A DISSERTATION ON

Quantitative estimation of total Amino acids in blood serum samples using Isatin paper-based microfluidics and digital scanner

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING & INFORMATION TECHNOLOGY
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF TECHNOLOGY
IN BIOTECHNOLOGY

BY
SHALINI SINGH
M. Tech Biotechnology (IV Semester)
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UNDER THE SUPERVISION OF

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DECLARATION FORM

I, **Shalini Singh**, a student of **M.Tech. Biotechnology** (2^{nd} year/4^{th} semester), Integral University have completed my six months dissertation work entitled **"Quantitative estimation of total Amino acids in blood serum samples using Isatin paper based microfluidics and digital scanner"** successfully from Integral University and KGMU under the able guidance of **Dr. Punit Kumar Singh .**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Name and Signature of Student with Date

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CERTIFICATE

Certificate that Ms **Shalini Singh** (Enrollment Number 2100103267) has carried out the research work presented in this thesis entitled **"Quantitative estimation of total Amino acids in blood serum samples using Isatin paper based microfluidics and digital scanner"** for the award of **M.Tech. Biotechnology** from Integral University, Lucknow under **Dr. Punit Kumar Singh** supervision. The thesis embodies results of original work and studies carried out by the student herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her

M.Tech. Biotechnology degree.

I wish her good luck and bright future.

Dr. Punit Kumar Singh
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CERTIFICATE BY INTERNAL ADVISOR

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I wish her good luck and bright future.

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TO WHOM IT MAY CONCERN

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I wish her good luck and a bright future.

Dr. Alvina Farooqui
Professor and Head
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Date: **Shalini Singh**

TABLE OF CONTENT

Abbreviation

ABBREVIATION	FULL FORM
1. PADs/mPADs	Microfluidic Paper-based Analytical Device.
2. PADs	Paper-based Analytical Device.
3. POC	Point of Care
4. Approx.	Approximately
5. Etc.	Et cetera
6. WHO	World Health Organization
7. ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Deliverable
8. ALT	Alanine aminotransferase
9. IUPAC	International Union of Pure and Applied Chemistry
10. UV	Ultra violet
11. TB	Tuberculosis
12. HIV	Human Immunodeficiency Virus
13. ANOVA	Analysis of Variance

List of Figures

List of Table

s

List of Equations

1. INTRODUCTION

A new generation of analytical instruments based on cellulose materials, known as Microfluidics paper-based analytical devices (PADs), has emerged in the last ten years. This field will change how diseases are diagnosed and how a wide range of biological, chemical, and biochemical phenomena are sensed. Litmus paper has been used as a pH indicator for many years of experimentation, and Whitesides and colleagues introduced the first Microfluidics paper-based analysis device (PAD) in 2007. The field of "PADs" has kept growing exponentially, having a noticeable impact on the academic and business realms. These instruments use cellulose as a substrate and function as paper-based analytical devices (PADs) for forensic investigations, biosensing, environmental monitoring, clinical diagnostics, and point-of-care diagnosis. Paper has also been utilized extensively in chemical and biochemical analysis, including paper chromatography, paper-based colorimetry, paper-based filtration and purification, pH testing, and home pregnancy tests. The appeal of PADs is predicated on a number of benefits, including their low cost, powerlessness when using small volumes of samples due to cellulose fibre networks, capacity to store reagents, simplicity of building and operation, portability, and disposability. The µPADs are used in various fields, including environmental monitoring they of contaminations, food safety, health diagnostics, biodefence (micro-organisms sensing), and drug discovery as well as a biomarker and single cell detections. In heath diagnostics it has application in- Analysis of biomolecules such as proteins, hormones, neurotransmitters, etc. Analysis of small molecules such as glucose, uric acid, etc. Nucleic acid analysis. Immunoassays for infectious diseases and cancer detection. Pregnancy tests. Blood typing and blood filtering. Drug sensing. In environmental it has following application in- Water, soil, air analysis, metal detection etc. Food and Beverage control, pesticide, foodborne detection. Water and wine quality analysis etc. The selection of a paper is largely dependent on the application and construction method. In the last years, Whatman® grade 1 filter, which is one of the standard grade filters, has widely been used in the construction of sensors and microfluidics, in large part because of their suitable flow rate, porosity, and particle retention. The wax printing method is a low-cost and simple approach for the construction of the hydrophobic barriers. The "PADs" are still in the early stages of development, but they will soon surpass other gadgets in popularity and usability. In conclusion, it should be emphasized that the PADs will, in the very near future, transform the

direction of pharmaceutical research and development and medical sciences, opening a new frontier in drug discovery and diagnostics.

The analysis then looks at the primary applications and detection techniques for PADs put forth over the previous five years. Seven different detection methods are discussed, including colorimetric detection, fluorescent detection, electrochemical and photoelectrochemical detection, chemiluminescent detection, electro chemiluminescent detection, nanoparticle-based detection, and spectrometry detection.

OBJECTIVES

1.To optimize the fabrication process' essential process parameters. The choice of the melting temperature and melting time necessary to produce impermeable hydrophobic barriers is covered.

2.Determination of the total amino acids content in blood serum samples using paper based microfluidics.

2. REVIEW OF LITERATURE

The science and technology of devices manufactured from paper or other porous membranes that control small (10-6 to 10-9 L) quantities of fluids by capillary action is known as paper-based microfluidics. With a wide range of applications, it introduces a cutting-edge platform technology for fluid handling and analysis that promotes cheap cost, simplicity in construction and operation, and equipment independence. The classic definition of paper is a flexible sheet comprised of an interwoven web of pressed cellulose fibres. Paper is defined more broadly in the context of paper-based microfluidics as any porous membrane that wicks fluids by capillary action. Since the invention of litmus paper in the 19th century, paper has been extensively utilized as a platform for conducting analytical experiments. Paper made of cellulose and nitrocellulose membranes are two of the most often utilized varieties of porous membranes for tests. Paper derived from cellulose fibres is hydrophilic by nature and has a high density of hydroxyl functional groups and few carboxylic acid groups. The two types of cellulose-based paper that are most frequently utilized for the creation of microPADs are filter paper and chromatography paper. By nitrating cellulose, or changing the hydroxyl groups in the cellulose molecule with nitrate groups, nitrocellulose is created. The resultant polymer is subsequently cast into controlled-porous membranes. The lack of flow control is the problem of paper-based analytical devices, which generates obstacles for marketing and slow down the transition of paper devices from the laboratory into the consumers' hands. Paper's fibrous and porous structure provides Capillary action-leading to the transportation of the liquids without a need of an external force, absorbency -enabling the storage of the reagents inside the paper, air permeability -removing the air bubbles problem, a network structure-enabling the filtration of the sample and a high source to volume ratio-increasing the number of possible reagents immobilized, causing a considerable fall in the time for the analysis. Reagents' strong biocompatibility (which is crucial for the samples), biodrigradibility, disposability, and chemical and biological inertness enable them to be immobilized naturally. mPADs have a number of benefits, including the following: (1) they can transport liquids via capillary forces without the aid of outside forces; (2) they are compatible with numerous chemical, biological, and medicinal applications; and (3) they are widely available and exceedingly inexpensive cellulosic materials. Liquid flow can be

controlled since it is contained within the channels created by creating Microfluidics channels on paper. The paper has been used to produce a variety of 2D and even 3D Microfluidics channels that can move liquids along predetermined paths. Rising technology, at the current state of its development, is equipment-free and therefore particularly useful to enhancing disease screening in healthcare in developing countries. However, it also has disadvantages as a material for diagnostic devices: lack of flow control, it is also not well suited for absorbance measurements because the paper fibres leads to large amounts of light scattering, the wicking rate of paper is not always perfectly uniform even within a single sheet of paper.

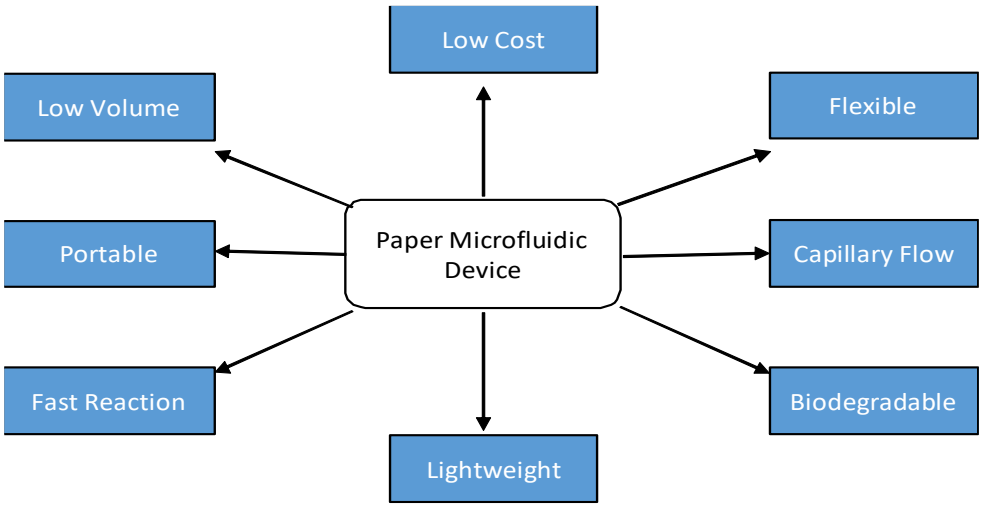


Figure 1. Key points of Microfluidics paper-based analytical devices (μPADs)

2.1 Application

The primary use of paper-based Microfluidics devices is to offer an inexpensive, simple-to-use, and portable analytical platform for assays that are multi-analyte or semi-quantitative (or even quantitative), in order to give people in the developing world access to affordable disease diagnosis and environmental monitoring. μPADs are of critical importance since they

enable POC detection and can ideally be used massively in low-income countries, where people predominantly die of preventable or curable infectious diseases: lower respiratory infections, HIV/AIDS, diarrheal diseases, malaria and tuberculosis collectively, accounting for almost one third of all deaths in these countries. While significant progresses have been made in interventions to prevent and treat most of these diseases, often the effort are not widely reaching all population due to the lack of laboratory infrastructure, trained personnel and financial support. Therefore, affordable, equipment free, simple to operate, and robust diagnostic assays at the point of care would be considered as life savers under these resource-limited conditions. The aim of µPADs for POC is to obtain the results quicker in order to take clinical decisions instantly and to carry out the treatment plan immediately. Moreover, µPADs fulfil the World Health Organisation (WHO) ASSURED criteria for and ideal rapid test: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free, Deliverable to end users, delivering affordable POC diagnostic tests.

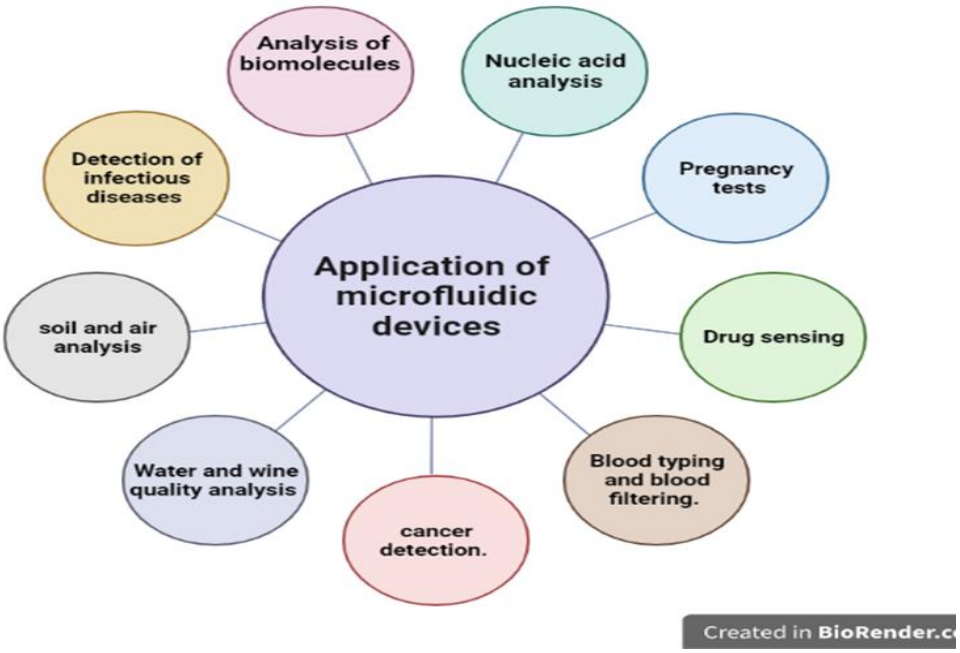


Figure 2. Application of µPADs.

Additionally, early diagnosis of diseases like cancer, where the detection and sometimes quantification of molecules and biomolecules like glucose, lactate, uric acid, alcohol, biomarkers for liver function, ATP, ALT, nitrite, nucleic acids among others is needed in physiological fluids like blood and urine, can be successfully accomplished by μPADs. The potential of μPADs is not only limited to healthcare; devices developed for environmental safety, detecting water, soil or air contamination, and for food and beverage control are also available at the laboratory state. In the resource-limited countries or after a natural disaster like an earthquake, to be able to determine drinking water quality with an easy to use and low cost Paper-based device would be very beneficial. Furthermore, the possibility of controlling the quality of food and beverages by the final consumer and ensuring the control over the whole food/beverages production chain would be an important advantage of farmers, production companies and sellers in order to generate a more dynamic market. Therefore, it is here where μPADP are realistic alternatives for low cost, mass production and marketable devices.

The amount of pyrrole blue, a special blue derivative formed when proline and isatin react, is inversely proportional to the amount of proline. Although this method is suited for the analysis of a wide variety of materials and allows for the relatively simple quantification of proline among the many amino acids found in biological samples, it is very occasionally employed because of the proline measurement range's limitations [21–23]. Proline content in sulfosalicylic acid-prepared plant extracts can be found via colorimetric analysis. Using isatin to create a particular chromogen, proline has been quantified in the presence of extra amino acids and hydroxyproline. Maximum sensitivity and recovery-determining factors are given. After acid hydrolysis, the technique has been effectively used to assess the proline content of mammalian collagen. It has become possible to create a straightforward, better approach for determining proline using the colorant isatin. Other amino acids often found in bodily fluids and protein hydrolysate did not affect the outcomes. The technique has been used to accurately measure proline in serum and acid hydrolysed protein samples.

2.2 Fabrication:

The objective of every technique for patterning paper is to create well-defined patterns of hydrophobic barriers in a piece of paper to define hydrophobic channels and zones.

It is a simple and inexpensive method for fabricating Microfluidics device in paper using a bronze stamp and hot plate. The stemp is heated on the hot plate up to an optimized temperature then it is stamped on Whatman filter paper beneath which a wax coated Whatman filter paper is placed. By using this technique, paper is completely hydrophobic, defining hydrophilic channels, fluid reservoirs, and reaction zones. Hydrophobic agents are directed to the selected area of a sheet of paper to hydrophobized these areas; areas not receiving patterning agents remain hydrophilic. Wax is a cheap hydrophobic material, which has been widely used in the fabrication of mPADs. Due to the porous structure of the filter paper. These hydrophobic agents change the wetting property of paper by filling the paper pores or absorb on the filter surface.

For the purpose of defining hydrophobic micro-channels on paper, a variety of hydrophobic materials have been used, ranging in price from relatively expensive photoresist SU-8 ($0.1 for patterning filter paper of 100 cm2) to less expensive photoresist ($0.01 for patterning filter paper of 100 cm2) to extremely cheap alkyl ketone dimer (AKD, $0.00001 for patterning filter paper of 100 cm2).

The paper patterning principles of these techniques can be categorized into three groups based on the binding states of hydrophobic agents to paper: physical blocking of the paper pores (using materials like photoresist and polydimethylsiloxane (PDMS)), physical deposition of a hydrophilizing reagent (e.g., polystyrene or wax), and chemical hydrophilization. No chemical reactions between the hydrophobic substances and the cellulose fibres are involved in the physical pore blockage or physical Fiber surface modification; instead, these substances are physically impregnated in the paper pores or deposited on the Fiber surface. These substances alter the paper's ability to bind liquids, which enables the development of hydrophilic-hydrophobic patterns in paper.

Using the bronze stamp, the patterns were imprinted on Whatman No. 1 chromatography paper. The bronze can print an 8.5 in. sheet of paper in approximately 45 s. The wax melted and spread through the thickness of the paper. The patterned paper was ready for use and

allowing it to cool to room temperature (<10 s). Another economical stamping technique was also introduced where the design of the Microfluidics structure was patterned in a lightweight and portable stainless steel stamp for rapid prototyping of mPADs, using paraffin over a chemically modified paper substrate. The additional procedures of preheating the stamp and oxidizing the paper surface could be seen as disadvantages. Although stamping techniques are generally thought of as being inadequate for mass manufacturing, they are perfect for the quick and portable creation of mPADs. Nevertheless, it needs to be considered that they are good device candidates to be used in field and under resource limited settings.

There are various types of fabrication methods, some are explained below;

2.2.1 Paper Microfluidics device: Wax Printing

Due to its speed (5–10 min) and low cost, it is suited to produce numerous paper analytical instruments in a single batch. The two main steps in the fabrication process are printing patterns of wax (100 μm width) on the paper surface and melting the wax into the paper to form hydrophobic barriers. There are few different ways to realize wax patterning, and direct printing by a wax printer is the most convenient and efficient method. Painting with a wax pen is an alternative that is also viable, although some people prefer to print patterns using a regular inkjet printer first, then trace and paint with a wax pen to improve the resolution of the printed pattern.

2.2.2 Paper Microfluidics device: Inject Printing

Inkjet printing is a new fabrication method that associates sizing chemistry with digital inkjet printing technique. To create a difference between the hydrophobic barrier and the hydrophilic flow channel is the primary goal in this process of fabricating Microfluidics channels. Drop-on-demand technology (DOD), which enables the jetting of ink droplets onto cellulose paper dot-by-dot only when necessary, is used by inkjet printing systems used to create patterns. This make inkjet printing a technique that allows for rapid and flexible high

resolution. The Inkjet printing can accurately deliver biomolecules and indicator reagents into the Microfluidics patterns, thus forming biological/chemical sensing zones within the patterns. The cellulose Fibers can be covalently changed by using sizing agents like rosin, alkyl ketene dimer, or alkenyl succinic anhydride to define channels on the paper substrate. The use of AKD for the Microfluidics patterning of paper by inkjet printing makes the paper substrate more hydrophobic as a result of the hydrophobic reagents.

2.2.3 Paper Microfluidics device: Photolithography

This technique creates an image of a pattern by projecting light through a mask, much like a negative image in traditional photography. The hydrophobic regions that make up the patterns in the easy, rapid, and affordable method of photolithography are constructed of polymeric barriers. Photolithography-produced channels have a high background, but wax-printed channels, for example, have a very low background. However, the setup of photolithography is slightly more difficult due to the need for pricey photoresists, organic solvents, and photolithography equipment. A fast method for laboratory prototyping of Microfluidics devices on paper is Fast Lithographic Activation of Sheets, a variant technique based on photolithography. The only equipment needed is a UV lamp and hotplates, and patterning can even be done outside in the open air if a UV lamp and hotplate are not available. No clean room or other special equipment is needed.

2.2.4 Paper Microfluidics device: Flexographing Printing

This patterning technique is based on the hydrophobicity of the paper substrate, which is achieved via flexographic printing of polystyrene, a polymer. This technique leads to the formation of liquid guiding boundaries and layers on paper substrates. The hydrophobizing inks then partially or totally penetrate the entire depth of the paper substrate, forming hydrophobic barrier structures. The paper substrate is then partially or completely penetrated by the hydrophobizing inks, creating hydrophobic barrier structures. A great advantage of

flexographic printing is that biomolecules and other reagents required in analytical and diagnostic tests can easily be transferred by it on paper substrates. Flexographic printing makes it possible to create paper Microfluidics analytical devices in a single roll-to-roll process, which makes this technique perfect for mass production.

2.2.5 Paper Microfluidics device: Wax screen-printing

Wax screen-printing is a low-cost and simple method for fabricating paper Microfluidics analytical devices. It's simple fabricating process includes printing patterns of solid wax on the surface of paper using a simple screen-printing method. Using a hot plate, the printed wax is then melted into the paper to create hydrophobic barriers. As was already said, wax is an inexpensive material that can be acquired anywhere in the world and is also good for the environment. A wax printer and easily accessible printing screens are needed for this technique. Additionally, the wax screen-printing technique does not require a sterile setting, a UV lamp, organic solvents, or complex equipment. Another significant benefit of this technology over earlier ones is that it simply needs a typical hot plate (or comparable surface) and a typical printing screen, both of which can be created anywhere in the world, making it perfect for producing PADs in poor nations. Finally, both colorimetric and electrochemical detection methods can utilise this construction technique.

2.3 Future prospect

Paper diagnostics are manufactured with the help of cellulosic materials as well as paper in order to quantify and identify chemical agents as well as biomolecules which are hazardous to the health. Due to its ease of use, hydrophilicity, sterility, biocompatibility, combustibility, biodegradability, ease of engineering, availability, and affordability, paper diagnostics are in higher demand. Paper diagnostics are in demand as a result of the need for cost-effective solutions. Also, a rise in the usage of pregnancy test kits and diabetes test kits are driving the growth of the market. In addition, factors including the rise in obesity, poor diet, smoking, and other lifestyle choices are anticipated to lead to greater market expansion. It is also due to rise in the incidence of diseases such as cardiac diseases and diabetes. The product category comprises lateral flow tests, dipsticks, and paper-based microfluidics. The market for paper diagnostics is growing at the fastest rate for lateral flow assays. It is because more people are using pregnancy test kits and because they are being used in more items. Additionally, during the course of the projected period, an increase in infectious disorders like HIV, TB, and pneumonia is anticipated to spur market expansion. The expansion of the market is being driven by increased investments and efforts made by healthcare institutions and the government to treat and identify TB.

The devices type segment includes monitoring devices and diagnostic devices. Diagnostic devices have the largest market share in the paper diagnostic market. These gadgets are mostly used for blood separation and glucose testing. The introduction of new products, the frequency of diagnoses, the quality of the gadgets, and growing consumer awareness all contribute to the market's expansion. Wax patterning technology has higher adaptability and flexibility which result in increasing the demand for diagnostics devices. Additionally, there is a rise in market demand due to the rising demand for urinalysis.

North America, Europe, Asia-Pacific, the Middle East and Africa, and South America make up the market segments. North America has the highest growth due to a strong technological distribution network along with presence of major headquarters of the key players. The government's increased efforts to promote improved diagnosis are helping the market in the area expand. Some of the notable players in the global paper diagnostics market are Acon

Laboratories Inc., Bio-Rad Laboratories, ARKRAY Inc., Siemens healthcare Gmbh and Abbott.

Although new fabrication methods of paper-based Microfluidics devices will continually be reported in future, the practicality of the existing and future methods will be judged by the POC (point of care) and diagnostic market in terms of the material and fabrication costs, their potentials for mass productions, their reliance upon any other equipment in order to function, their reliability in producing assay results that are simple to understand and compatible with telemedicine, particularly when it comes to mobile phone transmission or test result interpretation.

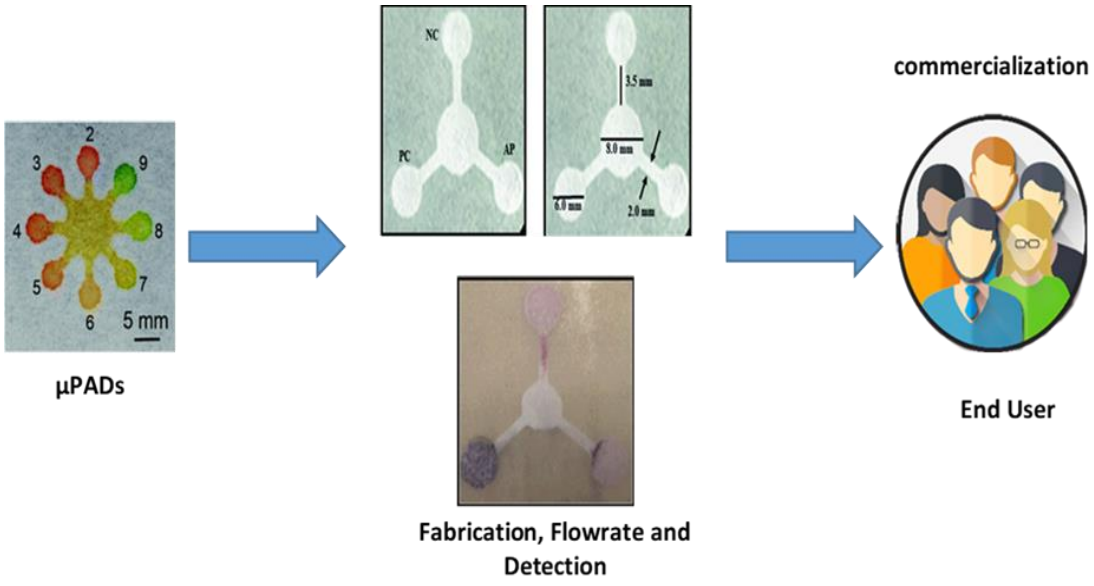


Figure 3. Commercialization of µPADs.

3. MATERIALS AND METHODS

3.1 Fabrication of Microfluidic Device:

The fundamental principle underlying these fabrication techniques is to pattern hydrophilic hydrophobic contrast on a sheet of paper in order to create micron-scale (i.e., hundreds to thousands of micrometres) capillary channels on paper. Researchers and manufacturers should think about a variety of issues, such as equipment accessibility, material costs, the ease of production, and the intended uses of paper-based Microfluidics devices while deciding on the best technique. Due to the low cost of patterning chemicals and the simple, quick fabrication procedure, AKD ink jet printing and wax printing may now be the most promising approaches.

Steps involved in fabrication of device:

1. Wax sheet preparation: A piece of filter paper was immersed in liquid paraffin and solidified at room temperature. This Wax sheet (p-paper) was then placed on the native paper (n-paper) surface.

2. Stamping: A metal stamp was preheated and brought in contact with the paraffin Coated paper to stamp the Microfluidics structure on the n-paper, which transferred the paraffin from the p-paper to the n-paper and formed the hydrophobic barriers. As shown in figure 4 below.

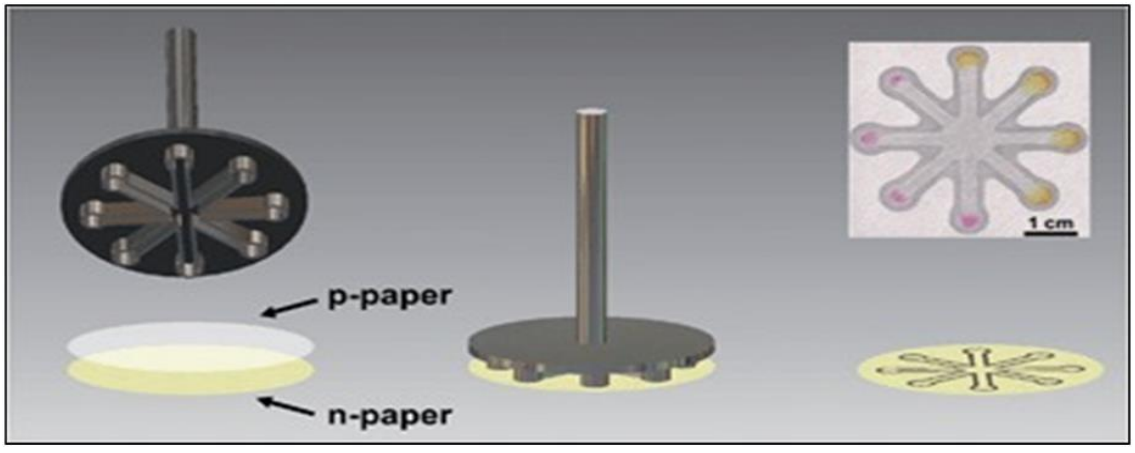


Figure 4. Illustration of fabrication of device.

3.2 Wax sheet preparation:

The Whatman filter paper was dipped into paraffin wax (melting point > 65°C), after it was set dry another fresh Whatman paper was placed upon the waxed coated sheet and it is then pressed by the heated stamp to create a hydrophobic barrier. The area of the paper penetrated by the wax is hydrophobic, whereas the paper itself is hydrophilic. Functionalized paper based Microfluidics device were dried at room temperature and used the same day.

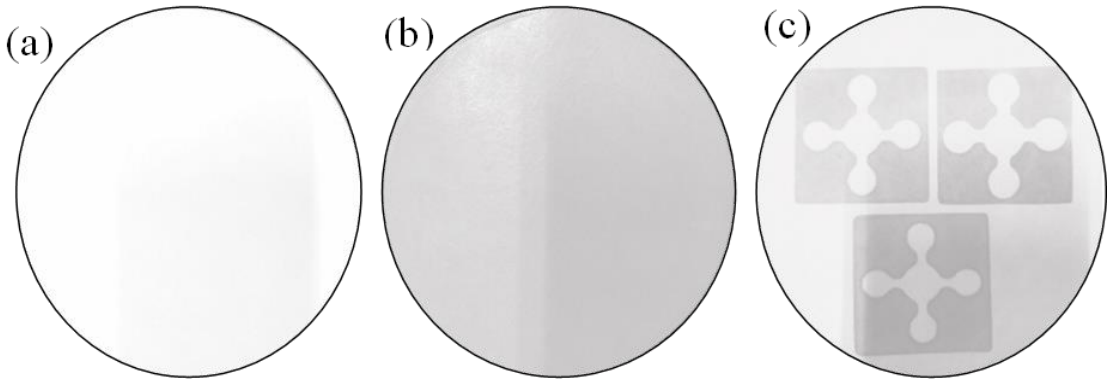


Figure 5. (a) shows Whatman filter paper (n-paper), **(b)** shows coated Whatman filter paper (p-paper), **(c)** fabricated sheet having reservoirs obtained by stamping.

3.3 Stamping:

Every paper patterning approach aims to define hydrophilic channels and zones in a piece of paper by forming clearly defined patterns of hydrophobic barriers. A Microfluidics paper-based analytical device (μPAD) was fabricated by stamping the hydrophobic barrier pattern

onto a laboratory filter paper. The µPAD has a central zone from which an applied sample flows into four surrounding narrow channels to which had been added the standard solutions. A circle that is filled with the reagent and attached to each channel. We showed that paper-based microfluidics may be made with just contact stamping by utilizing the filter paper's capacity for absorption. Pressure is required for thermal transfer of paraffin onto surface of filter paper. A stainless-steel stamp that was machined was used to pattern the Microfluidics structure's design. A sheet of native paper (n-paper) was placed between a filter paper sheet that had been paraffin-impregnated. A filter paper sheet was impregnated with paraffin and sandwiched with a native paper (n-paper) sheet. Using a hot plate (Fig-7B) The metal stamp was preheated at 75°C and then brought in contact with the paraffined paper (p-paper) to enable the thermal transfer of the paraffin to the n-paper, thus forming the hydrophobic barriers by applying pressure for 45 s.

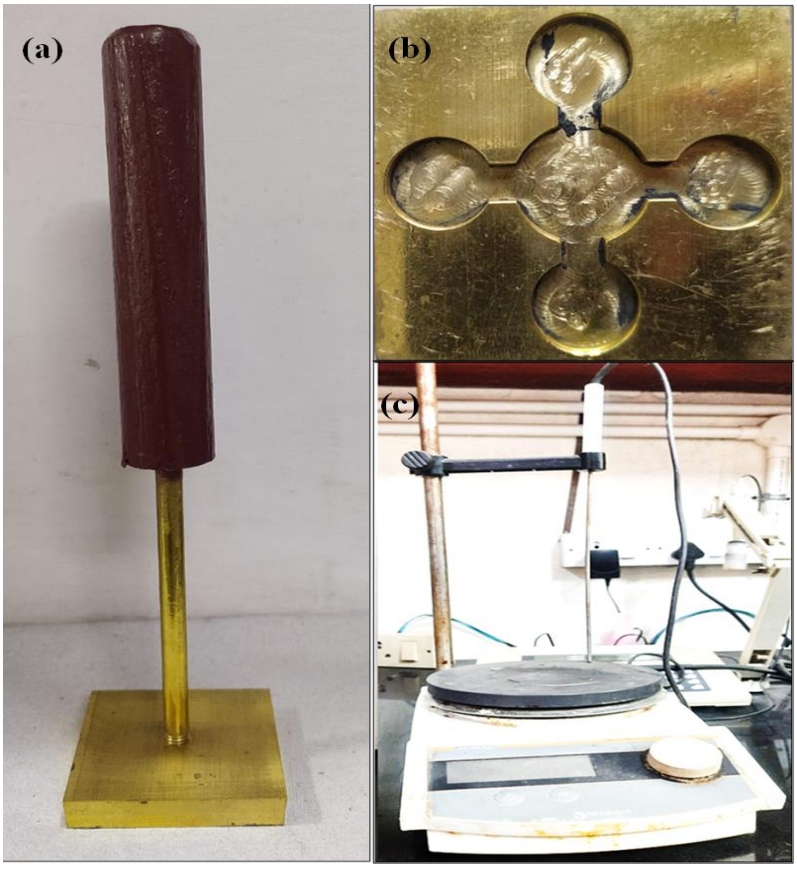


Figure 6. (a) Bronze Stamp, **(b)** Engraved channels on bronze stamp.
(c) Hotplate

3.4 Serum Sample Preparation-

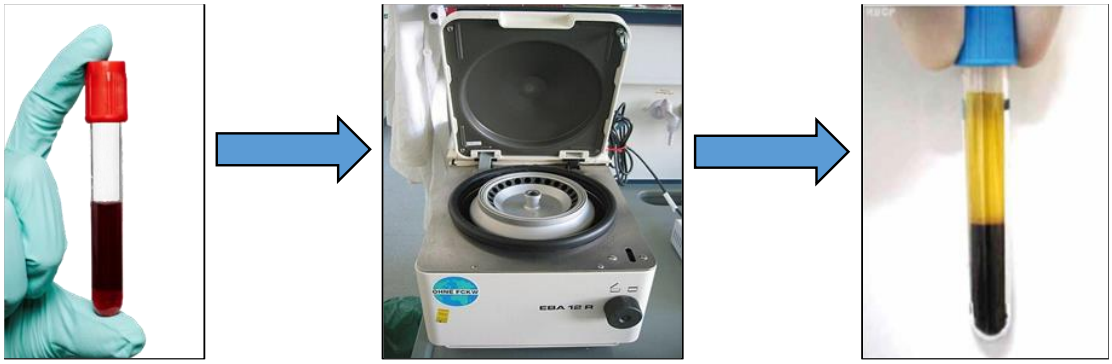


Figure 7. Illustration of serum sample preparation.

Collected blood in a micro centrifuge tube taken out of different patients. After collection of the blood, the blood was allowed to clot by leaving it undisturbed at room temperature. This usually takes 15-30 minutes. By centrifuging for 10 minutes at 1,000–2,000 rpm, the clot was removed. The resulting supernatant is designated serum.

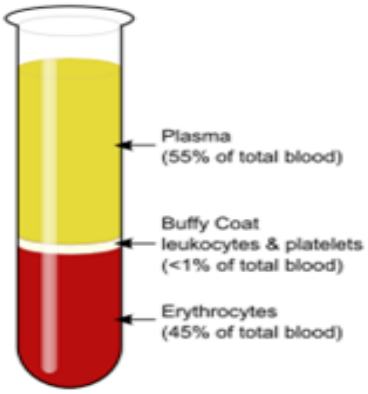
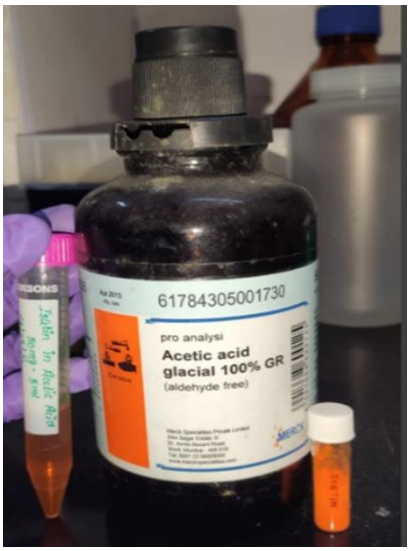


Figure 8. Components of blood sample separated after centrifugation.

3.5 Determination of amino acid:

3.5.1 Material-

All reagent were used of analytical grade and distilled water was used. The 1% 0f isatin solution was prepared by mixing 100g of isatin in 10 mL of distilled water. Proline was used as the reagents for preparing the standard solution of amino acids. A proline stock standard solution of 1.0mg/mL was prepared by dissolving 10mg of proline with distilled water and diluted to 10 mL. The proline working standard solutions were prepared by appropriate dilution of the stock solution with distilled water.



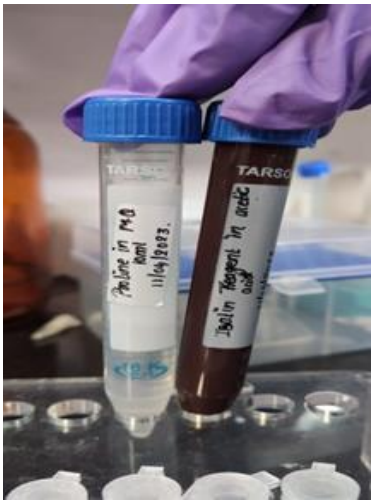


Figure 9. (a) proline **(b)** isatin

3.5.2 Procedure-

Steps were followed to determine amino acid using isatin test

1. Place a beaker of approx. 40 mL capacity on the experimental table, and then put the dry fabricated paper-based device on the top of the beaker.

2. Spotted 70 μL of 1% isatin on the circle zone in the center of the dry paper-based device. The isatin solution flows into the detection zones within approx. 100 seconds.

3. Allow the device to dry (approx. 9 minutes).

4. Spot 10 μL of each 7 standard solutions proline (.0,10,30, 60,90,120,180 μg/mL). Let the solutions dry for 5 minutes at room temperature.

5. Heated the device on a hot plate at 80°C for 15 minutes such that the amino acid reacts with the isatin to form a purple-coloured complex in the detection zones.

6. Taken an image of the device covering all detection zones using a scanner.

7. Analysed the test zones for the mean grey value using ImageJ software.

8. Obtained a calibration curve. The final mean grey values of standards used while creating the calibration curve are obtained by subtracting the grey value of blank.

9. Obtained linear equation for the correlation.

10. Calculated the total concentration of amino acids in sample solution according to the mean grey value in the sample detection zone and the linear equation.

3.6 Software Used For Analysis

3.6.1 ImageJ:

The National Institutes of Health developed ImageJ, free image processing software that is available to everyone. Scientists from a variety of fields, including astronomy and medicine, can use it as a research tool because of its strength and adaptability. Raster (row and column) image data can be displayed, annotated, edited, calibrated, measured, analysed, processed, printed, and saved. It can read text-based raw data files from spreadsheets and the majority of popular raster picture formats.

Steps involved in analysis using ImageJ:

Step 1- Image was uploaded as shown in figure 11.

Step 2- Region of interest (ROI) was selected using suitable shape to select the area as shown in figure 12.

Step 3- Further selected the option “Analyse” then in analyse chose the option “measure” which gave results including the area of selected region along with its mean as shown in figure 13.

Step 4- Saved the result and opened it in spreadsheet.

Step 5- Mean grey value was calculated by subtracting the value of blank from all other calculated values.

Step 6- The graph was plotted using scattered graph type and obtained a straight line graph by joining the dots.

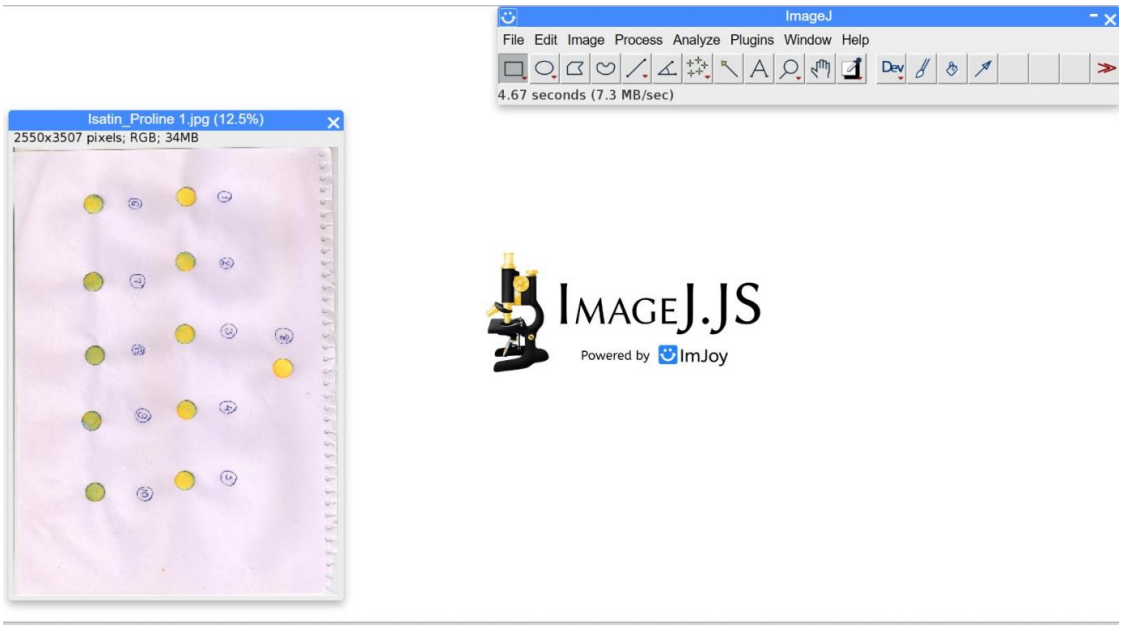


Figure 10. The image was uploaded.

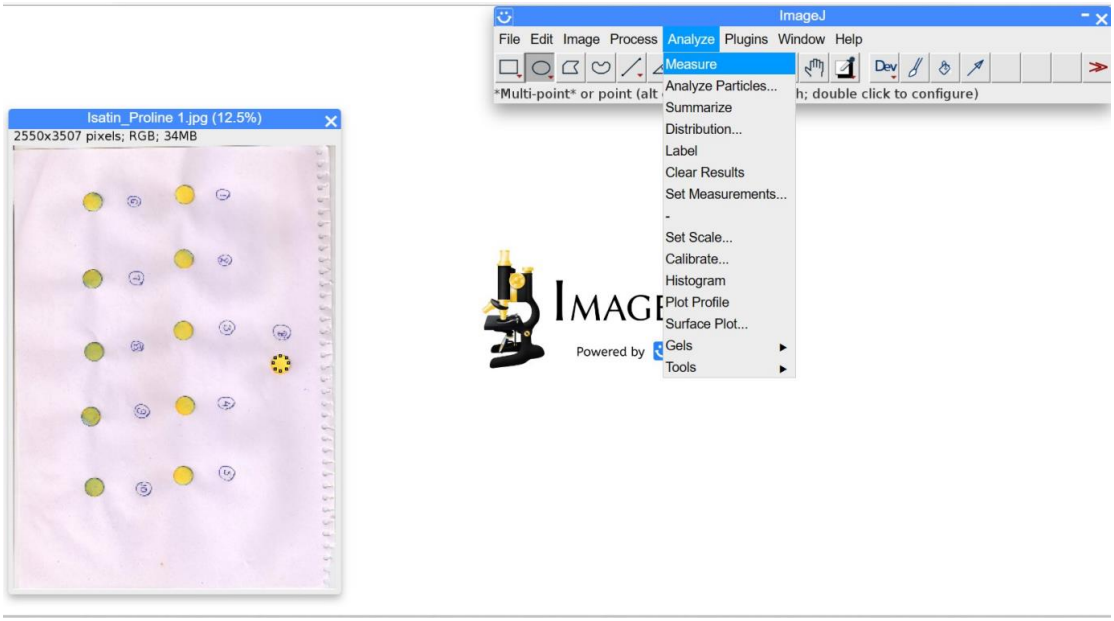


Figure 11. Selection of region of interest. While selecting the region the area must remain same of all other reservoirs.

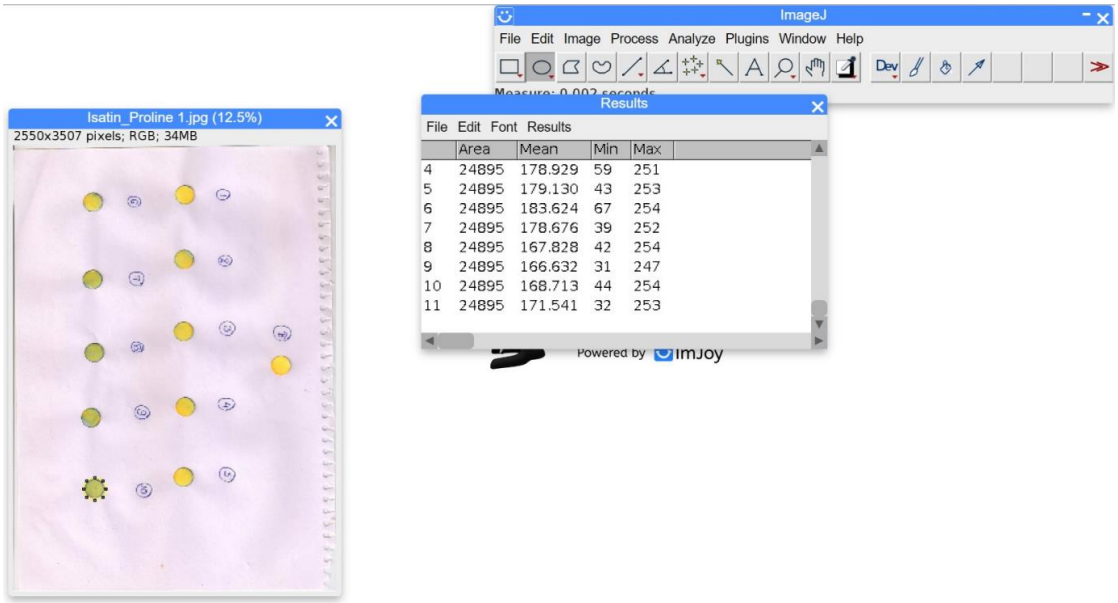


Figure 12. Obtaining the computed result providing mean values for all samples.

3.7 GraphPad Prism:

Scientific graphing, thorough curve fitting (nonlinear regression), comprehensible statistics, and data organizing are all combined in GraphPad Prism. Prism makes it simple to carry out fundamental statistical tests that are frequently used by laboratory and clinical researchers, but it won't replace a powerful statistics application. The analysis of contingency tables, one-, two-, and three-way ANOVA, nonparametric comparisons, t tests, and survival analysis are all available in Prism. Choices for analysis are offered in plain language without superfluous statistical jargon.

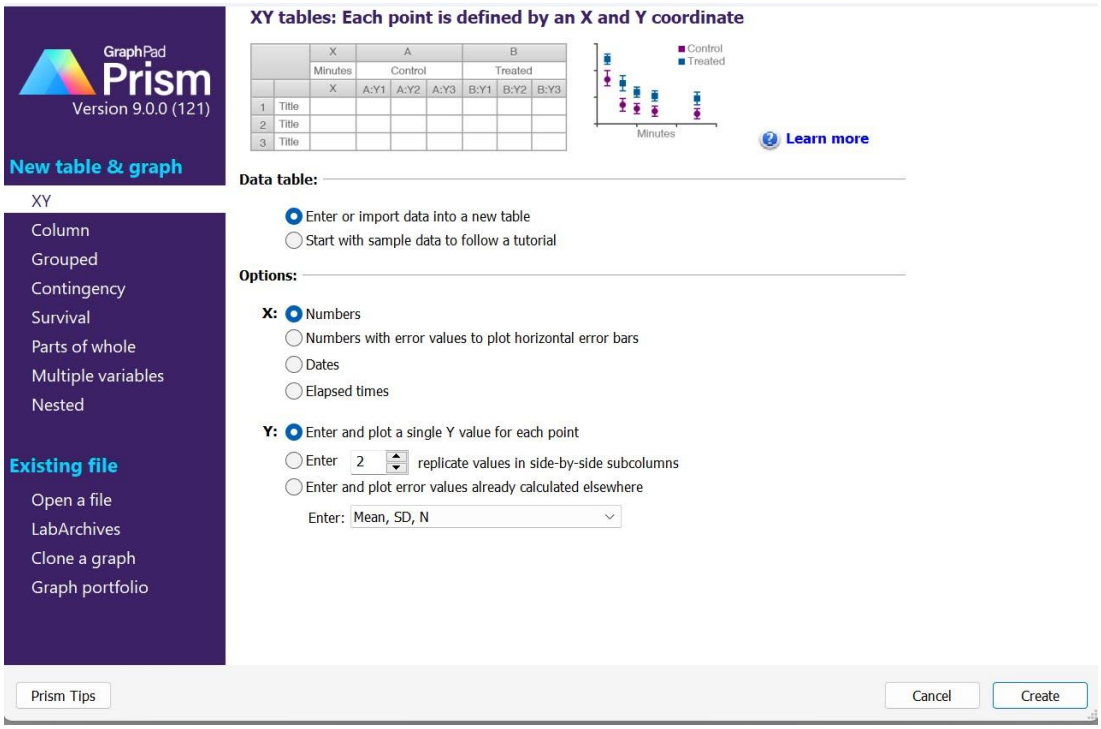


Figure 13. Cover page of GraphPad Prism.

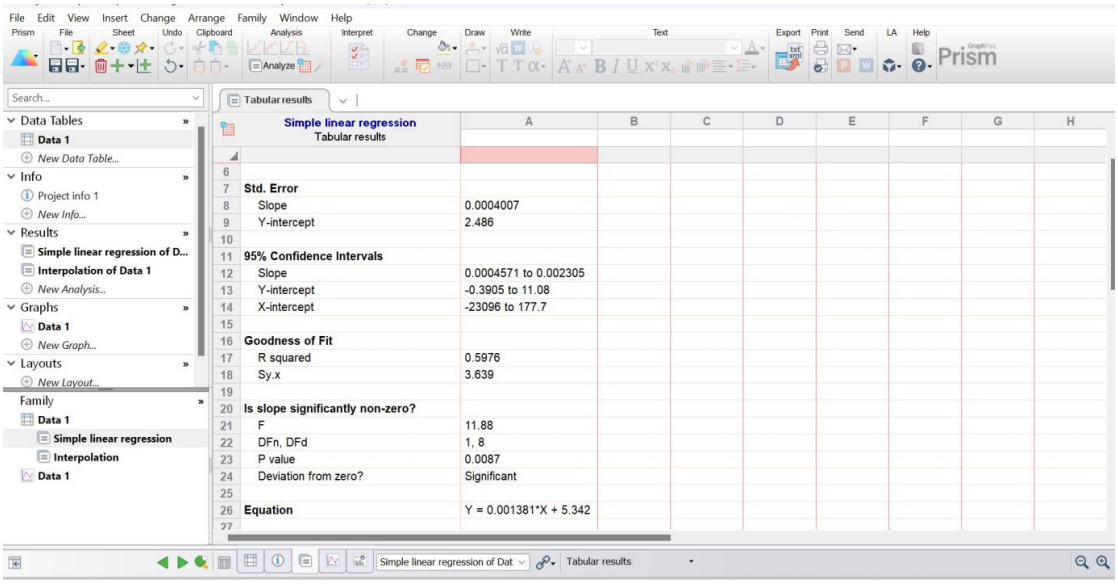


Figure 14. Equation obtained through graph plot of standard solution of glutamic acid.

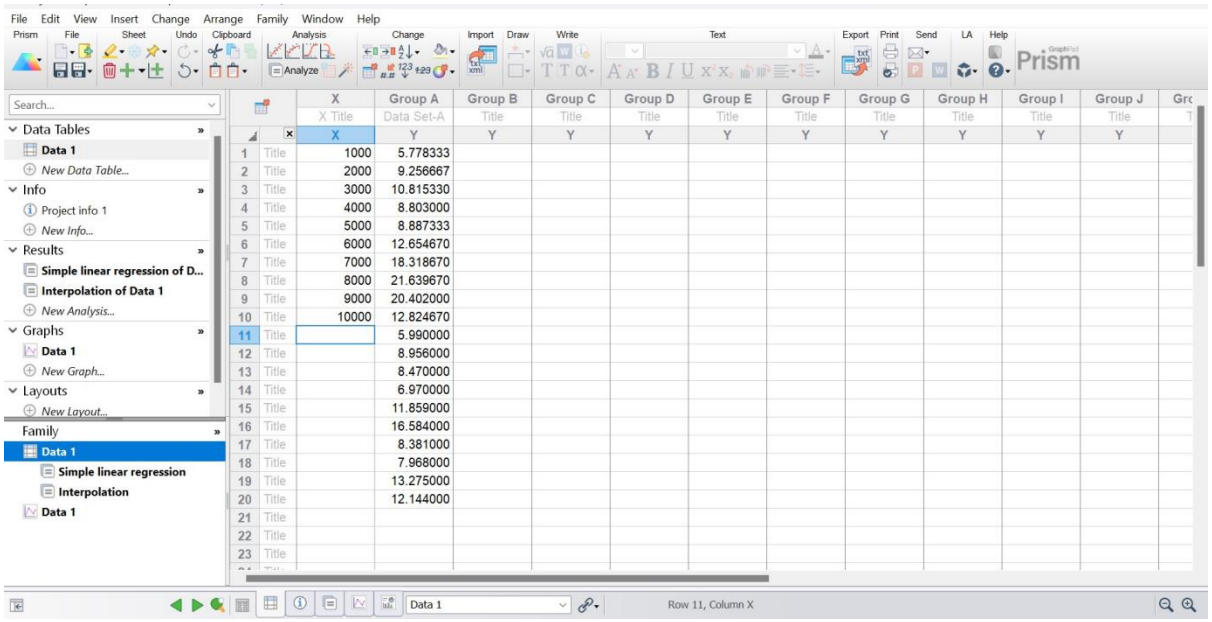


Figure 15. Unknown concentration of serum samples and mean grey value of serum sample calculated using ImageJ.

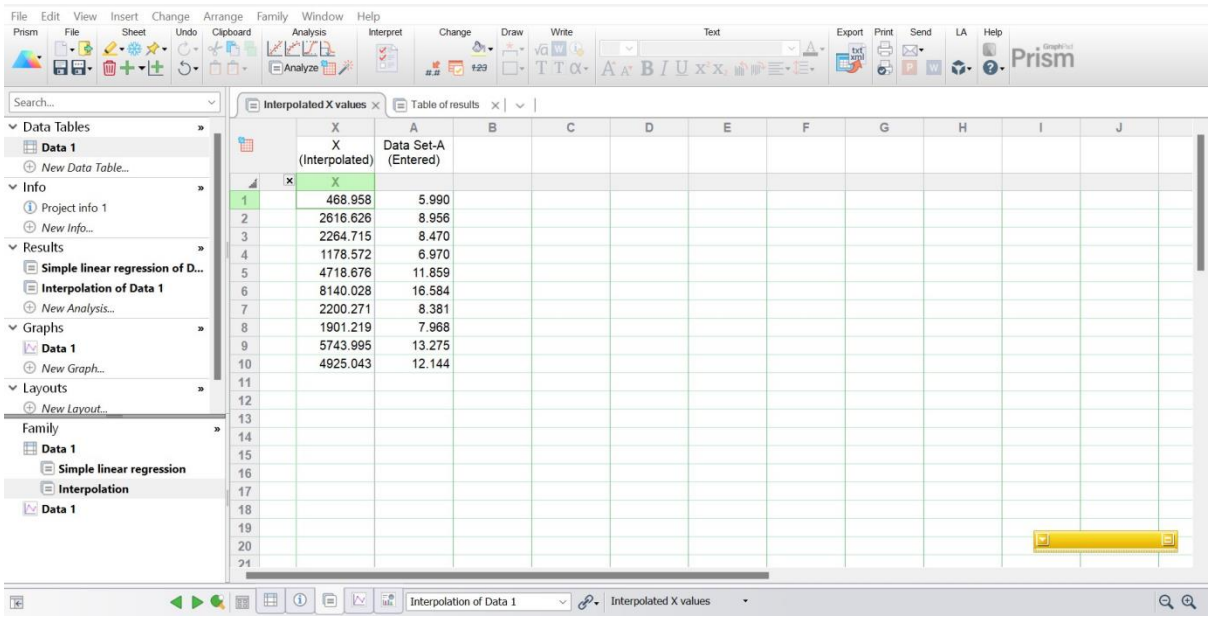


Figure 16. Concentration of serum sample calculated by inter plotting mean grey values of serum sample into standard graph plot.

4. RESULTS AND DISCUSSION

4.1 Objective 1:

To optimize the process parameters critical to the fabrication process. To optimize the process parameters critical to the fabrication process. The choice of the melting temperature and melting time necessary to produce impermeable hydrophobic barriers is covered.

Properties of paper for Microfluidics applications: surface characteristics, surface area, capillary flow rate, pore size, porosity and thickness. The use of the test determines the grade of paper that is chosen. Filter paper formed paper plates, preventing the spot from spreading over a sizable surface. Forming confined spaces also allowed uniformity in both area and tint of the spot, thus enabling the estimation of the concentrations from the intensity of coloration. Paraffin wax was suggested for the formation of water-repellent zones on filter paper due to its general inertness to chemical reagents and its practicality for the formation of diverse patterns.

We used Whatman no. 1 chromatography paper in most of this study because it is hydrophilic, homogeneous, pure, reproducible, biocompatible, and available. It is also relatively inexpensive. It is available in sheets of 460 mm × 570 mm. Filter paper was impregnated with paraffin to demonstrate preferential elution of pigment mixtures.

We used a bronze stamp to print a wax in the filter paper. The stamp dispenses wax from wax coated sheet beneath the filter paper giving imprints of apex on the surface of the paper, where they cool and solidify instantaneously without further spreading. We heated the paper using a digital hot plate because it offers a flat, evenly heated surface. Wax patterning can also be done with the use of other heat sources like ovens or heat guns.

4.1.1 Optimized Time and Temperature:

The area over which the paraffin wax spreads could be controlled by varying the temperature of the printing tool, the pressure, the time of contact and the pore size of the filter paper. It was observed that the matrix in which the analyte is to be detected has not perfectly formed the hydrophobic barrier at temperature 60°C and 70°C with pressing of stamp time for 30s,

45s and 60s, but at temperature 75°C with pressing of stamp time for 45s completely impermeable hydrophobic barrier with a hydrophilic zone enclosed inside it.

Temperature and time optimization to obtain desired reservoirs:

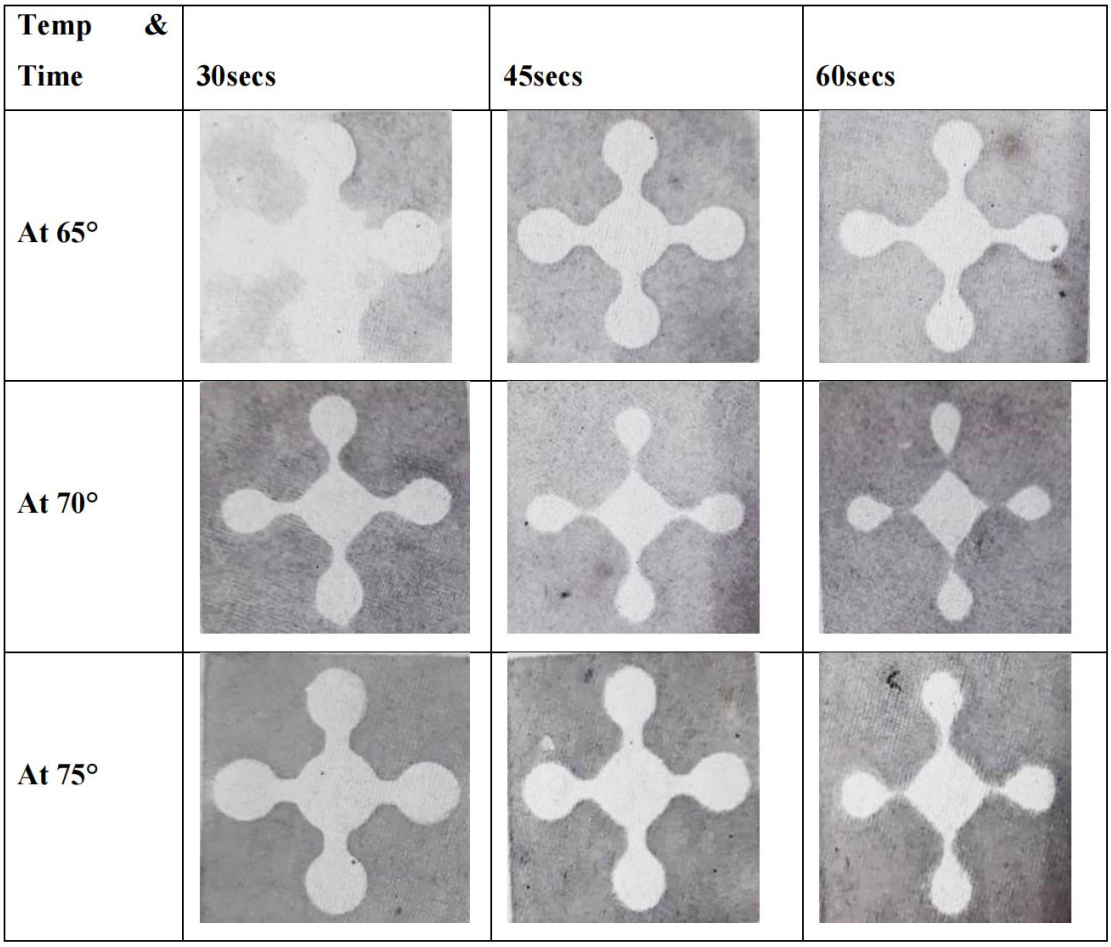


Figure 17. Stamping was performed at different time and temperature to get the desired reservoirs. It was stamped at 60°C, 70°C and 75°C for 30s, 45s and 60s. The optimised time and temperature for stamping is at 75°C for 45s.

4.1.2 Device Design:

The paper-based Microfluidics device is fabricated with a piece of filter paper, wax and a stamp. The device is consisting of four detection reservoirs and a central sample loading zone connected by distribution channels. All distribution channels are of same length and width

and all detection zones have the same diameter to ensure the solution spotted in the circle zone flows equally along all four channels and into the detection zones. Thus the detection reservoirs contain equal amounts of ninhydrin after adding the ninhydrin solution in the device. The three detection reservoirs are spotted with various concentrations of glutamic acid as standard amino acid.

4.2 Objective 2:

Determination of the Total Amino Acid Content in blood serum samples using Paper based microfluidics.

The amino acids in the serum sample were determined by the procedure described above. The colorimetric Isatin test was performed and with increasing concentration of amino acids the colour intensity of colorimetric test was increasing as shown in Figure 18. The image of the colorimetric assay for both standard and serum samples were scanned and stored in JPEG format (Figure 20 and 22). The JPEG images were opened with ImageJ software in RGB colour format. The image was then inverted and the mean grey values in detection zones were obtained by subtracting the blank value. Data were imported into GraphPad Prism to obtain a linear correlation (Equation 1) and a standard graph between mean grey intensity and amino concentrations (Figure 21). The mean grey intensity of serum samples was then interpolated in standard graph to obtain the concentration of amino acids (Table 2).

4.2.1 Colour intensity:

Figure 18. (a) The assay design: the device was incorporated with proline (10 µl of concentration 1 mg/ml) at the four reservoirs after being fabricated (grey region represents the hydrophilic area of the device); **(b)** isatin solution was introduced from central inlet zone; it penetrated into each detection zone and triggered different colour changes (pyrrole blue colour). The arrow in figure b shows the increasing amino acid content.

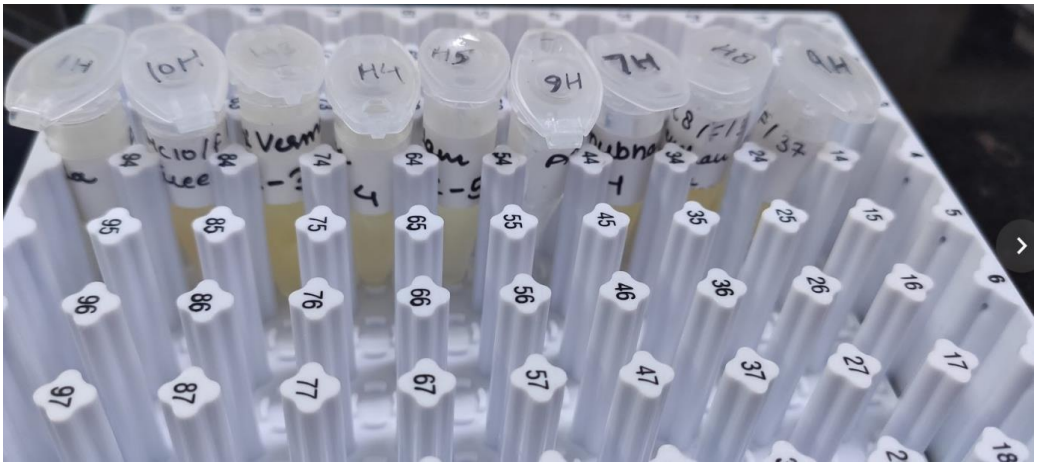


Figure 19. Blood Serum Sample

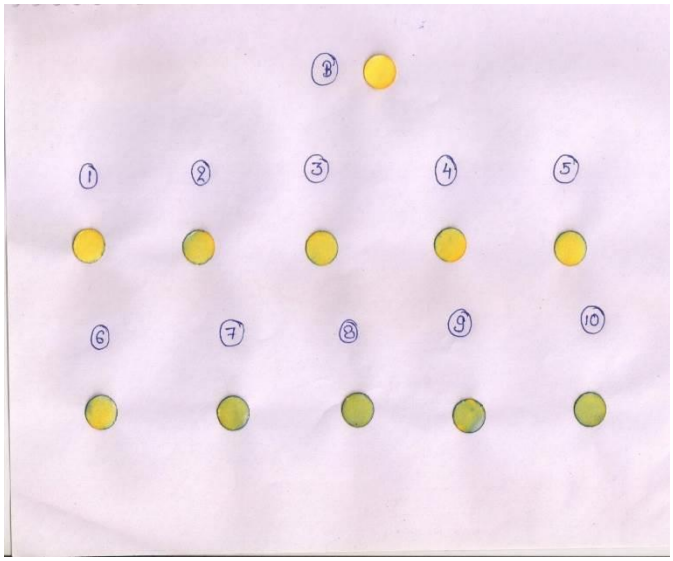


Figure 20. Image showing results of amino acid assay on the paper based device with varied concentrations

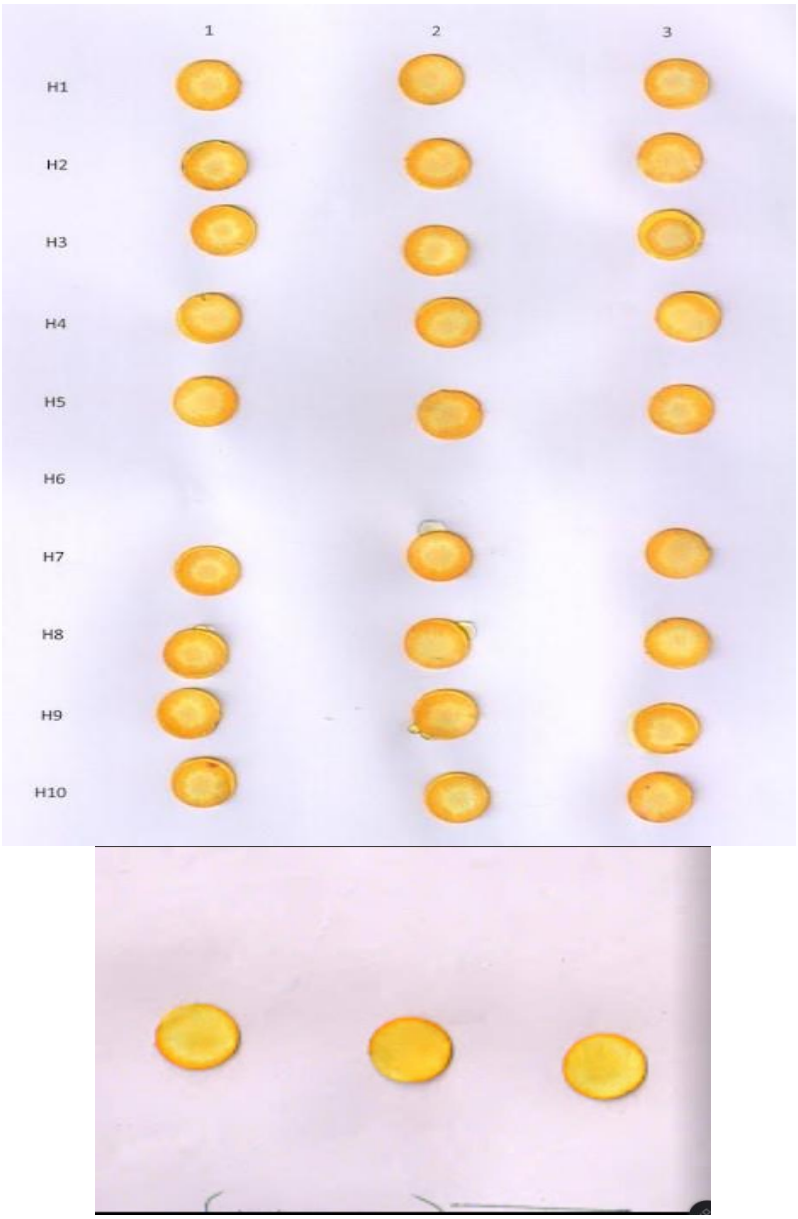
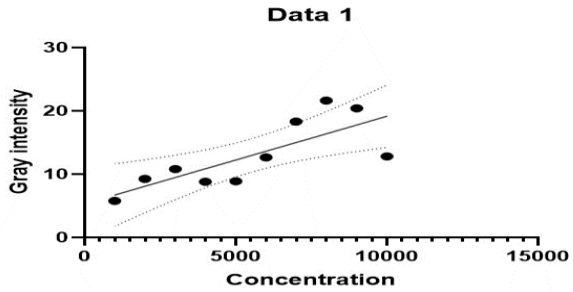


Figure 21. Blood serum samples tested with isatin giving different colour due to different concentration of proline present in the sample.

S.No.	Concentration (µg/ml)	Mean Gray Value
1.	Blank	-
2.	1000	5.558
3.	2000	11.777
4.	3000	10.435
5.	4000	10.234
6.	5000	5.74
7.	6000	10.688
8.	7000	21.536
9.	8000	22.732
10.	9000	20.651
11.	10000	17.823

Table 1. Mean grey value varies as a function of glutamic acid concentration obtained from data of figure 20. The mean grey values were obtained by subtracting values of mean provided by the ImageJ software with the blank value.



. **Figure 22.** Graph plot of concentration against mean grey value of samples

Straight line equation :

Equation 1 Y = 0.001381*X + 5.342

The graph was plot against concentration to obtain the straight line of proline standards. Straight line equation was obtained by GraphPad Prism.

S.No.	Samples	Concentration(µg/ml)	Mean g value
1	S1	468.957	5.99
2	S2	2616.625	8.956
3	S3	2264.715	8.47
4	S4	1178.571	6.97
5	S5	4718.675	11.859
6	S6	8140.028	16.584
7	S7	2200.270	8.381
8	S8	1901.219	7.968
9	S9	5743.995	13.275
10	S10	4925.043	12.144

Table 2. As the result was obtained when tested with isatin as shown in (figure 20)

The result suggested that these images were then scanned using printer scanner and was uploaded on ImageJ for analyses, obtained the computed values providing the mean values of all the samples and mean grey value was calculated by subtracting the highest mean value among the samples. Now as the equation for straight line was provided by GraphPad Prism (equation 1). We knew the values of Y (mean grey value of samples) and thus could calculate the value of X (concentration of samples)

5. CONCLUSION

We described a method for the determination of total amino acids in blood samples by using a simple paper-based microfluidics device. This device is easy to fabricate and could be applied to Microfluidics teaching especially in those less industrialized regions.

A fast (10 s), cheap and simple method to produce paper based Microfluidics devices by contact stamping using a bronze stamp has been developed. Paraffin wax ink can form effective hydrophobic barriers that constrain the diffusion of water within the boundaries of the wax pattern. Capillary flow rate is an important parameter for paper-based microfluidics. It is defined as the migration speed of a sample front moving along the length of the membrane strip. Depending on where the detection line or zone is located, the capillary flow rate is essential for achieving constant sensitivity. Whatman filter paper grade 1 has been used to produce the Microfluidics device because its porous, high flow rate and capillary action.

Compared to other reported paper-based Microfluidics device fabrication technologies, contact stamping is capable of providing an easy way to produce paper-based Microfluidics structures without sophisticated infrastructural requirements. For instance, in developing countries replicas of paper-based microfluidics for diagnostic purpose could be produce by non-especially trained staff employing the contact stamping technique, bearing in mind that only a hot plate, paraffin wax, filter paper and a bronze stamp with the the designed features need to be provide.

In order to develop paper-based Microfluidics devices into a more developed platform technology for diagnostic, point-of-care (POC), and environmental monitoring applications, major research efforts will be required in this area. Approaches towards instrument free detection of amplicons along with the development of PCR-on-paper or nitrocellulose, whole cell detection on paper/nitrocellulose, tissue on paper and all-in-one assays will be important contributions to the field. Furthermore, the quality of paper after-long term storage, and time-consuming sensors (e.g. full colour development) requires further improvements. Having performance data does not always yield efficiency after deployment. Possibles trial must establish the feasibility and cost-effectiveness after scaling up. The ultimate test in the realisation of paper-based microfluidics depend on experts in the commercial diagnostic

industry and this may require evidence for acceptance. One challenge for the academic community is to justify the feasibility of the proposed technologies. The lateral-flow format is the only ubiquitous, universally applicable platform that can be utilised for simple, quantitave, low cost point-of-care applications, while also having enough capability to be utilised in highly sensitive, fully quantified, multiplexed assays. In developing world markets, some applications may be found, but even there purchasing decisions tend to go through the tender process from organizations like the WHO or other large foundations and governmental agencies, who look for performance as well as price. It may be expected that low cost on its own will not be enough to achieve market penetration. The value of diagnostics can only be realised if they meet the performance requirements, manufactured at high-volume and actions can be taken towards treatment.

6. REFRENCE

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