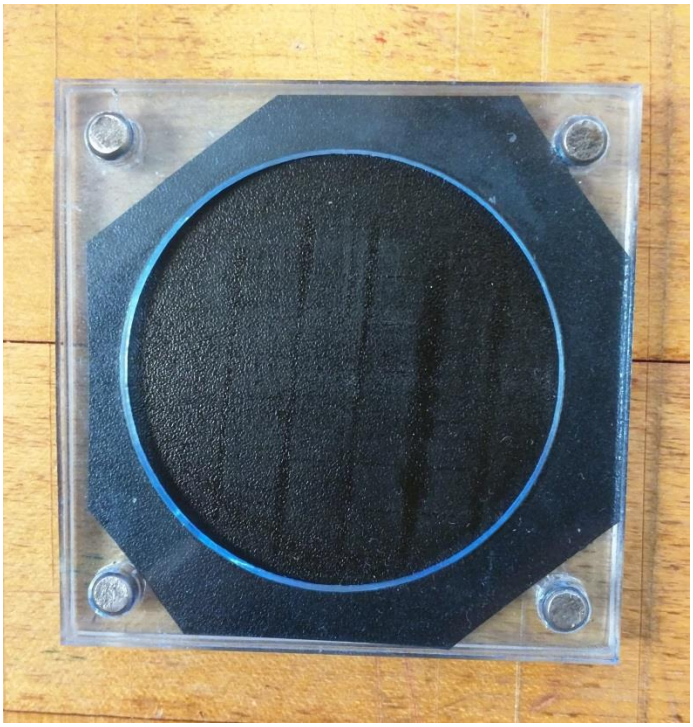


Figure 20: Nitrocellulose fixture for laser cutting

3.1.1.3 Wax melting

The wax printer only prints the wax ink onto the surface of the filter paper. To form solid hydrophobic wax barriers, the wax needs to be melted and absorbed through the thickness of the filter paper. The wax printed filter paper layers are heated for 25-35 seconds at a temperature of approximately 130 °C-140 °C in a Isotemp® Model 280A vacuum oven from Fisher Scientific. Before melting the back side of the wax printed filter paper remains white native filter paper. After the melting step, the black wax patterns can be seen absorbed all the way through the thickness of the filter paper as shown in figure 21. It is important to note that the printed wax structures will intrude laterally up to 0.5 mm into the native paper regions during melting and need to be compensated for in the initial design [19]. This expansion also happens to the wax

printed alignment markers used during laser cutting, which makes it important to laser cut the chips to size before melting as the markers will be far less precise post melting.

Before melting **After melting**

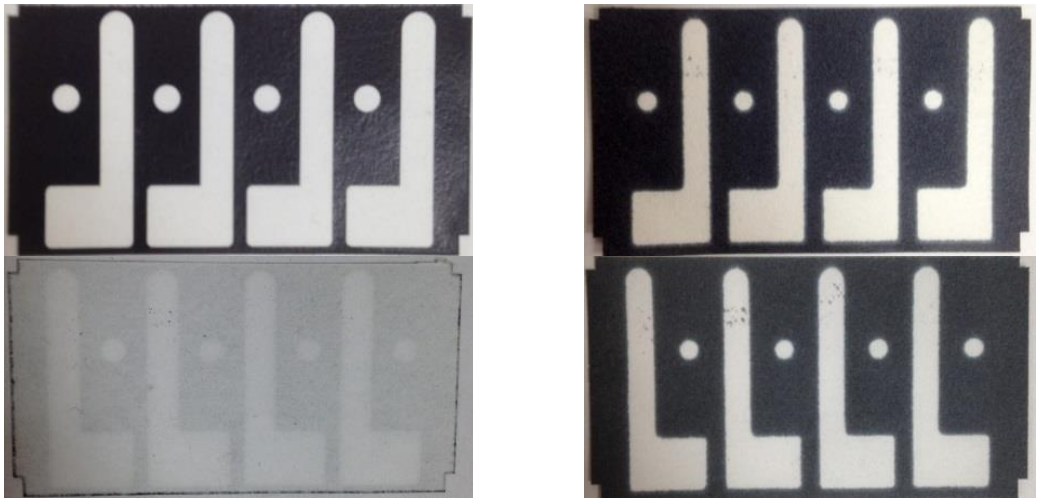


Figure 21: Before and after melting of top layer of protocol development chips. (showing the top and bottom sides of the filter paper) [19]

3.1.1.4 Chip assembly

Before assembly, designated spots on the hydrophobic layer need to be treated with a solution of allytrichlorosilane. This treatment makes these areas chemically hydrophobic but do not fill the filter paper pores like the wax. Surfactant is also deposited in assigned locations on the second filter paper layer. The combination of the chemically hydrophobic spots and the surfactant produce the fluidic valve described in section 2.3. After all the layers and materials have been prepared, the chip can be assembled. The edge of each layer was cut to fit a red alignment tool that can be seen in figure 22. The layers are combined, starting by inserting the blotting paper layer into the alignment tool. The third tape layer, which separates the fluid channels

from the second filter paper layer from the absorption pad, is aligned and pressed firmly onto the blotting paper in the alignment tool. The second filter paper layer is placed on top of that and is followed by the second tape layer and then the hydrophobic layer. The pieces of nitrocellulose and the conjugate pads are then precisely placed on designated locations on the hydrophobic layer. The first tape layer is then aligned and placed over the hydrophobic layer, securing the nitrocellulose pieces and conjugate pads. The first filter paper layer is then placed onto the first tape layer followed by the top clear tape layer, which is there to prevent evaporation and contamination from and to the chip.

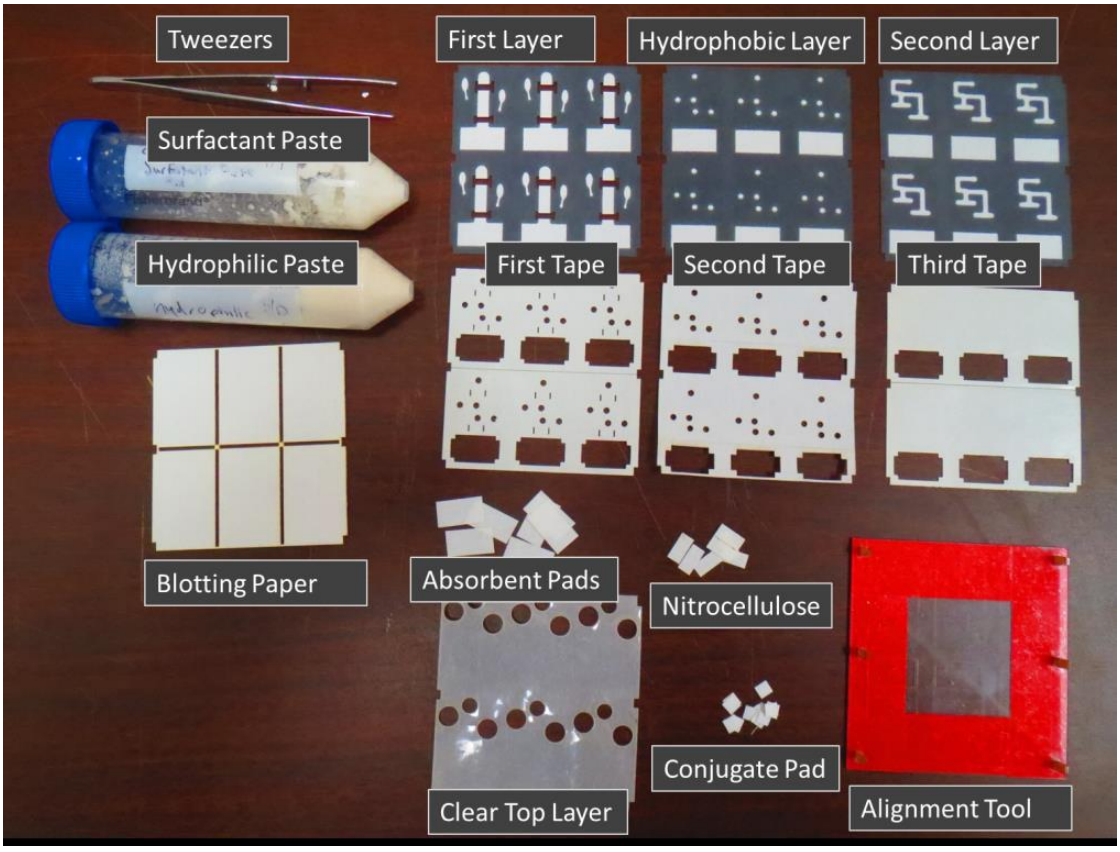


Figure 22: Chip layers and materials for double sided tape assembly [19]

3.1.2 Protocol development chip

A protocol development chip was developed as a way to focus on a biological test and its reagent interactions that only uses two fluids instead of three. As can be seen in figure 23, its functional components are filter paper channels, a single fluidic valve, a nitrocellulose detection area, and a blotting paper absorption pad. It is fabricated the same way as the three-fluid chip but requires only a single spot to by chemically treated with allytrichlorosilane to be hydrophobic. Because of the smaller size, eight paper based devices fit on each chip, two more than with the larger three-fluid design.

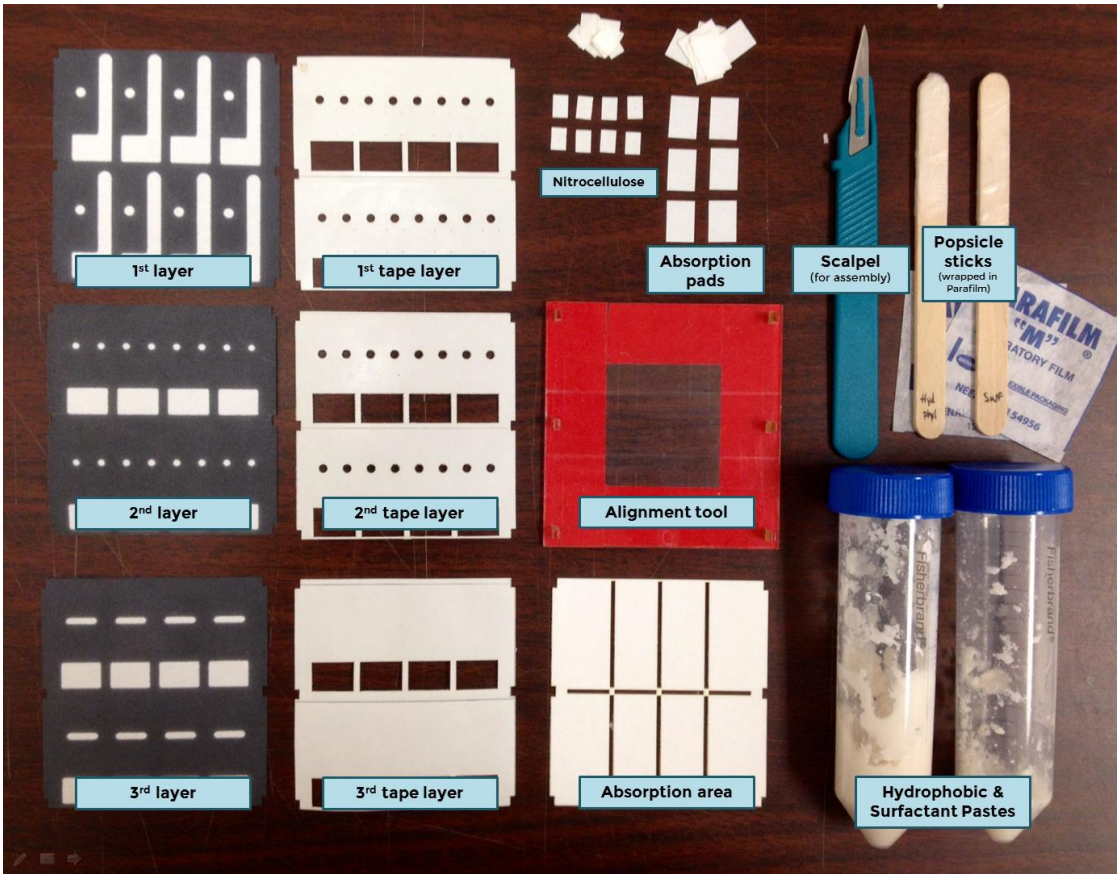


Figure 23: Chip layers and materials for protocol development chip assembly [19]

3.1.3 Spray adhesive method

A student from this research group, Benedikt Beermann, replaced the tape layers with a spray adhesive. This reduces the chip complexity and shortens the fabrication time by up to 80 % [37]. Figures 24 and 25 show the modifications made to the wax patterns on the filter paper layers. The surfactant is now directly spotted onto the fourth filter paper layer and a hydrophobic disc is used in place of the hydrophobic layer. To assemble the layers, the same procedure is used with the alignment tool except for the fact that the layers need to be sprayed with the adhesive before being attached to chip in the alignment tool. The adhesive is sprayed in two passes from approximately 1-2 feet away and as evenly across the chips as possible.

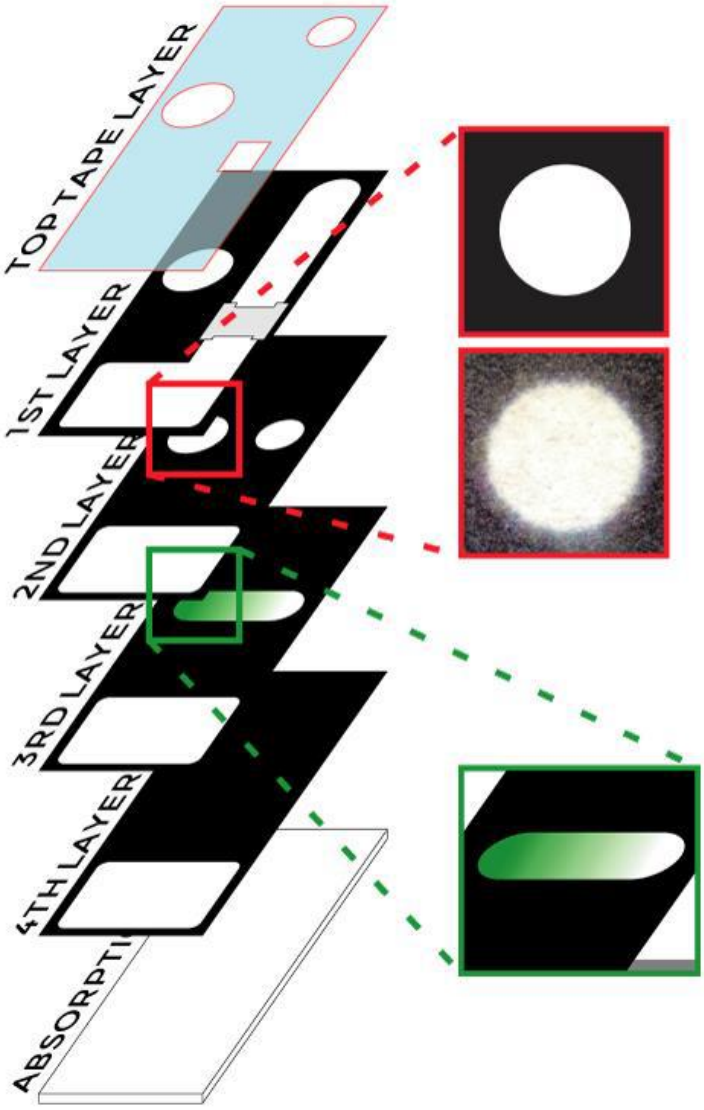


Figure 24: Design of protocol development chip with layers combined with spray adhesive [19]

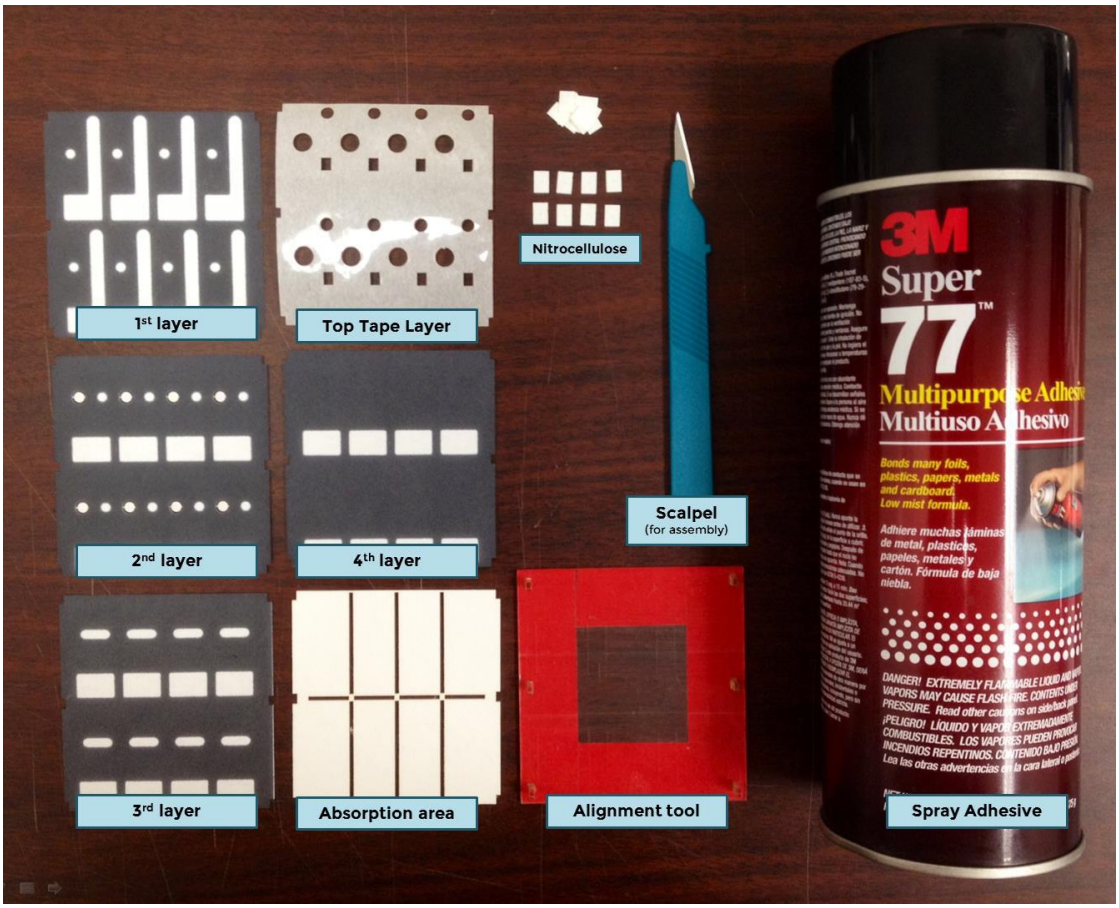


Figure 25: Chip layers and materials for assembly of protocol development chip using the spray adhesive method [19]

For the enzyme activity and enzyme inhibitor activity tests the chip was simplified even further. The second and third layers were removed from the design, essentially removing the reservoir and paper based fluidic valve from the chip, and turning it into a conventional strip test. This change was done to focus the research on the biological and chemical aspects of the chips. Lacking the automatic sequential loading of fluids to the chip, reagents for this simpler protocol development chip needed to be added to the sample inlet by hand. This avoids the need to optimize channel geometries for timing channels and fluidic paper valves, removes the chance of unknown effects from the surfactant, tween20, on the biological reactions, and

allows for variations in incubation times without having to optimize a new chip design. These simplified chips were also ideal for testing enzyme activity as it made it easy to vary reagent volumes without having to change the chip design.

3.2 Alkaline phosphatase activity

The enzyme, alkaline phosphatase (ALP), was used as an initial benchmark to measure enzyme activity with a paper based lateral flow device. It was chosen as it quickly converts the substrate, BCIP/NBT, into a dark purple precipitate that is highly visible when present in white nitrocellulose. Depending on the pore size of the nitrocellulose, the precipitate will also be physically hindered from flowing downstream in the chip.

Materials, equipment, and reagents needed for ALP activity test:

- Simplified single-fluid protocol development chips
- BioLabs® Biotinyated Alkaline Phosphatase (0.5 mg/mL)
- Sigma Fast™ BCIP/NBT (No. B5655)
- 1X PBS
- Deionized water (dH_2O)
- 1M Hydrochloric (HCl) in dH_2O
- 1M Sodium hydroxide (NaOH) in dH_20
- Sodium orthovanadate (Na_3VO_4)
- ImageJ® 1.49v (imaging software)
- Fujitsu® ScanSnap® iX500 scanner

The substrate, BCIP/NBT, comes in tablet form. One tablet is dissolved in 10 mL of ultra-pure dH_2O by vortexing. The volume of this solution can be made variable for a specific test but the concentration is usually kept the same for all experiments. The alkaline phosphatase (ALP) solution is aliquoted in 1X PBS from the stock 0.5 mg/mL solution. To test the enzymatic activity of ALP, concentrations of 5e-4, 1e-3, 5e-3, 1e-2, 5e-2, and 1e-1 mg/mL were used. To have a background measurement to compare the signal to, a separate solution was used with PBS with no ALP present (0 mg/mL ALP).

Each chip contains eight tests, one of which was assigned to be for the background measurement, where 1 µL of PBS was spotted. The remaining seven were spotted with 1µL of a defined concentration of ALP. Six different concentrations of ALP were tested using six chips (seven tests + one background per chip). After the spots were allowed to dry for 10 minutes, clear scotch tape was used to cover the nitrocellulose detection spot. A small gap was left at the sample inlet to introduce the substrate solution. 40 µL of the substrate solution was pipetted into each sample inlet. As the substrate flows passed the spotted ALP on the nitrocellulose detection spot, a color change can be seen in the center of the spot should the concentration of the ALP enzyme be high enough. It should be pointed out that conventional enzyme activity tests use a stop solution to stop the enzyme from converting the substrate at a specific time interval at which the optical density will be measured. For this lateral flow device a stop solution, such as a 3M NaOH solution, is not necessary because any unconverted substrate flows passed the detection spot into the waste area and is then out of reach of the immobilized enzyme.

After the substrate has finished flowing, the chip is allowed to dry for an hour and is then scanned in color at 600 DPI. Figure 26 shows an example scan of a single chip with seven tests (plus one background at the bottom right). The image is opened using the ImageJ software and the colors are inverted. In ImageJ and using a Red, Green, Blue (RGB) color scheme, the signal is measured per pixel as a brightness value in the range from 0 to 255. A circle is drawn around the detection spot (yellow circle in figure 26) and the pixel values within this area are averaged and converted to a mean gray value. The equation used to find the mean gray value from the three color measurements was the standard V=(R+G+B)/3. White has a mean gray value of 255, while black has a mean gray value of 0. It should be noted that the brightness values of the colors can be weighted to optimize the signal strength should the signal be of a specific color other than white. An ImageJ macro to adjust the brightness value weighting can be found in appendix A. The mean grey values of the detection spots and the background are recorded and plotted.

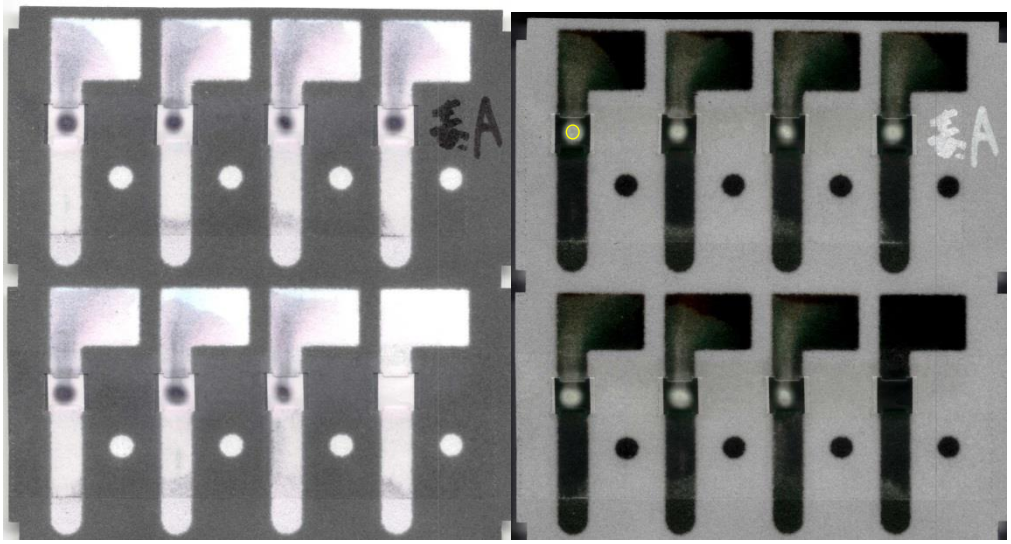


Figure 26: Example of scanned chip with positive signal of enzymatic activity (left) and with its colors inverted (right).

3.3 Sodium orthovanadate inhibitory activity

In this section sodium orthovanadate (Na_3VO_4) is used in solution form to inhibit the enzyme activity. This is to test how varying concentrations of an inhibitor in a sample solution will block the enzyme from converting substrate.

To make the sodium orthovanadate solution, the following steps need to be followed:

1. Dissolve 3.68 g of Na_3VO_4 (moleculare weight = 183.91 g/mol) to 90 mL ultrapure dH_2O. Once it is dissolved, bring the volume up to 100 mL.
2. Depending on the pH of the solution, slowly add tiny amounts of either 1 M NaOH or 1 M HCl while stirring to adjust the pH to 10. Adding HCl will lower the pH but turn the solution yellow.
3. To make the solution clear and colorless, boil the solution by heating in a microwave for 5-15 seconds.
4. Allow it to cool on ice until it reaches room temperature.
5. The pH will have risen above 10 and will need to be readjust to 10 by mixing in small amounts of the 1 M HCl.
6. By repeating steps 3-5 a few times, the pH should stabilize at ~10 and adding more HCl will result in little to no yellow color.
7. Aliquot and store activated Na_3VO_4 at -20 °C.

The chips that are used for this test are the same simplified protocol development chips used in the ALP activity experiment. An ALP concentration of 0.05 mg/mL was chosen as it gives a high signal, meaning high ALP activity, yet is not oversaturated (from ALP activity experiment results section 4.1). The idea is that with increased

inhibitor concentrations, the signal will decrease. The sodium orthovanadate solution is aliquoted in 1X PBS into concentrations of 1:1, 1:10, 1:100, 1:1000, 1:10000, and 1:100000. As a control, PBS is used without any Na_3VO_4 present, which should give a high signal, meaning high enzyme activity and low inhibitory activity. For the background signal, some detection spots have pure PBS spotted, without the presence of ALP. This should give a low signal, meaning low enzyme activity and can be compared to high inhibitory activity.

To run the experiment, 1 µL of the 0.05 mg/mL ALP solution is spotted on the nitrocellulose detection spot. After allowing it to dry for 10 minutes, clear scotch tape is applied to cover the detection spot, leaving a gap for the sample inlet. Next, 40 µL of the inhibitor aliquots are introduced to the chips at the sample inlets. Each chip has a designated Na_3VO_4 concentration associated to it. For this test, two chips with eight paper devices each are used for each inhibitor concentration. After the inhibitor solution has fully wicked through the chip (seen by the depletion of the inlet drop), the chips are allowed to dry/incubate for 10 minutes. Once the 10 minutes are over, 40 µL of the BCIP/NBT substrate solution is introduced to the devices at the sample inlets. As the substrate solution passes the nitrocellulose detection spot, a purple color may be seen depending on the concentration of inhibitor used on that chip. The chips are then allowed to dry for one hour before being scanned in to collect the quantitative data.

The same scanning procedure is used as with the ALP activity experiments in the previous section. The chips are scanned in color at 600 DPI, their colors are inverted using ImageJ, and a mean gray value is measured from a circular area chosen

in the detection spot. The difference for these chips though is that a high signal means low ALP enzyme activity and thus a high Na_3VO_4 inhibitory activity, while a low signal means high ALP activity and thus a low Na_3VO_4 activity.

3.4 ALP substrate volume test

For this experiment, the effect of the substrate volume was investigated. The idea was to see, if at low ALP enzyme concentrations, whether increasing the BCIP/NBT substrate volume would increase the signal. The chips and reagents were set up the same way as in the ALP enzyme activity test in section 3.2. The only differences were the volume of substrate introduced and the enzyme concentration. The substrate volumes for this experiment were 20, 40, 60, 80, and 100 µL and the ALP enzyme concentrations were 0.1, 0.01, and 0.001 mg/mL ALP in PBS. It takes approximately 20 µL of fluid for the substrate to even reach the nitrocellulose detection spot. Therefore, when looking at the data, signal will only start appearing with volumes higher than 20 µL for all tests. After the chips were dried following the same procedure as the other ALP enzyme activity tests, the chips were scanned and the data analyzed using ImageJ.

3.5 Trypsin activity

The next tests were done as a precursor to the full-fledged sepsis test. For sepsis, the concentration of the enzyme inhibitor, inter alpha inhibitor protein (IαIP), is measured. This inhibitor blocks the enzyme activity of trypsin from converting the

substrate, BAPNA. Before testing the activity of the inhibitor, the enzyme activity of trypsin first needs to be tested.

The materials, equipment, and reagents needed for trypsin enzyme activity tests:

- Simplified single-fluid protocol development chips
- Sigma® TPCK-treated Trypsin (0.2 mg/mL in 1 mM HCl)
- Sigma® BAPNA (N-Benzoyl-L-arginine)-p-nitroaniline HCl (21 mg/mL in DMSO)
- 1X PBS
- Deionized water (dH_2O)
- 1M Hydrochloric (HCl) in dH_2O
- 1M Sodium hydroxide (NaOH) in dH_20
- ProThera® Inter-alpha Inhibitor Protein
- ImageJ® 1.49v (imaging software)
- Fujitsu® ScanSnap® iX500 scanner

Before any trypsin enzyme activity experiments could be carried out, the following reagents needed to be prepared:

Trypsin solution (50 mL 0.2 mg/mL Trypsin in 1 mM HCl) [20]

1. Mix 50 µL of 1 M HCl with 50 mL dH_2O
2. Weigh 0.01 g of Trypsin and add 50 mL of 1 mM HCl
3. Mix by swirling
4. Aliquot 400 µL of resulting solution into small vials.
5. Store at -20 °C

TEA Buffer (100 mL 0.2 M TEA pH 7.8) [20]

1. Weigh 3.714 g of TEA (in powder form)
2. Add approximately 90 mL of dH_2O to TEA and allow to dissolve
3. Use a pH meter or litmus paper to measure pH of solution
4. Add small amounts of NaOH to the solution to raise the pH to 7.8
    - Using a 1 M NaOH in dH_2O solution can speed up this process
5. Once the desired pH is achieved, add dH_2O until the solution volume is 100 mL.
6. Mix well using vortexer.

BAPNA solution (1 mL 21 mg/mL L-BAPNA in Dimethyl Sulfoxide) [20]

1. Weigh 0.021 g of L-BAPNA.
2. Add 1 mL of dimethyl sulfoxide to L-BAPNA
3. Vortex solution to mix well.
4. Aliquot 200 µL of solution in small vials and store at -20 °C.

Hide Powder Azure substrate solution (1 mL 4.17 mg/mL) [38]

1. Weigh 10 mg of Hide Powder Azure.
2. Add 2.4 mL of Tris Buffer (pH 7.8) to Hide Powder Azure.
3. Vortex solution to mix well.
4. Store at -20 °C.

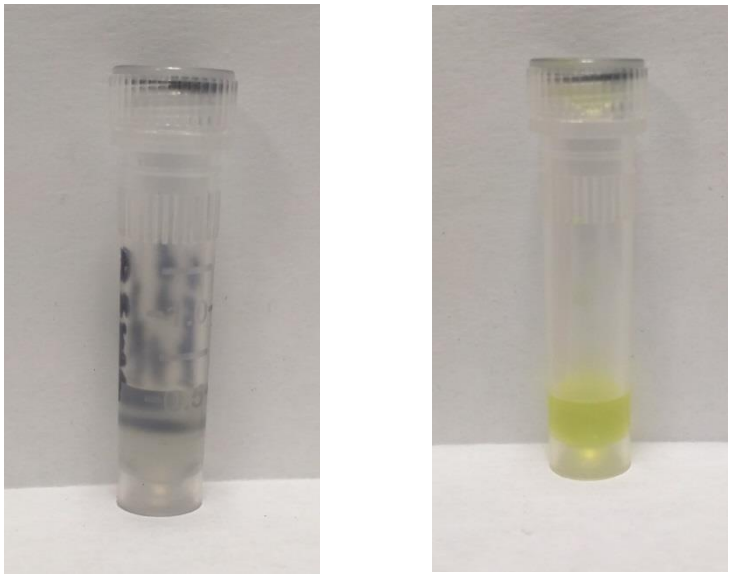


Figure 27: Unconverted (left) and converted (right) BAPNA solution using trypsin as the catalyzing enzyme.

Before carrying out a model enzyme activity test on the simplified single-fluid protocol development chips, 200 µL of a 1:3 trypsin in TEA buffer solution is mixed with 200 µL of 1:5 BAPNA in dH_2O dilution in a vial to test whether the enzyme is in fact active. After allowing the solution to incubate at room temperature for 30 minutes, the mixture should have turned a bright yellow. It is compared to an unconverted BAPNA solution in figure 27.

Just like in the ALP enzyme activity experiments, 1 µL of the enzyme solution, which in this case is a 1:3 trypsin in TEA buffer solution, is spotted on the nitrocellulose detection spots in the simplified single-fluid protocol development chips. Varying concentrations of trypsin are spotted as a way to model varying enzyme activity denoted by the unit 'U'. The unit 'U' is defined as the amount of enzyme that catalyzes the conversion of 1 µM of substrate per minute. After the spots are allowed to dry for 10 minutes, they are covered by clear scotch tape, covering the

nitrocellulose detection spot and the main fluid channel but leaving a gap for the fluid inlet at the opposite end of the fluid channel. A 40 µL volume of a 1:5 dilution of the premade BAPNA solution to dH_2O is then added at the fluid inlet and any color development is observed at the detection spot under the clear tape. The conventional trypsin activity tests use a stop solution, such as Basic Pancreatic Trypsin inhibitor (BPTI) or concentrated acetic acid ($C_2H_4O_2$), to stop the trypsin from continuing the cleaving of BAPNA substrate during the optical density measurements. For lateral flow devices a stop solution is not necessary as any unconverted substrate will have flowed into the waste pad, away from the immobilized trypsin enzyme. After the chips are allowed to dry for an hour, they are scanned and the detection spots are analyzed using ImageJ and the same procedure for the ALP tests described in section 3.2. The weighting of the RGB colors can be tuned to detect a yellow color as opposed to the dark purple color in the ALP tests. When the colors are inverted in RGB, a yellow color becomes blue. For a blue signal, the RGB weighting equation is:

$$V = (0 \times R) + (0 \times G) + (1 \times B)$$

3.6 Inter-alpha inhibitor protein inhibitory activity

Once concentration curve has been made for trypsin to signal strength, a concentration is chosen with the highest consistent signal that does not show signs of oversaturation. A new reagent, an inter-alpha inhibitor protein (IαIP) solution, is introduced to the experiment. This protein, IαIP, is a biomarker for various stages of sepsis and its concentration may be detected using this experiment. Each IαIP molecule has the ability to block the activity of a trypsin molecule from converting the

BAPNA substrate into the yellow dye. The more of the IαIP present in the solution, the more trypsin enzymes are deactivated, which in turn would produce less of a yellow color after the BAPNA solution is introduced.

To begin the experiment, 1 µL of the chosen concentration of the trypsin solution is spotted onto the nitrocellulose detection area and allowed to dry for 10 minutes. Clear plastic tape is then used to cover the detection area, leaving a gap for the fluid/sample inlet at the opposite end of the main fluid channel. Next, 40 µL of the varying concentrations of IαIP are added to the fluid inlet and allowed to flow through the chip and incubate for 10 minutes. Afterwards, the 1:5 dilution of the premade BAPNA solution to dH_2O is added at the fluid inlet and any color development is observed at the detection spot. The chips are then left to dry for one hour and then scanned. Using ImageJ, the chips are analyzed using the same procedure as in the previous test, section 3.5, and using the same RGB mean gray value method and color weighting for an inverted yellow to blue signal. The same test was done again using only the highest trypsin concentration available and increasing the substrate volume to 400 µL, pipetting the substrate 20 µL at a time to avoid the fluid from overflowing.

4 Findings

The first thing that needed to be tested was to see whether a basic enzymatic activity test could be performed using a paper-based lateral flow device. To prove that such a test is possible, a well-known enzyme, alkaline phosphatase (ALP), with respective substrate, BCIP/NBT, and inhibitor, sodium orthovanadate, combination was selected to be tested using a simplified version of the protocol development chip fabricated in-house. This simplified chip was developed as a way to focus the research on the biological test and its behavior in a porous substrate, which in this case is nitrocellulose. Using this chip avoided interactions with other aspects of the chip such as the surfactant and chemicals, which are used for paper sizing, present in the paper-based fluidic valves. As there was only the one single fluid channel in this design, the timing between the introductions of fluid reagents as well as incubation times were all done manually. This is advantageous when testing new unproven biological tests as the incubation times still need to be optimized and can then be easily adjusted. Should the original protocol development chip be used for such tests, the chip design would have to be altered every time the timing of the fluid reagents needs to be changed by, for example, changing the fluid channel lengths.

4.1 Alkaline phosphatase activity

To model different levels of enzymatic activity of alkaline phosphatase (ALP), concentrations of 0, $5e^{-4}$, $1e^{-3}$, $5e^{-3}$, $1e^{-2}$, $5e^{-2}$, and $1e^{-1}$ mg/mL of ALP in 1X PBS were spotted and allowed to dry on the nitrocellulose detection spots. After the drying and

taping step, the substrate solution of BCIP/NBT is introduced to the chip and allowed to flow past the detection spot. The immobilized ALP cleaves the substrate molecules with one of the product molecules being a dark purple precipitate. Depending on the amount of ALP present, a varying amount of the substrate is converted, which can then be detected by the amount of color change. The chips are scanned, the colors are inverted, and the detection zones are analyzed using ImageJ. The mean gray value of all the pixels in this zone is calculated where the maximum signal strength has a value of 255 (white) and minimum signal strength of 0 (black). For a the dark signal in this test, the equation used for the mean gray value is: $V = (R + G + B) / 3$. Figure 28 shows a selection of two chips with the color inverted that show an example of high signal and one of low signal. A complete set of all the images of scanned chips can be found in appendix B.

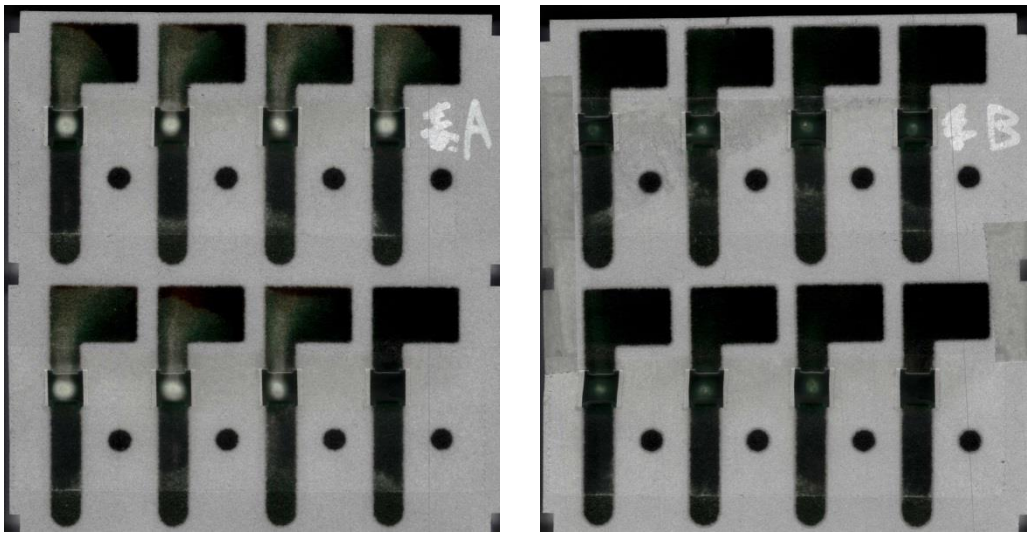


Figure 28: Example of scanned chips with inverted colors, one with a visually high signal (left) and one with a visually low signal (right).

Each chip contains eight paper based lateral flow devices, seven of which are had the spotted ALP and the eighth was used to measure the background signal as no ALP was spotted there. The mean gray values of the seven tests of each concentration were averaged together and the standard deviation calculated. For the error bars that will be used to graph the data, a 95 % confidence interval was used, which equates to two times the standard deviation, plus and minus the average of the data points. The average of these data points can be seen in table 2 and the expanded version containing all of the data in appendix C.

	Background	**$5e^{-4}$**	**$1e^{-3}$**	**$5e^{-3}$**	**$1e^{-2}$**	**$5e^{-2}$**	**$1e^{-1}$**
Avg. MGV	16.40	12.99	20.64	26.48	45.34	70.74	78.82
95% confidence	8.34	3.83	6.60	5.37	8.60	11.49	18.35

Table 2: The average mean gray value (MGV) and 95 % confidence interval of different concentration of ALP in mg/mL shown as RGB brightness values.

Figure 29 shows a comparison of the signals produced by these various ALP concentrations. A distinct trend can be seen that with an increased ALP concentration, the signal increases as well. At an ALP concentration at or below $1e^{-3}$ mg/mL the measured signal goes below the measured signal of the background. Interestingly enough, when visually looking at the spot at such low concentrations, the signal can still be seen like in figure 30, yet the measured values sink within the error range of the background. It should also be pointed out that the error bars increase in size with an increase in enzyme concentration.

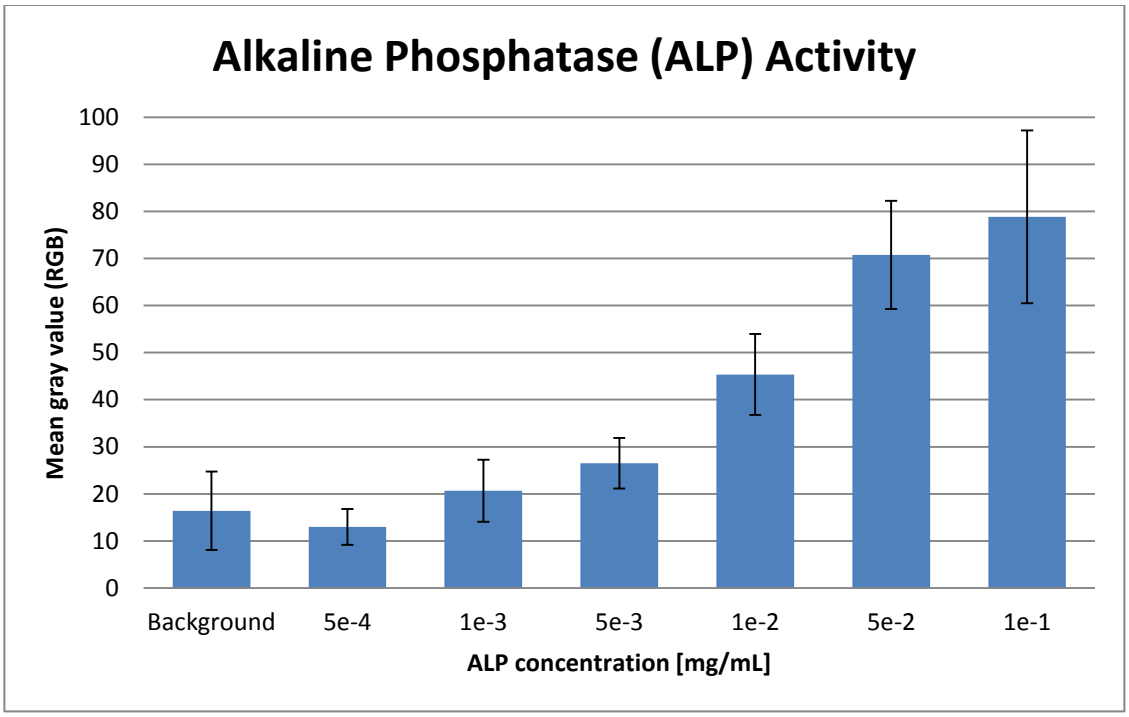


Figure 29: Model of enzyme activity using various concentrations of alkaline phosphatase.

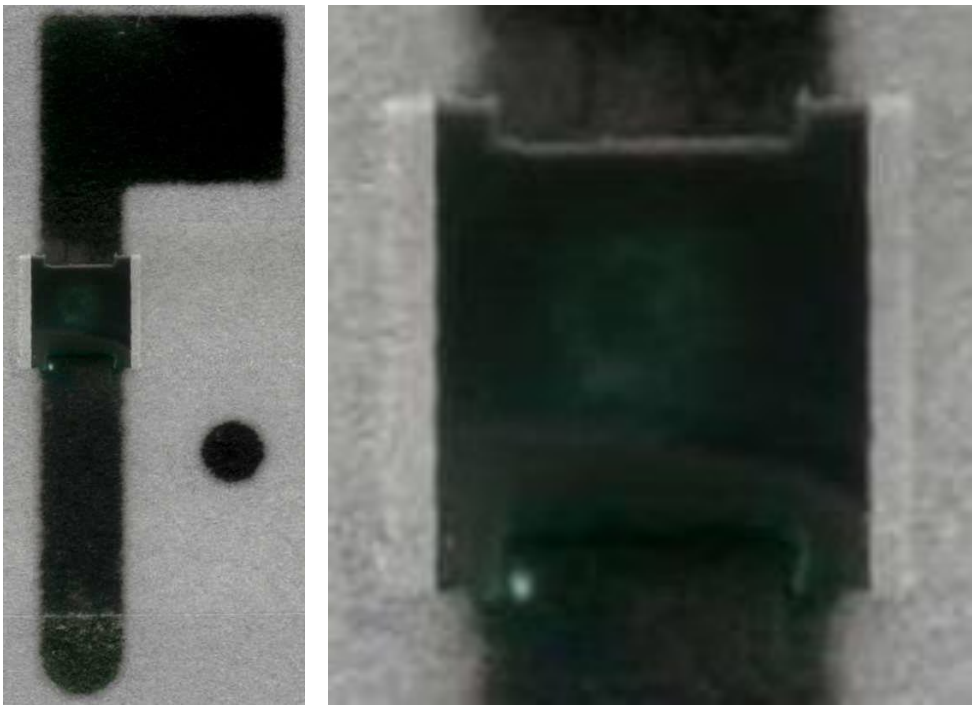


Figure 30: Protocol development chip with $1e^{-3}$ mg/mL ALP concentration with the colors inverted (left) and a closer view of the detection spot (right).

The stock ALP solution was acquired having a concentration of 5 mg/mL. The reason that the highest concentration shown in the data set in table 2 and figure 29 is $1e^{-1}$ mg/mL, is that above those ALP concentrations the chip becomes oversaturated. At such high enzyme concentrations, the ALP converts the substrate far too quickly and the resulting precipitate clogs the porous substrate. This clogging forces the remaining substrate fluid to wick around the sides of the detection area, where no enzyme is present, and thus will not be converted. Figure 31 shows an example of such a case, where at the upstream edge of the spotted enzyme, there is a high concentration of visible white signal but has a dark band downstream that is darker than the corresponding background signal on the edges of the detection area. It is assumed that because the unconverted substrate wicks around the sides, the center of the detection spot does not encounter any fluid with substrate and thus is the local area with the lowest brightness values. Because the center of these spots are so dark (in the inverted image), the mean gray value, calculated from all of the pixels within the detection spot, will have a much lower value than expected.

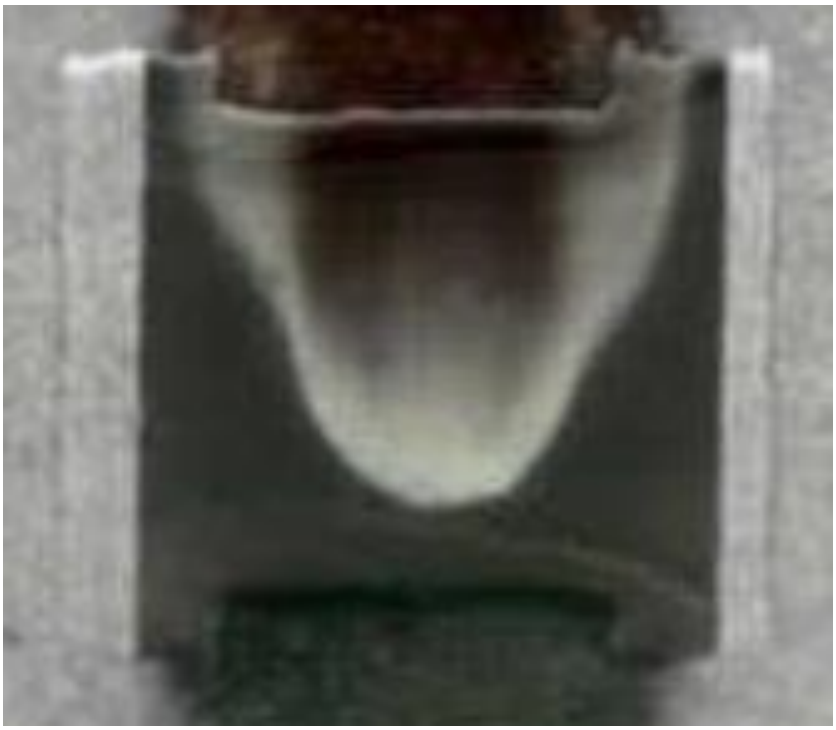


Figure 31: Detection spot with a 0.5 mg/mL ALP concentration. High signal (white) is visible on the upstream edge of the v spot and no signal (black) is pocketed within that edge.

4.2 Sodium orthovanadate inhibitory activity

The next step was to discover the effect of an inhibitor, in this case sodium orthovanadate (Na_3VO_4), on the enzyme, ALP, when the reagents interact in the porous nitrocellulose. After a rudimentary signal to enzyme concentration curve was made from the ALP activity test in section 4.1, an ALP concentration of 0.05 mg/mL was chosen as high color brightness values were measured using this concentration and at that concentration there was no danger of oversaturation the detection area. A high starting brightness value is important for this test because the introduction of an inhibitor was expected to decrease these brightness values. After the immobilizing of the 0.05 mg/mL of the ALP solution, the inhibitor solution with various concentrations

of sodium orthovanadate were introduced to the chip at the fluid inlet and allowed to wick through the chip and across the detection spot. In that step the activated inhibitor is expected to deactivate a certain amount of the enzymes depending on the concentration of inhibitor introduced. The more enzymes that become deactivated, the fewer the enzymes that will be active when the substrate is introduced, decreasing the rate at which the substrate is converted into the detectable precipitate. Table 3 shows the average mean gray values measured for each corresponding inhibitor dilution. For the control tests, in place of the inhibitor solution, PBS was used to keep as many parameters as constant as possible. Theoretically, the control spot should have the highest average brightness value as none of the enzymes are deactivated by the inhibitor. To have a background measurement to compare the results to, some tests were done that replaced both the inhibitor solution and the spotted ALP solutions with PBS. The background should theoretically have the lowest average brightness value as there is no enzyme to convert the substrate. The 95 % confidence interval was calculated as two times the standard deviation of all the data points for each respective inhibitor concentration and was used for the error bars in figure 32.

	Control	**Background**	**1:1**	**1:1e^{1}**	**1:1e^{2}**	**1:1e^{3}**	**1:1e^{4}**	**1:1e^{5}**
Av. MGV	105.37	29.28	24.13	41.09	56.52	88.28	104.97	109.89
95% conf.	15.15	3.69	3.17	6.91	9.62	13.52	19.89	22.17

Table 3: The average mean gray value (MGV) and 95 % confidence interval of different dilutions of Na3VO4 as RGB brightness values.

In figure 32, the tabulated numbers can be visually compared and it is easily recognizable that with an increased concentration of inhibitor the measured mean gray value decreases as expected. At inhibitor concentrations below the $1{:}1e^{3}$ dilution, the range in the error bars start to blend together. At the highest inhibitor concentration the measured mean gray value sinks below the measured mean gray value of the background tests. It should also be pointed out that with an increase in inhibitor concentration, the size of the error bars also increase. Possible solutions to these phenomena will be discussed in chapter 5.

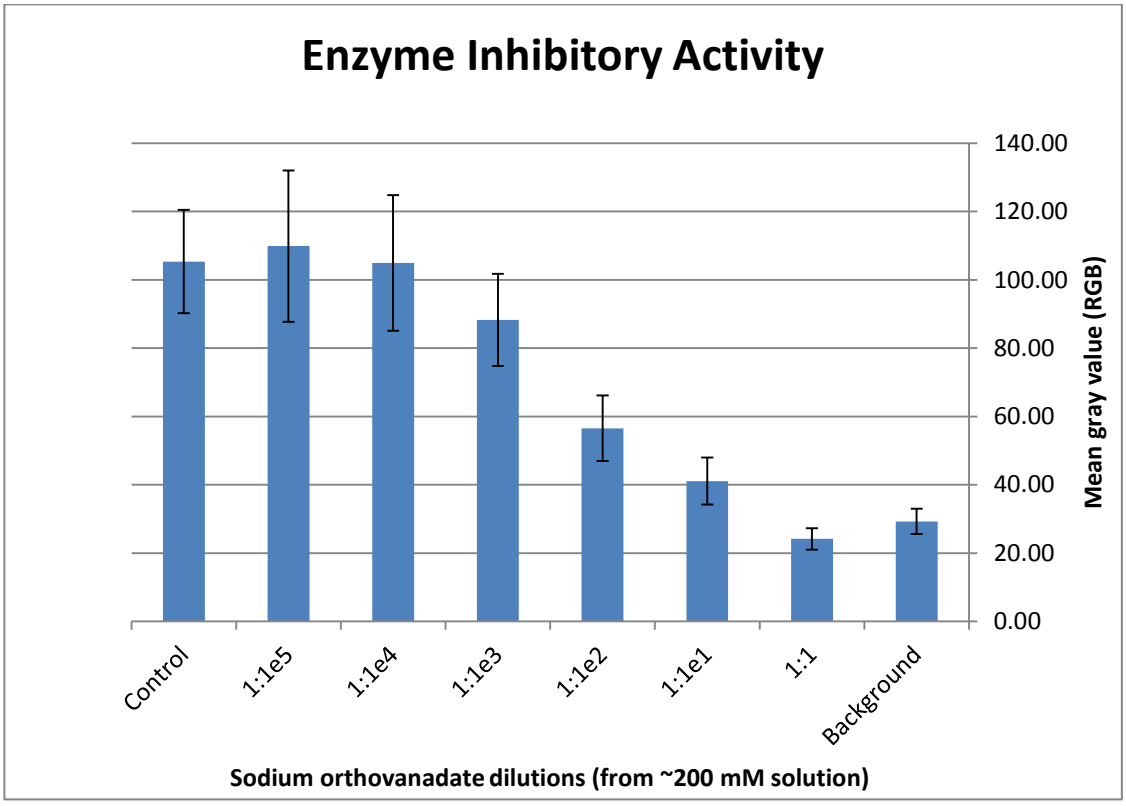


Figure 32: Model of enzyme inhibitor activity using various dilutions of sodium orthovanadate.

4.3 ALP substrate volume test

Because it is the substrate that is converted into the detectable signal, and because there is a wide range of volumes that potentially could be used for these devices, an appropriate volume needed to be found for such tests. The idea was that by increasing the volume of substrate passing the detection area, the signal would also increase proportionally, because the enzymes immobilized on the surface of the nitrocellulose detection spot continually convert the substrate as it is flowing by. For this test, ALP concentrations of 0.1, 0.01, 0.001 mg/mL, and one 'background' with no enzyme present were used. For each of these concentrations, substrate volumes of 20, 40, 60, 80, and 100 µL were added. Table 4 shows the data from this experiment.

	20 µL	**40 µL**	**60 µL**	**80 µL**	**100 µL**
0.001 mg/mL					
Avg. MGV	2.43	32.69	38.92	43.41	41.89
95% conf.	2.12	4.76	5.71	4.89	13.71
0.01 mg/mL					
Avg. MGV	5.67	70.77	81.18	83.12	80.53
95% conf.	6.94	7.28	4.62	15.79	9.09
0.1 mg/mL					
Avg. MGV	15.54	126.75	151.12	165.71	172.41
95% conf.	5.77	18.96	4.32	11.88	11.60
Background	4.094	39.53	39.74	37.29	34.66

Table 4: The average mean gray value (MGV) and 95 % confidence interval when various substrate volumes are used for three ALP enzyme concentrations.

A visual representation of this data can be seen in figure 33. It is immediately noticeable that there is a huge jump in signal from 20 µL to 40 µL. This is due to the length of the wicking channel before the substrate reaches the nitrocellulose detection

area where the enzyme is immobilized. It takes approximately 20 µL for the fluid front to reach the detection spot so very little of the substrate solution crosses the detection spot and is converted. For each substrate volume tested, an increase in enzyme concentration also increased the measured signal. When looking at each enzyme concentration individually, an increase in substrate volume from 40 µL to 100 µL only made a noticeable change at the high enzyme concentration line of 0.1 mg/mL. The signal measured at the lowest concentration of 0.001 mg/mL did not have a quantitative signal above the brightness value measure from the background. These phenomena and potential solutions to problems are discussed in chapter 5.

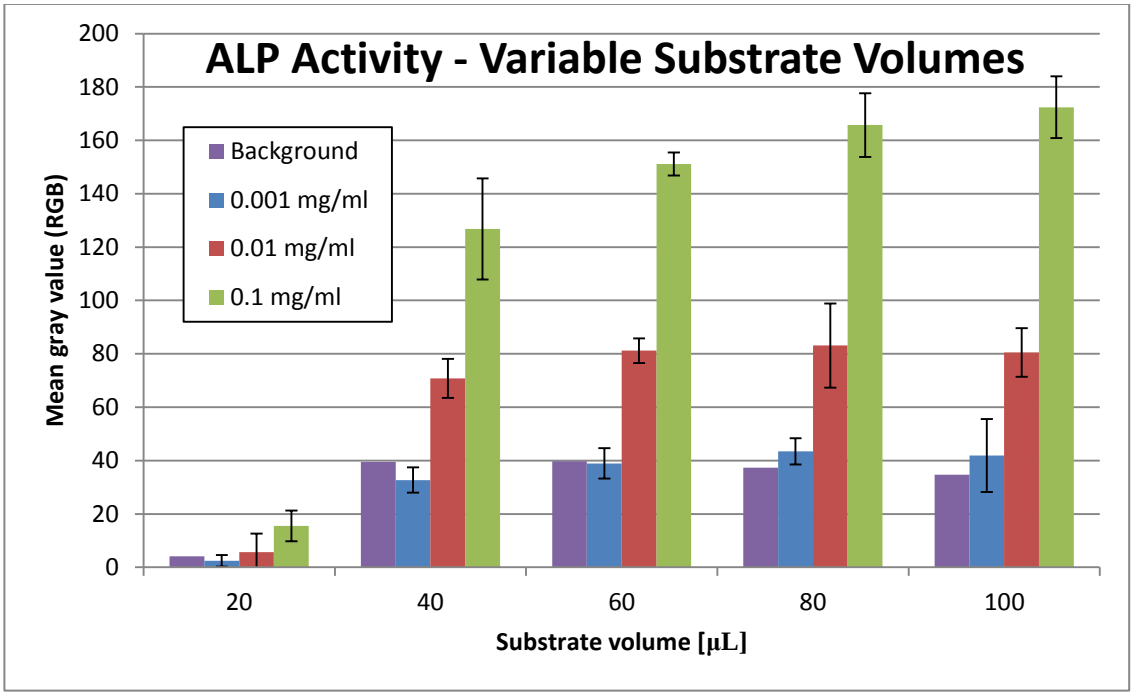


Figure 33: ALP enzyme activity test showing the effect of a change in substrate volume on the measure signal on three different enzyme concentrations.

4.4 Enzymatic and inhibitory activity of trypsins

Inter-alpha inhibitor protein (IαIP), an inhibitor for the enzyme, trypsin, and a biomarker for sepsis, is the next analyte of interest to be detected using an enzyme inhibitor activity assay. Because these reagents behave similarly to the ALP/sodium orthovanadate assay, the tests using trypsin and IαIP were developed the same way.

4.4.1 Trypsin activity

Before the detection of the inhibitor can be done, the enzymatic activity of the trypsin needed to be tested. Various concentration of the trypsin solution were spotted and immobilized on the nitrocellulose detection area and after it was allowed to dry, covered by a piece of transparent tape. A 40 µL volume of the substrate, BAPNA, was then introduced to the fluid inlet and allowed to wick through the paper-based lateral flow device and over the nitrocellulose detection area where the trypsin was immobilized. As the fluid crossed the detection area there was no visible change in color.

4.4.2 Trypsin activity with increased substrate volume

The same test was repeated with some changes in an effort to obtain even a very faint color change. For this test the highest concentration of trypsin available (0.2 mg/mL) was used. Also, the volume of substrate added to the chip was increased from 40 µL to 400 µL. To avoid the substrate fluid from overflowing, 20 µL were added to each test every 5 minutes until the 400 µL total is reached. When this test is done with the conventional 96-well microplate method, it takes over 30 minutes for the

BCIP/NBT substrate to be converted by the trypsin enzyme. Adding the 5 minute wicking time also gave the paper-based lateral flow method more time for the substrate to be converted. With 40 µL the fluid will stop flowing in less than 5 minutes, while with the 400 µL procedure, it takes ~100 minutes.

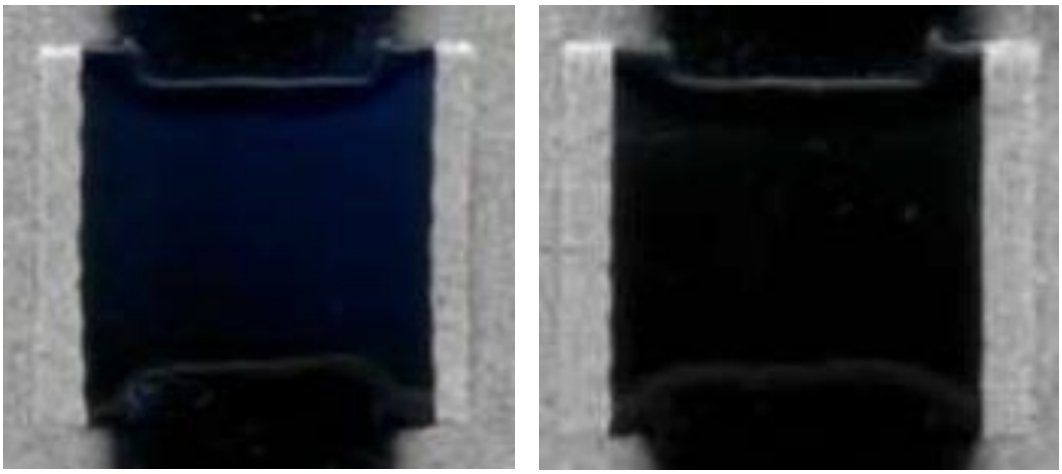


Figure 34: Inverted color scans of enzymatic activity test using 400 µL of BAPNA substrate solution over 0.2 mg/mL trypsin spot (left) and over TEA buffer spot (right).

Figure 34 shows a scan of the test where the highest blue brightness value was measured and a scan of a chip where no substrate was converted. The dark blue colored signal is difficult to see on the black background with the naked eye but can be measured using ImageJ. Figure 35 shows the measured values for all of the tests and shows that with trypsin there will be a slight color change but inconsistently. Only four of eight tests with the trypsin spotted showed a meaningful change in color. The rest had a color brightness value comparable to the tests done without any immobilized trypsin. The waste pads did show some signs of a faint yellow hue after drying, indicating the presence of converted substrate for both cases. It should be

pointed out that not many tests were done for this experiment and there are many uncontrollable parameters. There is a chance that these results may not be repeatable, though they could definitely be improved by controlling parameters, optimizing the volume of trypsin spotted, and the method of spotting the trypsin.

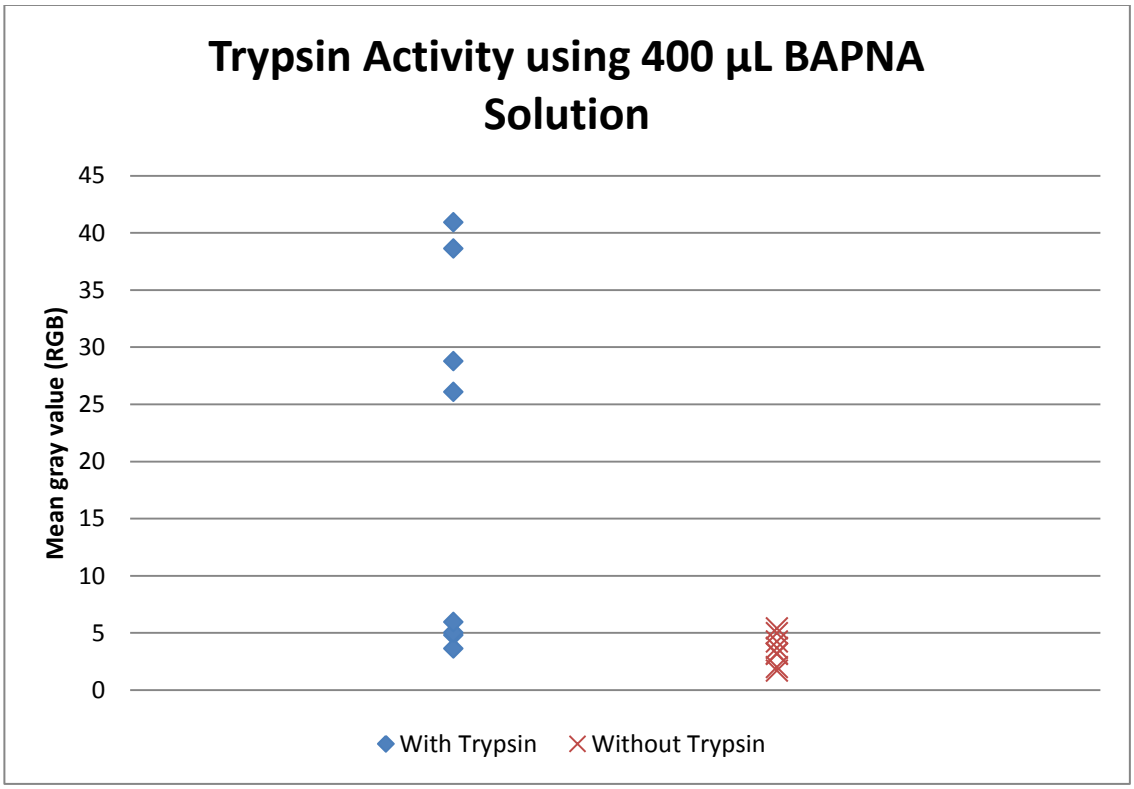


Figure 35: Enzyme activity test using 400 µL of BAPNA substrate solution over 0.2 mg/mL trypsin spot.

4.4.3 Inter-alpha inhibitory protein activity

For this test it is necessary to have a control and high starting signal as the role of the inhibitor in this assay is to decrease the enzymatic activity, which in turn reduces the resulting signal. Because the previous test (section 4.4.2) did not produce high enough signal, the experiments for this section could not be carried out.

5 Conclusion and Future Work

The research done into enzymatic activity and enzyme inhibitory activity assays on paper-based lateral flow devices progressed in several ways over the course of this research. The most important of these was the proof of concept that enzymatic activity and enzyme inhibitory activity tests are possible on a paper-based lateral flow device. The conventional method for an enzymatic activity and enzyme inhibitory activity tests are run using elaborate and expensive methods that require large amounts of sample fluids and reagents, expensive equipment, a laboratory, and a trained technician [20]. This new method potentially allows for the elimination of all of these requirements with some additional optimization and product design work.

5.1 ALP enzyme and inhibitory assay

For the alkaline phosphatase (ALP) enzyme, data was acquired showing the possibility of a signal to enzyme concentration calibration curve (figure 29 in section 4.1). The very rough method of spotting the enzyme, however, causes rather large ranges in measured values for tests with identical parameter sets. Once that has been optimized, a calibration curve useful for marketable products will be possible. For the enzyme inhibitory activity of sodium orthovanadate, using the ALP assay, the same conclusions can be made. A preliminary signal to inhibitor concentration curve (figure 32 in section 4.2) was achieved, yet the error range of this data also leaves much room for improvement through optimization and other engineering methods. This curve also

proves that the binding of the inhibitor to the enzyme is strong enough and will not be broken as the substrate solution flows by.

The shape of the spot that changes color is far smaller than the area that gets wetted when spotting the ALP enzyme solution, which indicates that the ALP molecules quickly immobilize on the nitrocellulose during the spotting. The volume and concentration of the enzyme spotted both have an effect on the final size and shape of the immobilized enzyme and cannot be measured or controlled before the tests are carried out. A new method for the deposition of the enzyme reagent needs to be found. Using an inkjet printer has proved promising to precisely deposit small volumes of biological liquid reagents [39]. Printing the enzyme as a test line instead of a spot will not only solve the problem of controlling the size, shape, and concentration of the immobilized enzyme detection area, but also the problems with uneven brightness values measured and the wicking of substrate fluid around the sides of the detection spot. Many of these problems are due to local areas of a highly concentrated substrate precipitate particles clogging the small pores in the nitrocellulose. If the enzymes were printed as a test line similar to conventional lateral flow tests, the substrate and other reagents will not be able to bypass the functionalized detection spot, preventing the loss of a large portion of the signal strength.

To improve these tests even further, a washing step can be introduced to the chip design. After the substrate solution is finished flowing, any unconverted substrate could be washed away with an additional fluid, because as the fluid dries, any unconverted substrate may cleave to give falls signal both in the detection area and on the background. Increasing the color brightness of a background measurement will

have a huge effect on the signal to noise ratio as the noise is calculated from the background brightness value. Also another enzyme, horseradish peroxidase (HRP) could be used along with an appropriate precipitating substrate like 3,3'-Diaminobenzidine (DAB), to perform similar enzymatic activity tests. As a potential application, the inhibitory activity of cyanide on the HRP enzyme could be quantitatively be measure with a similar PBD assay as the ALP enzyme inhibitory activity assay. HRP also has the advantage that it is also useable at a wider range of pH levels [40].

5.2 Trypsin activity

The enzymatic activity tests done using trypsin had far less promising results. The chemistry involved in this test does not fit well with paper-based lateral flow devices. There are several problems that need to be addressed. The first, the time required for the enzyme, trypsin, to convert the substrate, BAPNA, into its colored product, from which one receives the signal, take much longer than in the ALP activity tests. The substrate takes over 30 minutes to convert in the conventional 96-well microplate method, while it takes less than 3 minutes for the same volume of substrate solution to flow through the paper-based lateral flow device. Another issue is that the colored product is not a precipitate like in the ALP assay, but a soluble yellow dye. The advantage of a colored product that is a precipitate is that it will stay stuck between the pores of the nitrocellulose detection spot producing small areas of high concentration that can be easily detected. The soluble dye, however, will continuously be washed into the waste/absorption pad as fresh substrate solution enters the

detection area. The yellow color of the dye produced in the trypsin/BAPNA assay is also difficult to see and measure on the white nitrocellulose background. The simplest solution would be to find an alternative substrate that when converted by trypsin, turns a darker color. Hide-Remazol Brillian Blue R from SigmaAldrich® may be a substrate worth investigating for future tests. This substrate is converted from a blue color to orange (595nm), colors that contrast each other strongly and can easily be detected on the white nitrocellulose background. Ideally, a substrate that also precipitates when converted should be chosen. It is unclear at this point whether this substrate is a precipitate before or after it is converted. Eventually the paper-based lateral flow devices with the trypsin/IαIP assay should produce results comparable to the conventional 96-well microplate method like in figure 36, which shows the optical density with respect to the inhibitory activity of the IαIP.

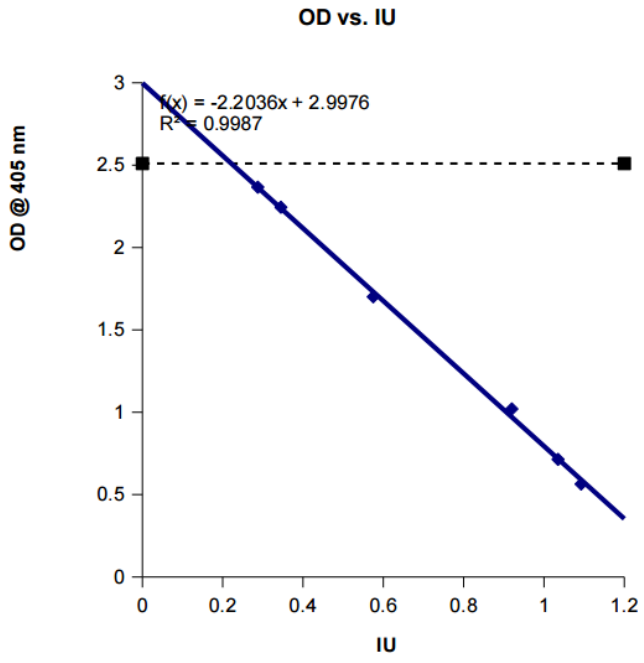


Figure 36: Optical density at 405 nm light for various inhibitory activities (i.e. various IαIP concentrations) [20]

After the reagent concentrations, volumes, and incubation times for these assays are optimized, a full 3d paper-based lateral flow device can then be designed with appropriate timing channels to account for the incubation times, and paper-based 3d fluidic valves to trigger and sequentially load various fluidic reagents. The assays done in this work all require a minimum of two fluids when they are run, the sample fluid containing the inhibitor solution (or a wash if only and enzyme activity test) and the substrate in a separate reservoir. The order and timing at which each of them enters the detection zone are automatically controlled by the chip. A protocol development chip, a two fluid design, as can be seen in figure 37, was designed to handle two fluids in a sequential loading scheme [19]. The sample fluid holding an unknown concentration of the enzyme inhibitor is added at the sample inlet. A defined volume of substrate will be added at the reservoir inlet and its flow will be stopped due to a hydrophobic fluidic valve in the layer beneath it. Once the sample fluid reaches the other side of the fluidic valve, it mixes with a surfactant dried in the paper and opens the valve, opening a path for the substrate solution to flow through the detection spot. The design would have to be optimized to account for incubation times but would roughly function as is.

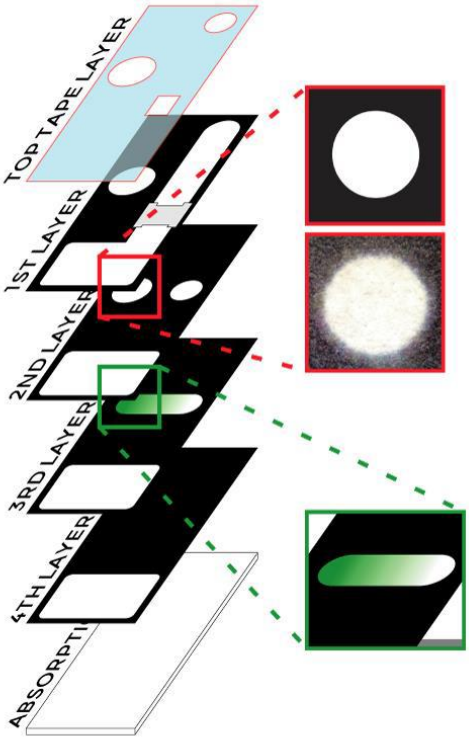


Figure 37: Design of two-fluid protocol development chip [19].

APPENDICES

Appendix A

"MeasureRGB" Macro for ImageJ to weight specific colors

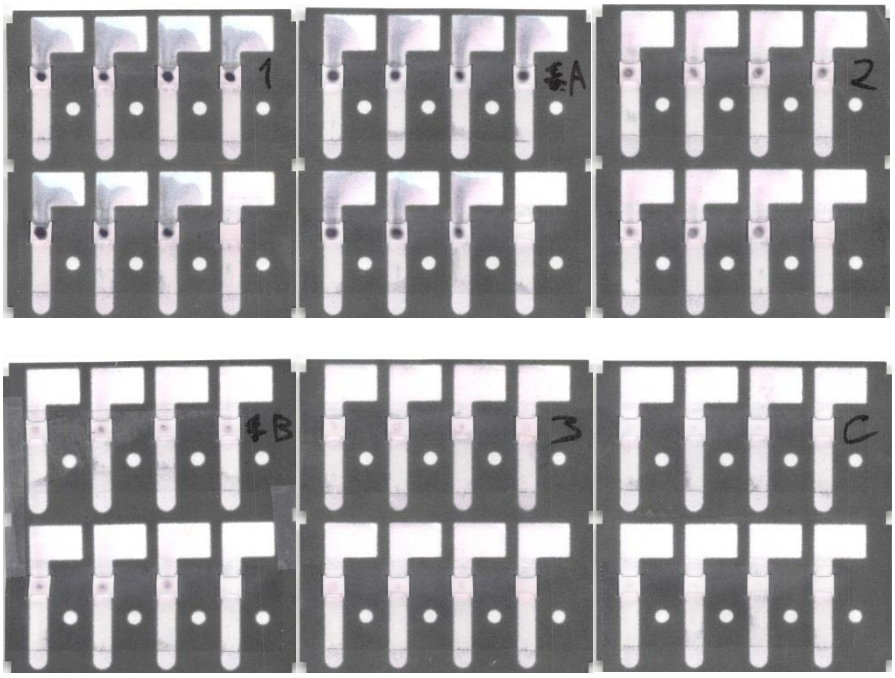
```
// "MeasureRGB"
// This macro demonstrates how to separately measure
// the red, green and blue channels of an RGB image.

  requires("1.35b");
  if (bitDepth!=24)
     exit("This macro requires an RGB image");
  setRGBWeights(1, 0, 0);
  run("Measure");
  setResult("Label", nResults-1, "Red");
  setRGBWeights(0, 1, 0);
  run("Measure");
  setResult("Label", nResults-1, "Green");
  setRGBWeights(0, 0, 1);
  run("Measure");
  setResult("Label", nResults-1, "Blue");
  setRGBWeights(1/3, 1/3, 1/3);
  run("Measure");
  setResult("Label", nResults-1, "(R+G+B)/3");
  // weights uses in ImageJ 1.31 and earlier
  setRGBWeights(0.299, 0.587, 0.114);
  run("Measure");
  setResult("Label", nResults-1, "0.299R+0.587G+0.114B");
  updateResults();
```

Appendix B

ALP activity test scans

ALP concentrations [mg/mL]					
1	A	2	B	3	C
0.1	0.05	0.01	0.005	0.001	0.0005



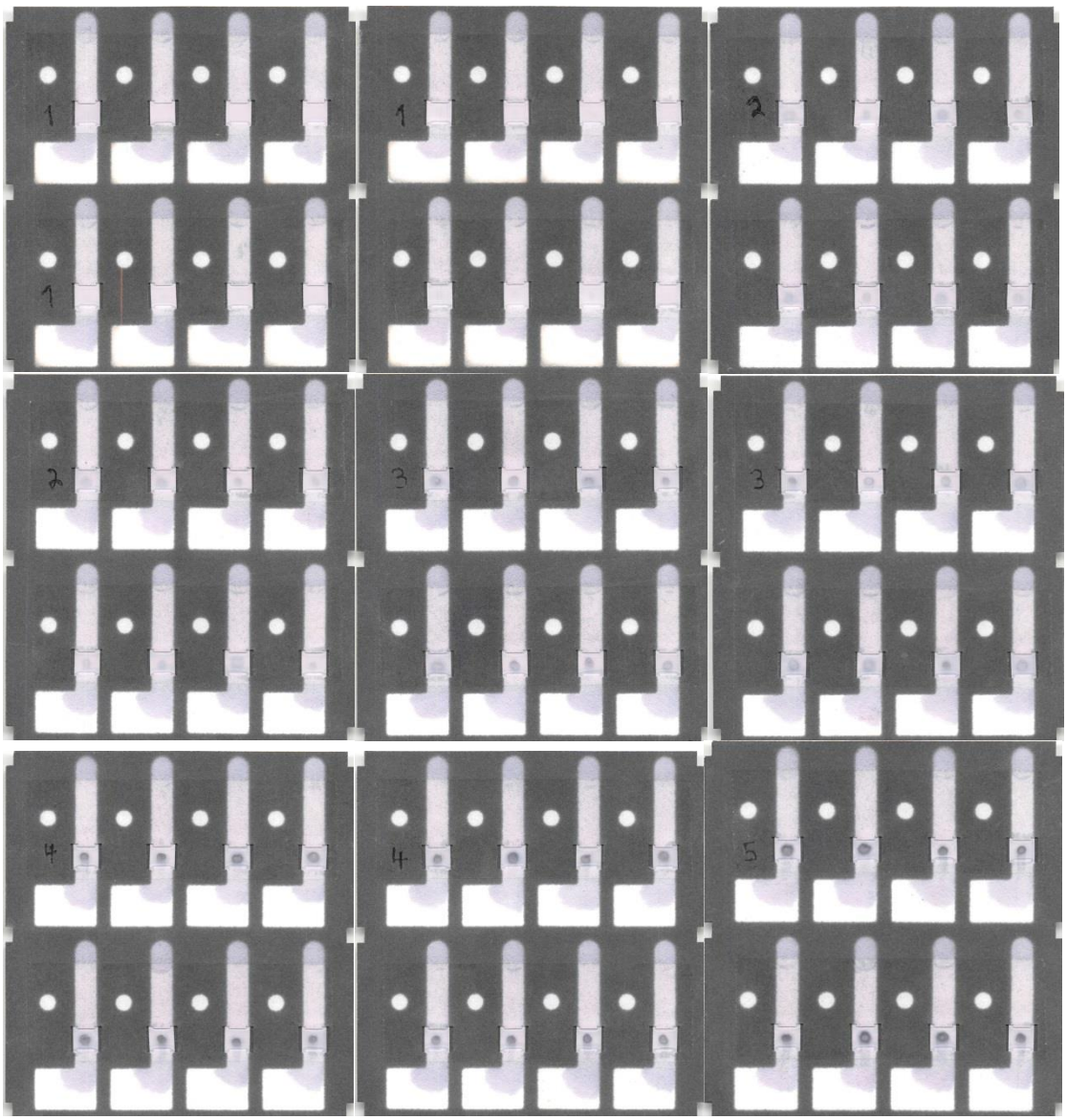
ALP activity test data in table format

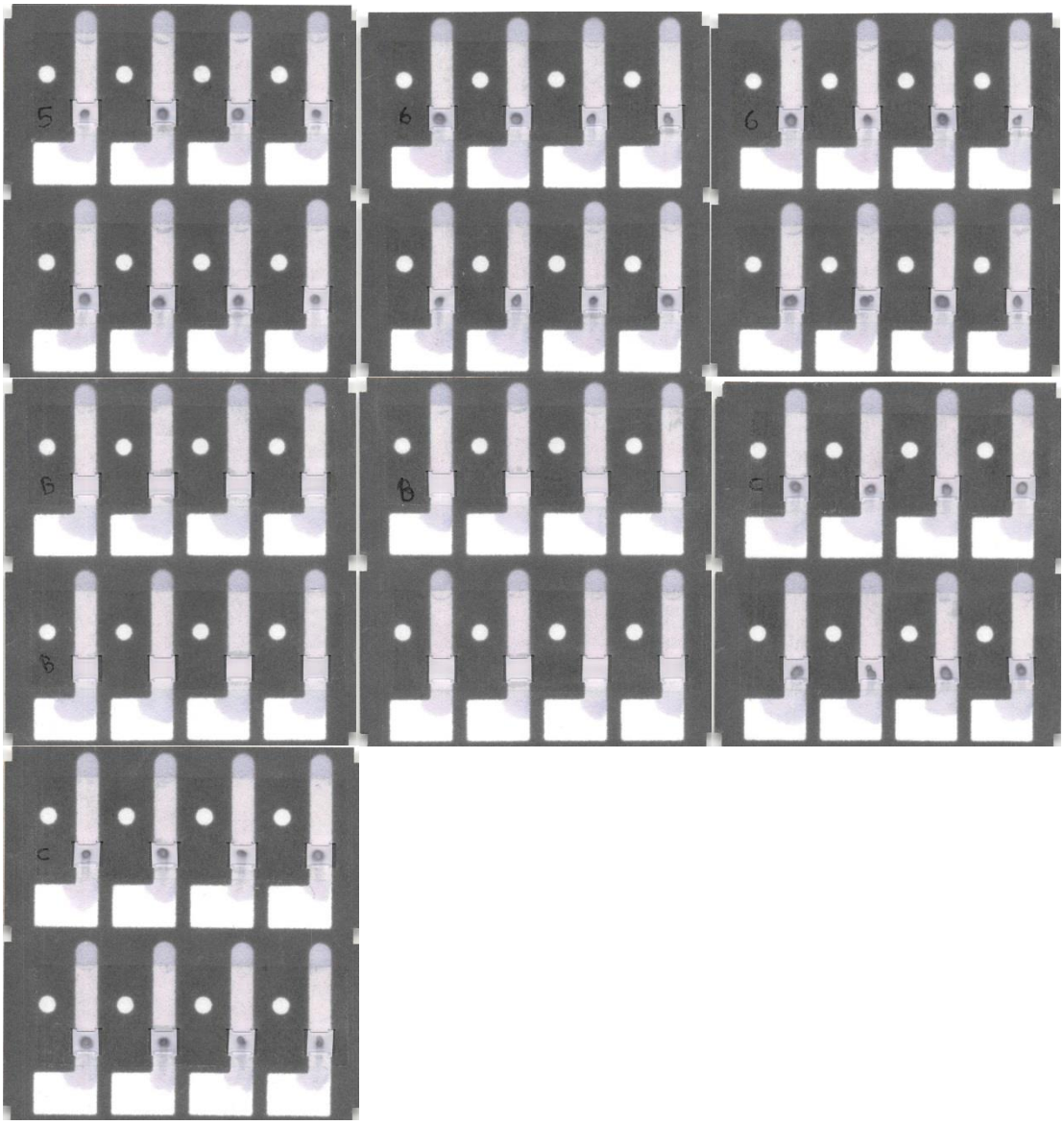
	ALP concentration [mg/mL]					
	1e-1	5e-2	1e-2	5e-3	1e-3	5e-4
Measured RGB brightness values	98.034	71.98	50.153	22.236	19.47	11.703
	73.034	66.075	48.813	30.824	17.146	13.011
	78.423	64.844	36.884	26.991	19.703	16.042
	78.405	71.786	44.613	24.086	20.295	12.78
	84.051	76.662	47.656	25.861	26.528	11.115
	69.591	80.087	41.815	26.181	24.359	10.783
	70.226	63.702	47.468	29.147	17.003	15.485

Appendix C

Sodium orthovanadate inhibitory activity test scans

Sodium orthovanadate concentration (from ~200mM solution)							
1	2	3	4	5	6	B	C
1:1	1:1e-1	1:1e-2	1:1e-3	1:1e-4	1:1e-5	Background	Control




5
6
6
B
B
C
B
C

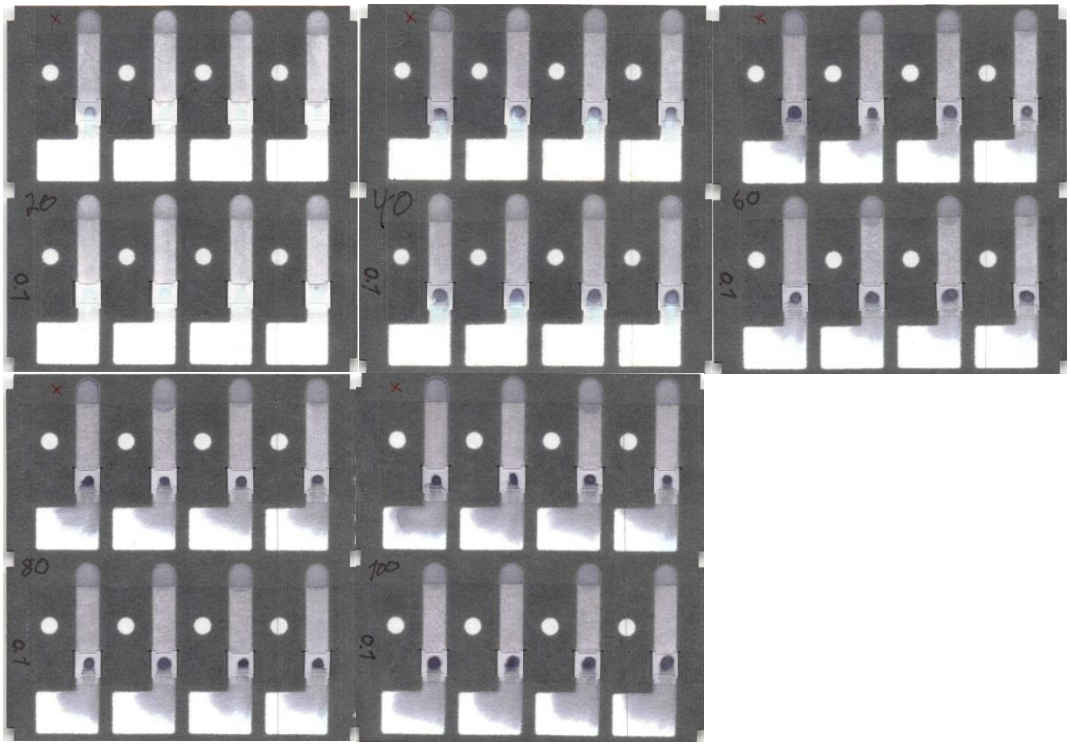
Sodium orthovanadate inhibitory activity data in table format

	Sodium orthovanadate concentration (from ~200mM solution)							
	Control	BG	1:1	1:1e1	1:1e2	1:1e3	1:1e4	1:1e5
RGB brightness values (ranges from 0-255)	109.75	29.00	27.92	41.04	60.15	80.49	107.73	119.17
	106.35	29.95	22.64	35.78	51.09	86.41	108.89	113.81
	106.57	30.89	23.14	48.19	64.11	99.11	94.04	100.27
	97.33	31.96	22.51	40.50	52.37	82.67	87.79	95.48
	108.52	28.09	23.18	45.58	57.19	97.10	103.30	100.27
	99.82	29.51	24.11	39.73	58.34	95.91	117.54	109.87
	110.21	27.49	24.31	44.74	58.21	87.99	113.54	105.61
	112.54	32.09	23.82	40.98	53.54	81.02	95.25	118.71
	101.46	27.01	23.80	40.00	57.56	83.24	93.78	117.93
	112.08	26.05	25.18	43.35	48.81	101.95	115.48	103.32
	91.18	28.67	26.27	39.24	48.94	87.12	114.50	128.00
	102.27	32.18	23.83	39.01	53.47	87.33	93.29	86.16
	110.26	28.83	26.61	38.24	59.98	81.58	108.38	119.95
	122.48	26.82	24.25	42.14	57.87	88.63	117.24	113.98
	99.40	29.98	22.55	44.35	66.32	91.38	114.16	123.85
	95.72	29.93	22.04	34.53	56.44	80.54	94.65	101.93

Appendix D

Substrate volume test scans

Substrate volume [µL] for ALP conc. **0.1 mg/mL**				
20	40	60	80	100

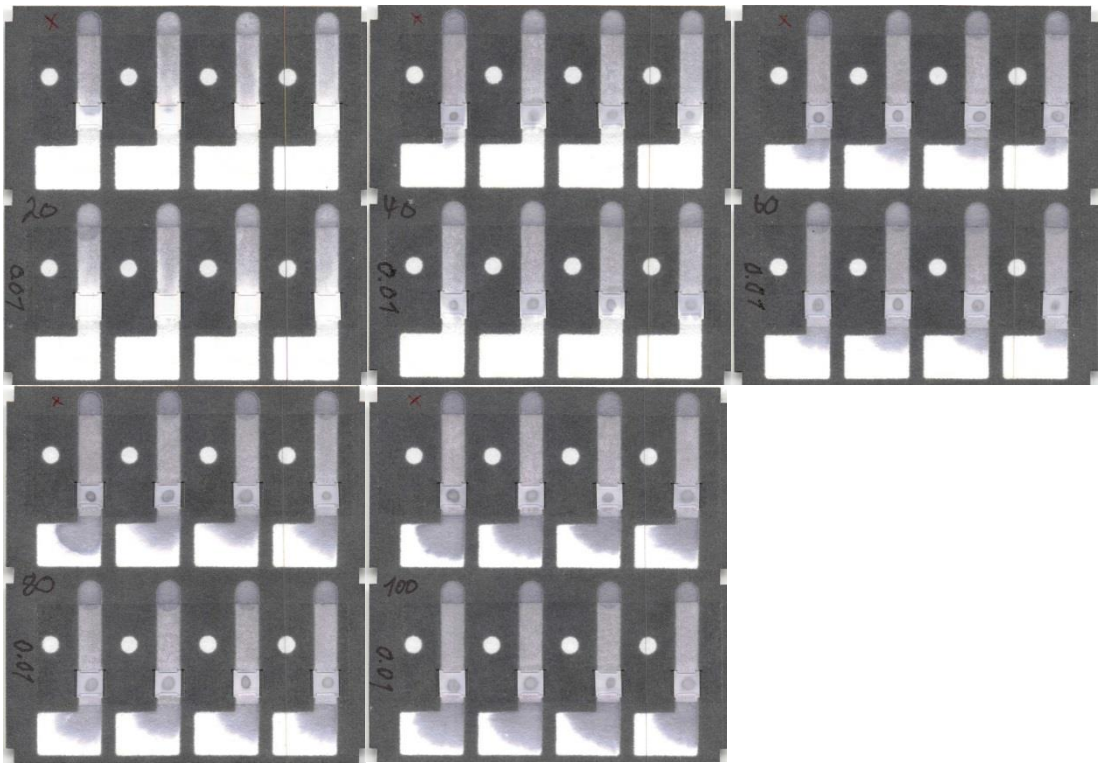


Substrate volume test data in table format

	Substrate volume [µL] for ALP conc. **0.1 mg/mL**				
	20	40	60	80	100
RGB brightness values (ranges from 0-255)	13.31	127.26	152.02	164.08	175.10
	18.98	109.31	151.42	162.55	172.53
	11.97	120.58	147.55	161.03	168.26
	14.62	130.84	150.10	160.37	177.79
	19.68	128.97	151.93	164.76	178.46
	17.41	143.02	149.81	179.02	174.30
	12.84	127.33	155.07	168.21	160.49

Substrate volume test scans

Substrate volume [µL] for ALP conc. **0.01 mg/mL**				
20	40	60	80	100

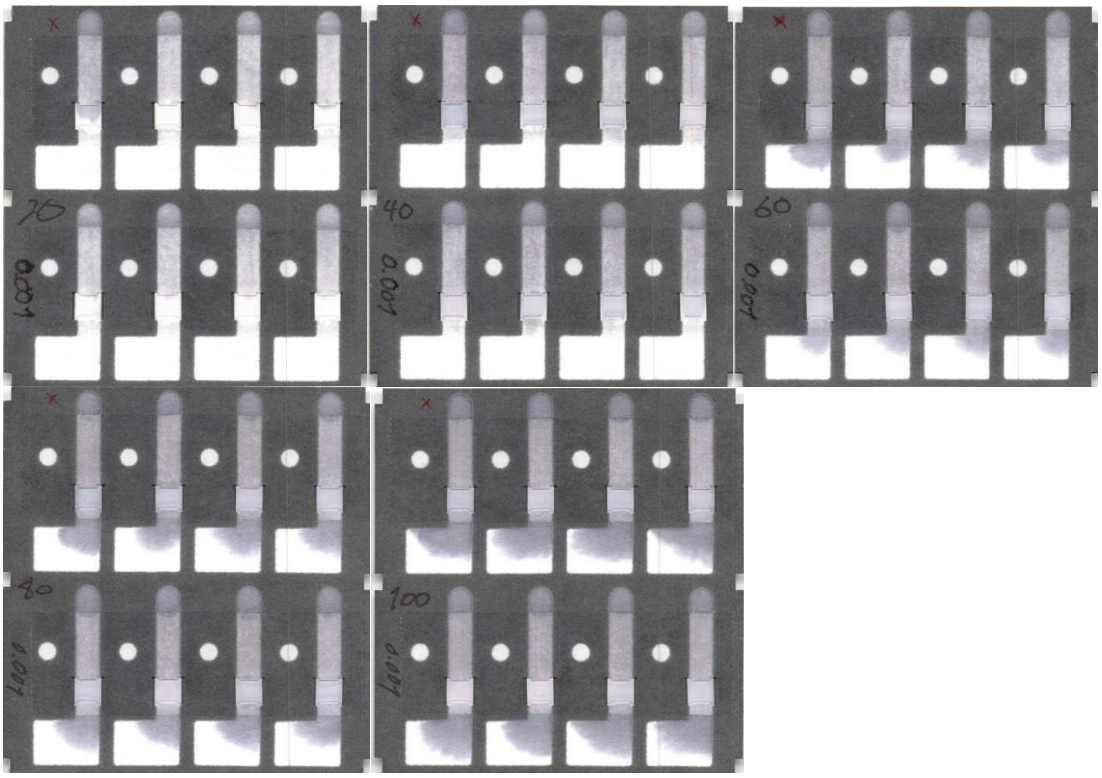


Substrate volume test data in table format

	Substrate volume [µL] for ALP conc. **0.01 mg/mL**				
	20	40	60	80	100
RGB brightness values (ranges from 0-255)	13.03	71.15	82.14	96.96	80.90
	4.87	71.11	84.71	91.88	84.89
	8.56	63.42	79.69	80.85	85.42
	3.48	71.07	82.83	78.07	79.67
	1.91	76.75	82.27	77.05	75.12
	6.59	72.15	77.38	83.92	84.65
	4.86	69.82	79.30	73.11	73.14

Substrate volume test scans

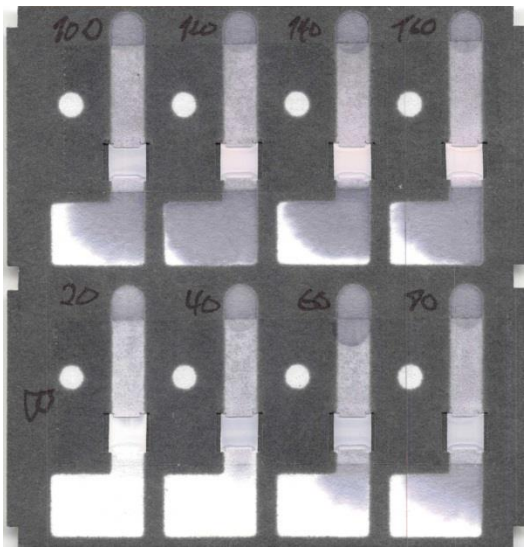
Substrate volume [µL] for ALP conc. **0.001 mg/mL**				
20	40	60	80	100



Substrate volume test in table format

	Substrate volume [µL] for ALP conc. **0.001 mg/mL**				
	20	40	60	80	100
RGB brightness values (ranges from 0-255)	2.68	31.55	39.02	45.70	45.20
	4.14	37.50	43.00	46.86	51.25
	1.00	29.76	34.90	42.40	47.16
	2.07	32.49	39.18	43.70	33.42
	3.00	31.03	36.61	38.95	30.54
	3.10	34.53	42.81	44.52	43.15
	1.04	31.99	36.95	41.79	42.56

	Substrate volumes [µL] for background measurements (ALP conc. **0 mg/mL)**							
RGB brightness values (ranges from 0-255)	20	40	60	80	100	120	140	160
	4.09	39.53	39.74	37.29	34.66	23.82	22.94	20.68



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