Summer 7-1-2019

A Microfluidics-Based Cross-Flow Filtration Platform for Rapid Processing of Amphiphilic Biomarkers from Blood

Kiersten D. Lenz

Kiersten Lenz
Candidate

Biomedical Engineering
Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

Harshini Mukundan, Chairperson

Steven Graves

Pulak Nath

A MICROFLUIDICS-BASED CROSS-FLOW FILTRATION PLAFORM FOR RAPID PROCESSING OF AMPHIPHILIC BIOMARKERS FROM BLOOD

by

KIERSTEN LENZ

B.A., BIOLOGY AND PSYCHOLOGY, UNIVERSITY OF DELWARE, 2011

M.A., SCIENCE EDUCATION, UNIVERSITY OF DELAWARE, 2013

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Master of Science
Biomedical Engineering

The University of New Mexico
Albuquerque, New Mexico

July 2019

Acknowledgements

The work presented in this thesis would not have been possible without the help and support from many individuals. I would like to first thank my committee members. Dr. Steven Graves, who gave me my first research experience at the University of New Mexico and helped me build a science foundation. Dr. Harshini Mukundan brought me up to Los Alamos National Laboratory and gave me the opportunity to further my research experience. I appreciate all of the wisdom and guidance you have provided me with throughout my graduate work. Dr. Pulak Nath, who made the time to mentor me on a daily basis, and gave me an invaluable engineering perspective on research. Dr. Jennifer Harris, who was my unofficial editor, helped improve my technical writing skills immensely.

I would like to also thank my coworkers and fellow students that I have worked with along the way. All of you have helped me succeed on this project, whether it be from providing assistance in the lab, giving feedback on practice talks, being a source of much-needed comic relief, or joining me for a caffeinated beverage.

To my family and friends, who have all supported me in my decision to pursue a career in biomedical engineering. My path has not been a straightforward one, and your encouragement helped me to stick with it. To Andrew, who believed in my ability to complete this degree, and helped me with countless math problems along the way.

A Microfluidics-Based Cross-Flow Filtration Platform for Rapid Processing of Amphiphilic Biomarkers from Blood

by

Kiersten Lenz

B.A., Biology and Psychology, University of Delaware, 2011

M.A., Science Education, University of Delaware, 2013

M.S., Biomedical Engineering, University of New Mexico, 2019

Abstract

Early and accurate detection of bacterial infections can help save lives, prevent the spread of disease, and decrease the overuse of antibiotics. Our team at the Los Alamos National Laboratory has developed novel assays to detect bacterial biomarkers from patient blood at the point-of-care in order to facilitate a universal diagnostic platform. However, these biomarkers are amphiphilic in nature, and this biochemical property causes them to be sequestered by high-density and low-density lipoproteins (HDL and LDL) in the host's blood. Extraction of the bacterial biomarkers from the lipoprotein complexes is thereby required for the development and deployment of a diagnostic platform.

Accordingly, our team has developed a sample processing protocol to extract the biomarkers of interest; however, this procedure requires multiple pipetting, mixing, and

centrifugation steps that must be performed by hand in a well-equipped laboratory. It also utilizes several chemical reagents, including chloroform and methanol, as well as potentially-infectious human blood. For use in resource-poor settings with minimally-trained personnel, sample processing should be automated in order to guarantee proper treatment of the sample and safety of the staff involved.

Presented in this work is the development of a semi-automated microfluidic sample processing platform for the extraction of amphiphilic bacterial biomarkers. A fully automated system would require two phase separation steps and a re-suspension step to be integrated into a microfluidic platform. Herein, a novel cross-flow filtration scheme was designed to achieve phase separations on a microfluidic device. The devices were fabricated using a combination of laser-based microfabrication and lamination methods. We have taken a stepwise approach to determine the optimal combination of membrane material, membrane pore size, fabrication methods, and geometric design parameters that result in consistent performance. To demonstrate proof of principle, two major phase separations were performed: blood/serum separation, and biomarker extraction. Device materials and surface chemistry were determined to be suitable for this application, as measured by biomarker retention experiments.

This study provides the groundwork for a potentially fully-automated sample processing platform for amphiphilic biomarker extraction from whole blood. The cross-flow filtration platform is a promising design for the complete automation of sample processing because it requires only 90µL of whole blood, is modular, and does not interfere with amphiphile detection. It is simple to manufacture, disposable, pump-free, and does not require the dilution of blood.

Table of Contents

Chapter 1: Introduction

1.1: Biomarker Detection

1.1.1: Motivation

Early and accurate detection of bacterial infections is necessary to guide treatment decisions and prevent the evolution of antibiotic-resistant organisms.[1–6] Novel assays developed by our team at the Los Alamos National Laboratory are able to quickly detect biomarkers indicative of infection from patient blood, and can be adapted for use at the point-of-care. These biomarkers are lipid-based, and their amphiphilic biochemistry causes them to be sequestered by host lipoprotein carriers, including high-density and low-density lipoproteins (HDL and LDL).[7,8] In order to detect amphiphilic biomarkers in human blood, the sample must be processed to first separate serum from blood, and then extract biomarkers from serum.[7] Our team has developed a protocol for this separation and extraction, but the method requires highly trained personnel and a multi-step benchtop procedure. The automation of sample preparation will allow for this method to be effective for use at the point-of-care (especially in resource-poor settings), save time, and ensure user safety (which is of importance when handling potentially infectious human blood). It will also facilitate quicker diagnoses, leading to improved outcomes for patients. Early detection and proper treatment of bacterial infections is essential to help prevent the spread of infection, save lives, and lessen the burden of outbreaks.

1.1.2: Amphiphilic Biomarkers of Interest

Bacteria have traditionally been categorized into Gram-negative, Gram-positive, or Gram-indeterminate based on the structure of their cell walls/membranes, which causes them to stain differently under Gram staining techniques.[9] Each class of bacteria secretes different cell wall components in the host during the course of infection, and these biomarkers are recognized by innate immune receptors, resulting in cytokine signaling.[1] Thus, the variability in cell wall composition is responsible for differential immune responses in infected individuals.

Gram-negative bacteria are characterized by two cell membranes: a thin inner membrane containing peptidoglycan, and an outer membrane composed of about 70% lipopolysaccharide (LPS), a biomarker of interest, in addition to proteins and phospholipids.[9] Gram-positive bacteria lack this outer membrane, but contain a biomarker of interest called lipoteichoic acid (LTA) that extends from the cell membrane through the peptidoglycan wall.[9] Lipoarabinomannan (LAM) is a similar molecule found in *Mycobacterium tuberculosis,* a Gram-indeterminate bacteria that is the causative agent of tuberculosis.[10] LPS, LTA, and LAM are virulence factors that fall into the category of molecules called pathogen-associated molecular patterns (PAMPs). PAMPs are bacterial cell products that are recognized by the host's innate immune system during infection.[11] After the rapid response of the innate immune system, the adaptive immune system responds to eliminate the infectious agent.[12] Thus, LPS, LTA, and LAM are ideal targets for diagnostics because they are released early in infection, are indicative of active infection, and are more stable than protein biomarkers.[1]

The structure of some of these amphiphiles, such as LPS, have been well characterized. LPS will serve as an example structure for all three major amphiphilic biomarkers. Three distinct segments of LPS have been identified (Figure 1). The lipid A portion is a highly conserved endotoxin made of acyl chains of varying lengths and patterns. The core oligosaccharide region is covalently attached to lipid A and is sub-divided into an inner core, which is proximal to lipid A, and an outer core, which is distal to lipid A. The inner core is conserved and consists of a high percentage of rare sugars, especially 3-deoxy-D-manno-octulosonic acid and L-glycero-D-manno heptose. The outer core consists of common sugars such as hexoses. The third portion of LPS is the furthest from the bacterial surface and is called the O-polysaccharide. The O-polysaccharide varies between bacterial serotypes and consists of one to eight repeating glycosyl units. Indeed, the different O-polysaccharide structures are responsible for the hundreds of different serotypes of Gram-negative bacterial species. The O-polysaccharide is also referred to as the O-antigen, since it is the target of the host's innate immune system.[13] This structure is important for understanding the biochemistry of the target biomarkers and their interactions with components of host blood.

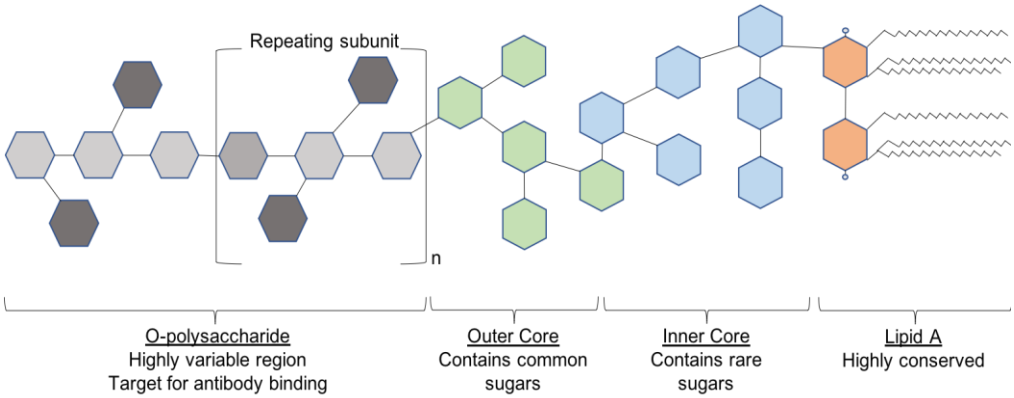


Figure 1: Schematic representation of lipopolysaccharide. Figure adapted from Erridge et al. 2002.

1.1.3: Inflammatory Response of Host

Upon exposure to a Gram-negative bacterial pathogen, LPS binding protein (LBP) present in human serum sequesters LPS from the bacterial membrane and delivers it to receptors for LPS.[13] Also, high-density and low-density lipoproteins (HDL and LDL) sequester circulating LPS in order to mediate macrophage activity. If left unconstrained, the inflammatory response could cause septic shock and even death. In this way, the host's lipoproteins serve as a form of protection against the overproduction of inflammatory molecules such as cytokines.[14]

As mentioned earlier, LPS is a PAMP and is recognized by the host innate immune response. This recognition is mediated by a family of transmembrane receptors called Toll-like receptors (TLRs) that initiate the subsequent signaling cascade.[12] The majority of serotype-specific LPS molecules are recognized by TLR4; however, two exceptions have been found to bind TLR2.[13] The focus of this overview will be on TLR4 receptors.

LPS bound to LBP is brought to extracellular CD14 proteins, which are associated with a TLR4 receptor on monocytic cells. CD14 cleaves large LPS micelles into monomers for presentation to the TLR4-MD-2 complex.[12] MD-2 is a protein that can non-covalently associate with TLR4 or form a complex with LPS in the absence of TLR4. Upon binding of LPS, TLR4 dimerizes, allowing intracellular Toll-interleukin-1 receptor (TIR) domains to begin mediating signal transductor adaptor proteins.[15] Binding of LPS to this complex activates a variety of cellular responses, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory factor 3 (IRF3), both of which affect DNA transcription for cytokine production.[12] Figure 2

illustrates the recognition of LPS by the TLR4-MD-2 complex. The rapid immune response produced by PAMPs are part of what makes them attractive for diagnostic applications.

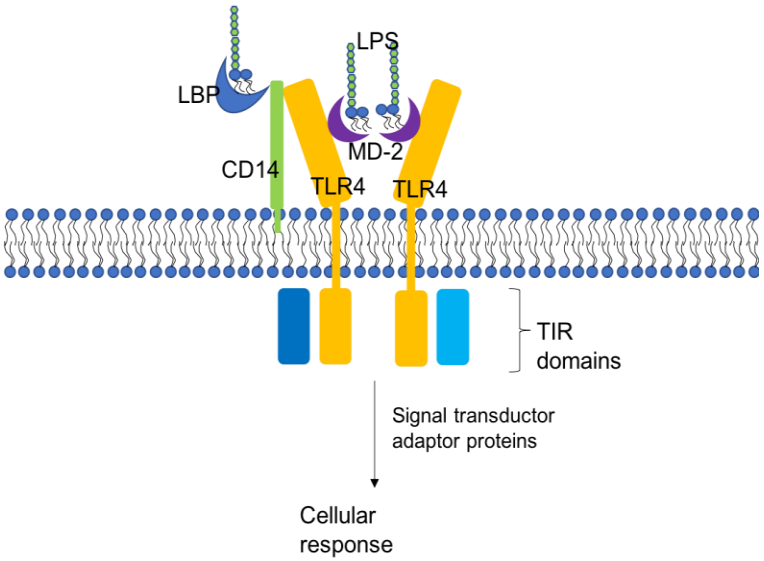


Figure 2: Illustration of TLR4-MD-2 complex recognition of LPS. Figure adapted from Lu et al. 2008.

1.2: Common Methods for Detecting Bacterial Infections

Presented in this section is an overview of commonly-used detection methods for bacterial infections. This overview is not an exhaustive discussion, as that is beyond the scope of this thesis.

1.2.1: Bacterial Cell Culture

Traditionally, bacterial infections of the blood have been detected by culturing and processing large blood samples. The blood samples are quickly transferred to a nutrient medium for bacterial cell culturing. Standard cultures require 48-72 hours to provide enough growth for analysis; therefore, treatment often needs to begin before a

final diagnosis is confirmed.[16] Some cultures, such as *Helicobacter pylori*, the bacteria that causes the majority of gastrointestinal ulcers, take five days to grow.[17] Even more concerning, *Mycobacterium tuberculosis* takes up to 6 weeks.[18–20] There are still more species of bacteria that are unculturable in a laboratory setting, knowledge of which is only known from molecular sequencing efforts.[21] The results from a blood culture are primarily used to discontinue or change the focus of antibiotic treatment, rather than guide initial treatment decisions.[5] This is of concern in a world of growing antibiotic resistant organisms, as overuse of these drugs further contributes to the evolution of resistance.[22] After bacteria have been cultured, they can be identified using a wide variety of clinical methods, which require further processing.

Bacterial cell culture requires laboratory facilities and highly trained personnel who must follow a strict protocol in order to identify truly causative agents. Clinicians must be aware of the potential for contamination and be able to discriminate between true pathogens and contaminant species.[16] The amount of time, training, and potential for misleading results does not make cell culture a viable option for a point-of-care diagnostic tool, especially for guiding treatment decisions in a timely manner.

1.2.2: The Gram Stain

The Gram stain, named after Christian Gram, is a staining protocol that can be done quickly and easily. It has been in use since 1884. Gram-staining can be performed to distinguish between the major groups of bacteria: Gram-negative, Gram-positive, or Gram-indeterminate. Since the structure of each group of bacteria is different, they respond to different types of antibiotics with varying degrees of success.[23,24]

Understanding the major group of bacteria that is causing an infection can greatly help to guide treatment decisions.

Gram-negative bacteria, with their LPS-rich outer membrane and thin peptidoglycan layer, do not retain the primary Gram stain during the wash step. Gram-negative bacteria will appear red under a light microscope due to retention of the secondary dye. Gram-positive bacteria, characterized by a thicker cell wall that is able to retain the primary dye, will appear purple under the microscope.[25] Gram-indeterminate bacteria (also called Gram-variable bacteria), either do not respond to Gram staining or appear both red and purple after Gram staining. Gram-indeterminate bacterial species may have varying levels of peptidoglycan during growth, which would lead to inconsistent Gram staining.[26]

While distinguishing between the major classes of bacteria is of the upmost importance for selecting antibiotic treatment, the requirement of cell culture beforehand makes Gram-staining an unlikely candidate for point-of-care diagnostics.

1.2.3: Enzyme-Linked Immunosorbent Assays

After amphiphilic biomarkers have been extracted from a host's blood sample, they can be detected *via* enzyme-linked immunosorbent assay (ELISA). ELISAs were developed in the early 1970's and are based on the immune response between antigens and antibodies.[27] There are many variations in ELISAs, but the overarching principle, which remains consistent between these, is described here. A microwell plate is coated with the antigen of interest, such as LPS. An epitope-specific primary antibody is then added to detect the antigen. The primary antibody can be conjugated to an enzyme, such

as horseradish peroxidase (HRP) for colorimetric or luminescent detection, or an enzyme-conjugated secondary antibody may be used for colorimetric detection.[28] Figure 3 shows the detection of LPS via ELISA in a schematic. ELISAs are colorimetric and use the color change upon the cleavage of the substrate by an enzyme conjugated to the targeting antibody as a read-out. Modified immunoassays which use fluorescence labels attached to recognition ligands and antibodies have also been developed, and sometimes can offer greater sensitivity over colorimetric assays.

While useful for the detection of amphiphilic bacterial biomarkers, there are some limitations associated with the use of ELISAs. Many amphiphilic biomarkers do not adhere well to ELISA plates, which are designed for protein-based hydrophilic interactions. This commands the need for sandwich assays: the requirement of two recognition ligands per target antigen, making the approach complex and not adaptable to small molecule detection. Low sensitivity and reproducibility are known to be issues when detecting amphiphiles via ELISA.[29] It is speculated that the amphiphilic biochemistry of these biomarkers causes inconsistent binding to microwell plates.

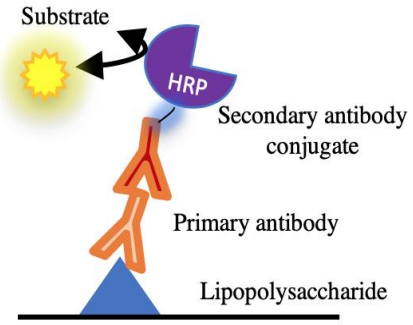


Figure 3: Schematic of an indirect ELISA with LPS as the antigen of interest. A microwell is coated with LPS, and a primary antibody is used to bind LPS. A secondary antibody conjugated to the enzyme horseradish peroxidase (HRP) is used for colorimetric detection when it reacts with the substrate.

Additionally, amphiphiles form micelles in aqueous solutions, which could block antibody binding via steric hinderance.[30] In order to detect physiologically-relevant concentrations of biomarkers, a more sensitive method should be used.

1.2.4: Polymerase Chain Reaction

Polymerase chain reaction (PCR) is another technique for the detection of bacteria; however, this method detects nucleic acids instead of amphiphilic biomarkers. PCR consists of three major steps: 1) denaturation of template DNA; 2) annealing of primers for new DNA synthesis at a specific site; and 3) extension of new DNA strands from the primers. Prior knowledge about pathogen-specific primers is necessary in order to get a detectable signal. Multiple rounds of synthesis are performed in order to maximize DNA yield. This process requires thermocycling to temperatures above which DNA polymerase denatures; thus, the discovery of DNA polymerase from thermophilic bacteria *Thermus acquaticus (Taq)* was key to the success of PCR.[31,32]

PCR can be performed on a variety of samples, including tissues, blood, and microorganisms. Only trace amounts of DNA are required for PCR, making it an extremely sensitive assay. It is also highly specific, as primers can be chosen to target a gene known to be associated with the species of interest. Additionally, the materials needed to perform PCR are inexpensive and fairly easy to use.[33]

While PCR is valuable, it does have limitations. Primer design is dependent upon prior sequencing data from the pathogenic species, and some primers can non-specifically bind to sequences similar to the target DNA.[33] For a point-of-care diagnostic tool, PCR may be too specific. Primers would need to be available for a variety of pathogenic

bacteria. With the evolution of antimicrobial resistance and the adaption of bacterial pathogens, the likelihood of PCR primers failing is a constant threat. Also, pathogen-specific nucleic acid sequences can sometimes be present in the absence of viable organisms, causing a false positive result. PCR cannot be applied for the detection of proteins. Detection of conserved amphiphilic biomarkers can allow for the fast detection of major classes of bacterial infections, yielding enough information for accurate treatment.

1.3: Waveguide-Based Biosensor as a Deployable Diagnostic Tool

1.3.1: Sensor Technology

A waveguide-based optical biosensor, developed by our team at the Los Alamos National Laboratory, has the ability to quickly detect amphiphilic biomarkers extracted from patient blood samples by using tailored and novel assay mechanisms. As an optical biosensor, the technology measures changes in light due to the binding of an analyte with a fluorescently-labeled reporter molecule.[2,8] The sensor technology is based on the total internal reflection of light when it passes through a boundary between materials with different refractive indices at a critical angle of incidence. LANL's biosensor utilizes a planar optical waveguide, comprised of a guiding layer that has a higher refractive index than the substrate layers.[8] Under total internal reflection, light travels along the guiding layer, and an electromagnetic field (the evanescent wave) is generated off the substrate layers. The evanescent field decays exponentially as distance from the surface increases to about 100nm.[34] It is within this evanescent field that biomarkers captured by fluorescently-labeled antibodies can be detected. The small area of detection minimizes

background signals, ensuring a more accurate diagnosis.[11] Figure 4 shows a cross-section of a planar optical waveguide with total internal reflection of light.

LANL's waveguide platform has been used to detect not only PAMPs (including LPS, LTA, and LAM), but other molecules of interest such as the carcinoembryonic antigen (a breast cancer biomarker), toxins such as the cholera toxin, and the protective antigen/lethal factor associated with *Bacillus anthracis* infection.[7,8,10,35–38] Because of the utility of this sensor, members of our team are developing a modular instrument for deployable applications. This work includes scaling down and ruggedizing the benchtop set up for field transport and use.

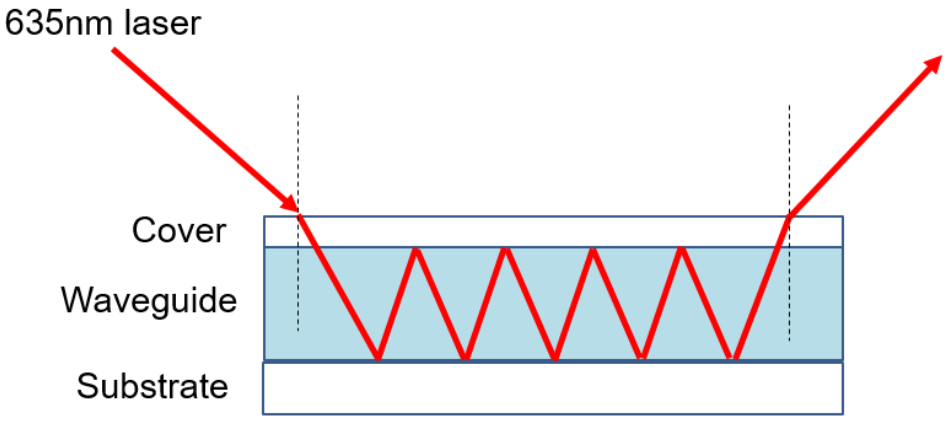


Figure 4: Cross-section of a planar optical waveguide. At a critical angle of incidence, total internal reflection of light occurs in the waveguide layer, and an evanescent field extends off the surface.

1.3.2: Waveguide-Based Assays

Two novel immunoassays have been developed at LANL for the detection of bacterial infections: lipoprotein capture and membrane insertion. Both assays can be performed on the waveguide-based optical biosensor, where biomarkers of interest are

immobilized on the waveguide surface and detected by fluorescently-labeled antibodies within the evanescent field. However, both assays can be adapted to other sensor platforms that offer adequate sensitivity for the detection of low prevalence pathogen biomarkers in complex samples.

Lipoprotein capture assays manipulate the natural affinity of amphiphilic biomarkers with lipoprotein carriers in blood. A functionalized lipid bilayer is applied to the flow cell of the waveguide, onto which an anti-lipoprotein antibody is conjugated. The sample is added, and lipoprotein-biomarker complexes are captured by the anti-lipoprotein antibody. A fluorescent anti-biomarker antibody, excited by the light coupled into the waveguide, serves as the reporter.[2] Since lipoprotein capture assays pull down already existing lipoprotein-biomarker complexes, there is no need to perform amphiphile extraction on the sample (our benchtop sample processing); however, prior knowledge about which lipoprotein (HDL, LDL, etc.) associates with the biomarker of interest is necessary. Additionally, biomarker epitopes could be embedded within the lipoprotein carriers, causing them to evade detection.

Membrane insertion assays are based on the amphiphilic chemistry of bacterial biomarkers. A lipid bilayer is applied to the flow cell of the waveguide to serve as a capture surface. After the biomarkers are extracted from their lipoprotein complexes, they are applied to the flow cell and naturally associate with the lipid bilayer. Only one antibody is needed for membrane insertion assays: a fluorescent anti-biomarker antibody.[11,38] While membrane insertion assays do require sample preparation, they are more sensitive and accurate at detecting bacterial infections. It is more likely that critical epitopes will be exposed for detection, since the biomarkers are unbound from their

lipoprotein carriers. Membrane insertion also requires less reagents, time, and lipid bilayer surface preparation.[11] Figure 5 compares the two assays as they would be performed on LANL's waveguide-based optical biosensor.

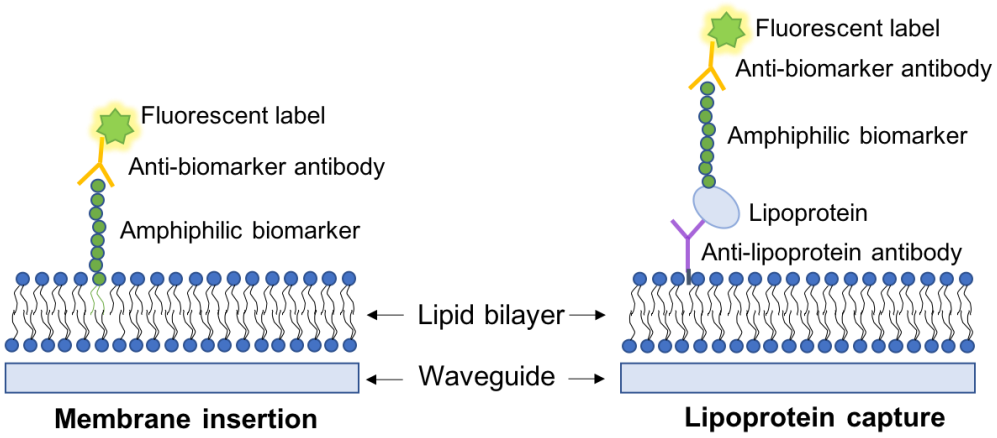


Figure 5: Two novel waveguide-based assays, membrane insertion (left) and lipoprotein capture (right) can detect biomarkers of interest from bacteria.

Because it is not always known which lipoproteins the bacterial biomarkers associate with in serum, membrane insertion assays are preferred for point-of-care use over lipoprotein capture assays. By performing sample processing before the membrane insertion assay, the amphiphilic biomarkers of interest can be directly detected. Figure 6 displays the overall processing and detection overview.

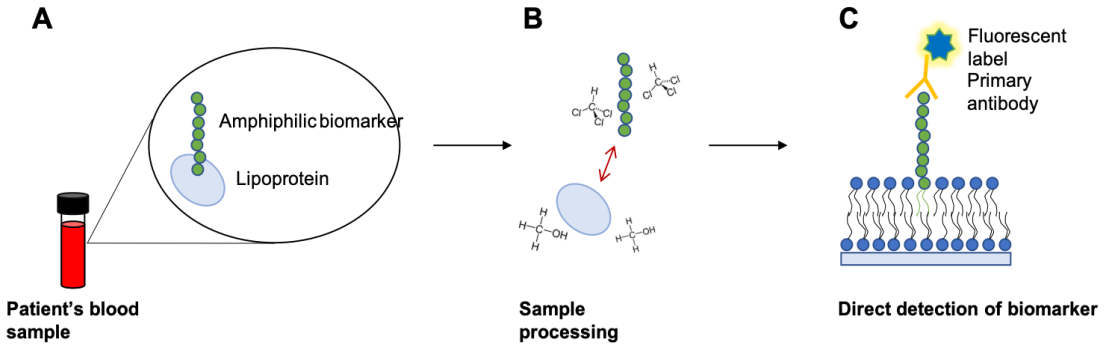


Figure 6: Processing and detection overview. A) Within a patient's blood sample, lipoproteins sequester amphiphilic biomarkers of interest. B) Sample processing liberates biomarkers from the lipoprotein complexes they form in serum. A mixture of chloroform and methanol is used for the process. Lipoproteins tend to associate with methanol, since it is the more hydrophilic solvent, while amphiphiles associate with chloroform, which is more hydrophobic. C) Our novel membrane insertion assays directly detect amphiphilic bacterial biomarkers. A lipid bilayer is used to capture the biomarkers of interest before adding a fluorescently-labeled primary antibody. Detection can be done via ELISA, LANL's waveguide-based optical biosensor, or otherwise.

1.3.3: Benchtop Sample Processing Methods

Before amphiphilic biomarkers can be detected from a host's sample, they must be extracted from the lipoprotein complexes they form in serum.[7] Our current sample processing method is a modification of the Bligh-Dyer lipid extraction protocol, a commonly used lipid extraction method from 1959. The Bligh-Dyer method relies on solvents to separate lipids from other biological molecules. An aqueous sample is mixed with chloroform and methanol in a volumetric ratio of .8:1:2, respectively. Bligh & Dyer (1959) found this ratio to be ideal to form a biphasic solution, which can then be manipulated to separate lipids from other biological molecules.[39] In comparison to methanol, chloroform is hydrophobic, and lipids associate with the chloroform layer.[40]

By choosing which layers of the biphasic solution to work with, one can successfully isolate molecules of interest.

As displayed in Figure 7, the modified Bligh-Dyer method for lipid extraction involves multiple mixing, centrifugation, and pipetting steps, as well as chemicals including chloroform and methanol. This protocol requires highly trained personnel to ensure successful biomarker extraction. The manual steps also introduce an opportunity for mistakes, which is of concern when handling potentially infectious blood. While useful for benchtop assay development, an automated biomarker extraction would be superior for deployable use.

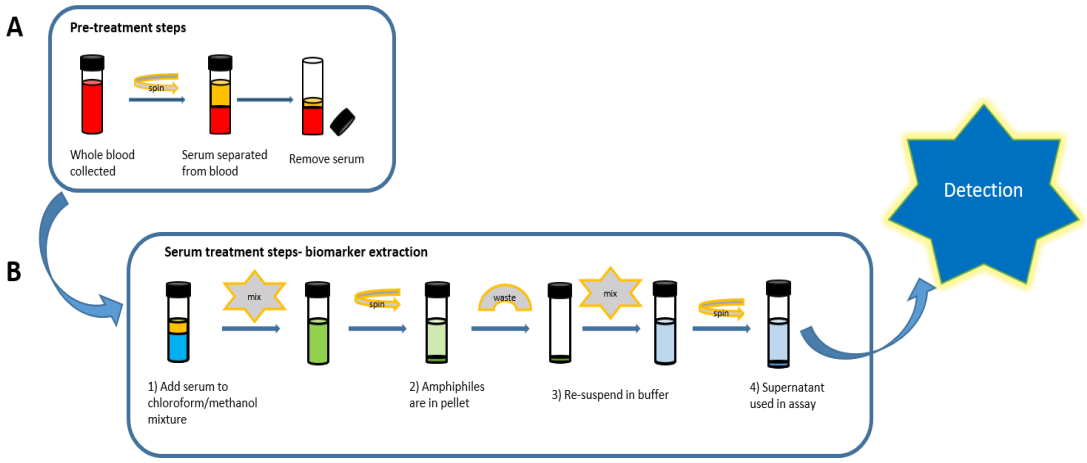


Figure 7: A detailed schematic of benchtop sample processing. A) The first major step of sample processing is to separate serum from blood. B) The second major step of sample processing is serum treatment for biomarker extraction. 1) Serum is added to a mixture of chloroform/methanol in a .8:1:2 ratio (by volume); 2) After mixing by pipette, the tube is spun at 5500RPM for 1 minute and the supernatant is discarded. 3) The lipid pellet is re-suspended in 1XPBS. 4) A short spin (5 sec) sends debris to the bottom of the tube, and the supernatant is used in membrane insertion assays.

1.4: Centrifugal Microfluidics

1.4.1: Centrifugal Microfluidics for Automation

Centrifugal microfluidics, also called "Lab-on-a-CD" or "Lab-on-a-Disc," is a promising field of research for the automation of a variety of biological processes, including assays and sample preparation. Mixing, metering, and phase separations can be automated by controlling the spin profiles of the device.[41] Other advantages include the fact that minimal instrumentation is needed, the devices are efficient at removing trapped air bubbles, and the centrifugal force is inherently effective at density-based separations.[42]

Centrifugal microfluidic designs are especially appealing for point-of-care and deployable diagnostics. Lack of clean water, dusty environments, unstable temperatures, and fluctuating power supplies are all limitations of resource-poor areas. Centrifugal microfluidic platforms can help overcome these limitations due to the low volume requirements, ease of use, and fast processing time of the device.[43] Because of this, centrifugal microfluidic platforms can expand the types of assays that can be performed in isolated (and often disease-burdened) regions of the world.

1.4.2: Introduction to centrifugal microfluidic techniques

Forces: Centrifugal, Coriolis, Euler

Multiple forces act on the contents of centrifugal microfluidic devices. These forces can be manipulated to control the flow of fluids within the device. The most obvious force is the centrifugal force itself, which acts radially outward during rotation of the disc.[42] A second force, the Coriolis force, acts on fluids when the device is spun at

relatively higher revolutions per minute (RPM).[41] The Coriolis force acts perpendicularly to the angular rotational frequency (ω) and the fluid velocity. The third major force is the Euler force, which acts proportionally to the rotational acceleration.[44] Figure 8 depicts a fictitious Lab-on-a-Disc and the forces acting upon it.

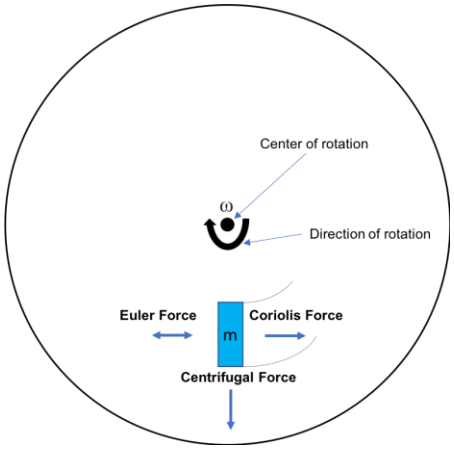


Figure 8: Schematic of a Lab-on-a-Disc and the forces acting on a mass (m) when the disc is spinning. Figure adapted from Strohmeier et al. (2015).

Unit Operations: Pumping, Valving, Mixing, Flow Switching

Unit operations refer to the basic functions that can be embedded within a Lab-on-a-Disc design to achieve the function of interest. The major forces (Centrifugal, Coriolis, and Euler) can be manipulated to achieve unit operations at a specific time.[42]

The centrifugal force can pump fluids from one location to another in a radial direction towards the outer perimeter of the disc. The rate of flow depends on RPM, location of the fluid reservoirs or channels, design geometry, and properties of the fluid itself.[41]

Valves can be used in order to further control the flow of fluid. Common valves include capillary, hydrophobic, and siphon valves. Capillary valves function by balancing capillary pressure and centrifugal pressure; if the centrifugal pressure is less than the capillary pressure, fluid will not flow through and the valve is considered closed. Conversely, when the centrifugal pressure exceeds the capillary pressure, fluid will flow through and the valve is considered open. Hydrophobic valves consist of a narrow hydrophobic channel, and also function based on the balance between capillary and centrifugal pressure. Hydrophilic fluid will only flow past the hydrophobic region when the RPM exceeds a critical value.[41] Siphon valves move fluid from a reservoir further from the center of rotation to a reservoir that is closer. These valves are based on capillary action, and rely on a hydrophilic surface.[45]

Mixing is a common unit operation needed in biological processes. Diffusive mixing alone is not usually sufficient for the small reagent volumes present in the microfluidic disc.[42] To adequately mix reagents, rapid oscillations of the disc, internal paramagnetic particles, or external magnetic stirring can help to homogenize solutions.

Another common unit operation is flow switching, which is changing the direction of specific fluids flowing into different chambers. This can be achieved by manipulating the Coriolis force with a Y-shaped channel. Fluids will differentially flow on either side of the Y-shape based on density.[41]

1.4.3: Challenges with Microfluidics at the Point-of-Care

While centrifugal microfluidic platforms offer a plethora of attractive solutions for point-of-care diagnostics, they also come with a set of challenges. The environment

inherent to resource-poor areas of the world imposes a set of limitations on the device. Temperature fluctuations can affect biological reagents, which must be properly stored in order to work effectively. Availability of a power supply cannot be relied upon; thus, backup power or solar power must be considered in the device design. In extremely remote areas, undertrained personnel are often responsible for sample procurement and device operation; therefore, these processes must be as failsafe as possible. It is also common to have low sample volumes available due to patient dehydration and illnesses.[43,45] Another consideration is the proper handling of waste: ideally, the waste chamber on the disc could be pre-loaded with disinfectant to avoid worker exposure to harmful chemicals or infectious patient samples.[43]

Beyond environmental challenges, design restrictions can limit the utility of centrifugal platforms for point-of-care diagnostics. Adequately mixing multiple fluids is difficult due to low fluid volume and viscosity. Diffusive mixing can be too slow for a diagnostic tool that needs to process samples and give results quickly. Many valving methods, such as siphoning, rely upon hydrophilic surfaces. This is often achieved by treating a previously hydrophobic surface.[45] Surface treatment can pose a problem for deployable devices because it imposes a shelf life on the disc that may not be long enough for a given application. Additionally, uneven surface treatment can affect the performance of the device. Both environmental and design constraints must be overcome in order to produce a fully functioning point-of-care diagnostic tool.

Chapter 2: Motivation and Overview of Project

The motivation for this work is to provide a safe, rapid, and automated microfluidic system for extraction of amphiphilic biomarkers from blood in order to accurately detect bacterial infections at the point-of-care. In the developing world, infectious diseases are one of the leading causes of death.[46] Many patients travel for hours or days to reach a clinic to receive medical treatment. If the diagnostic tool used at the clinic does not provide rapid results, and patients are asked to return later for outcome management, many will be unable to do so.[47,48] A timely diagnosis will therefore help more patients receive suitable healthcare and will reduce the burden of infectious diseases on resource-poor populations.

Antibiotic resistance is another issue that affects the developing world, perhaps more so than in developed countries. The overuse of antimicrobials is considered a major culprit in the evolution of antibiotic resistant organisms.[49,50] Broad spectrum antibiotics are often prescribed as a comprehensive way of treating bacterial infections in rural settings, where patients cannot wait for a definitive diagnosis.[47] Other factors that may contribute to antibiotic resistance is the ineffective regulation of pharmaceuticals, poor drug quality, and environmental factors such as high temperatures.[49] Rapid and accurate detection of bacterial infections may help to slow the spread of antimicrobial resistance.

Two specific aims will assist in the automation of blood sample processing, which will contribute towards the overarching goal of preventing human illness from bacterial infections: 1) automate and validate blood/serum separation on a centrifugal microfluidic disc; and 2) automate and validate amphiphilic biomarker extraction from serum for use with our membrane insertion assays. Figure 9 reviews the current sample processing

method. We identified multiple phase separations, waste and input steps, and a re-suspension step. In this case, a phase separation is defined as a pellet separated from a fluid phase. Table 1 displays the engineering considerations and challenges to take into account when translating this process to an automated system.

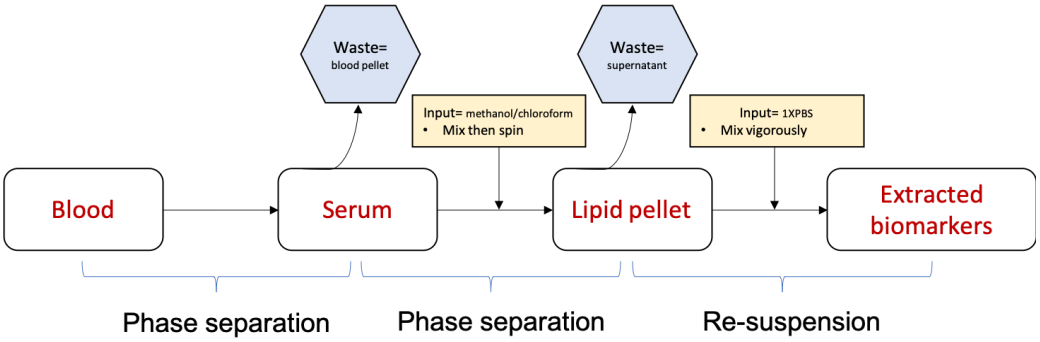


Figure 9: Schematic of biomarker extraction process to be transferred to an automated system. The process includes multiple phase separations, input, waste, and mixing steps, and a re-suspension.

Table 1: Summary of the engineering challenges associated with automated biomarker extraction and potential solutions to each

Challenge:	Potential Solutions:
1. Two phase separations	• Membrane-based separation • Centrifugal separation
2. Two waste steps	• Membrane to isolate waste • Siphon

3. Two buffer inputs	• Manually pipette into inlet holes
	• Pre-package buffers on device:
	  • Powder form
	  • Contained in reservoir w/valve for controlled addition

4. Two mix steps	• Mix manually by agitation • Programmable motor that can spin clock- and counter-clockwise

Chapter 3: Materials and Methods

3.1: Device Designs for Phase Separations

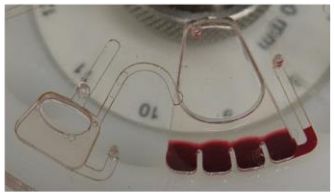
3.1.1: Adaptations from Previously-Published Works

Initial designs to separate blood from serum were adapted from previous studies.[51,52] Hin et al.[51] described a membrane-based device for the separation of serum from blood and subsequent analysis of C-reactive protein (CRP). A commercially-available Pall Vivid™ Plasma Separation Membrane was integrated into the inlet of a microfluidic cartridge. This specific membrane was chosen since it is designed to separate plasma from blood in a one-step process. Centrifugation was not required for blood/serum separation; the blood cells get trapped in the membrane due to the force of gravity.[53] Low-speed centrifugation then whisks away purified serum for further processing or analysis. This design may be suitable for point-of-care applications due to the small blood sample needed (90µL), minimal electrical power needed, and simple use. However, membrane integration was difficult to achieve consistently with this design, and serum yield was extremely low for our application (<10µL).

Amasia and Madou[52] developed a centrifugal microfluidic device for blood/serum separation that relies on a siphon design to isolate the serum from the blood pellet. Two milliliters of whole blood were added to the large sedimentation chamber. Blood/serum separation was achieved via a specific RPM profile, reaching 3800RPM as a maximum speed. Lower RPMs then prime and start the siphoning of serum into its own chamber. The isolation of serum is ideal for further processing or on-chip analysis. Plasma purity, measured by cell counting, was equal to or greater than plasma obtained from traditional

separation in a tube. While promising, this design relies on a hydrophilic surface achieved through oxygen plasma treatment in order for the siphon to function properly. We found that the effect of plasma treatment had an inconsistent shelf-life, which is not useful for a deployable device. Scalability was also an issue with this design, since it is more useful to start with smaller volumes of blood from patients at the point-of-care. When we scaled the design down, 350µL of blood was needed in order to yield 100µL of serum. This amount of volume may be too much for some patients to give. Table 2 compares the two previously-published designs and summarizes their advantages and disadvantages for our specific application of amphiphilic biomarker extraction.

Table 2: Designs adapted from previously-published works

Design modified from:	Madou & Amasia (2010)	Hin et. al (2018)
Photo of device:		
Advantages:	• Serum isolated in its own chamber • Easy fabrication process	• No centrifugation required for separation • Commercially-available membrane
Disadvantages:	• High blood volume required (350µL) • Not all serum available for further processing • Plasma treatment of surface required	• Difficult fabrication process • Low serum yield (<10µL)

3.1.2: Original Cross-Flow Filtration Design

A novel centrifugal microfluidic platform was developed based on the principals of cross-flow filtration. During the process of cross-flow filtration, a fluid passes tangentially across a filter. Tangential flow of blood is achieved via the centrifugal force acting on the centrifugal microfluidic platform. Fluid components smaller than the

membrane's pores are driven through the filter as pressure builds due to the centrifugal force, while larger components pass over the membrane without flowing through.[54] In contrast, dead-end filtration can result in clumping of particles that could clog the filter, since the force of fluid flow is perpendicular to the membrane's surface.[55,56] Cross-flow filtration decreases the chances of clogging, which in turn decreases the chances of red blood cell lysis (an important consideration for our process). Cross-flow filtration allows for decreased resistance across the filter, and therefore was chosen as a potential method of phase separation.[57] Figure 10 presents the concept of cross-flow filtrations as compared to dead-end filtration.[58]

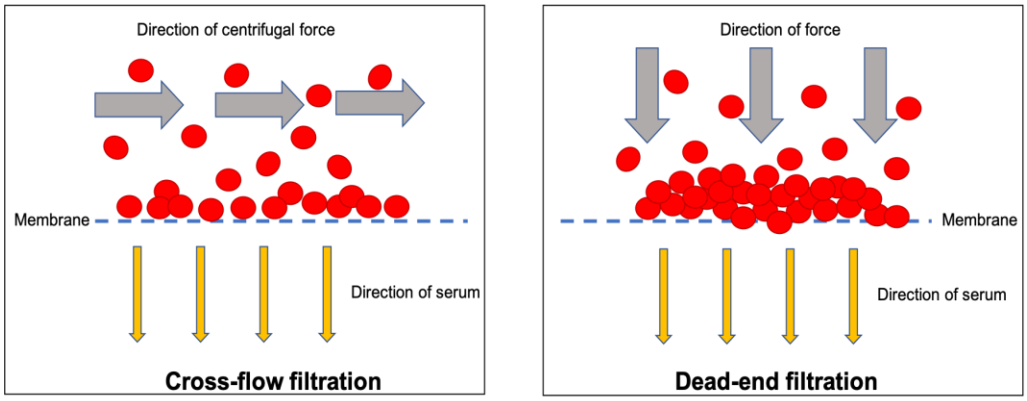


Figure 10: Cross-flow (left) vs. Dead-end (right) filtration for the separation of blood from serum. Cross-flow filtration allows for less clogging. Figure adapted from El-Safty & Hoa (2012).

3.2: Fabrication Materials and Methods

After the adaptation of previous designs, an original centrifugal microfluidic platform was developed for the separation of serum from blood and subsequent biomarker extraction from serum. The cross-flow filtration platform consists of five structural layers of plastic and one membrane layer held together by pressure-sensitive adhesive (3M^{TM}).

Layer schematics were drawn using SolidEdge 2D drafting software (Appendix 1). Alignment holes were included on all layers for assembly on a jig. The layers were cut using a CO_2 laser cutter (M360, Universal Laser System) from previously laminated stock acrylic (McMaster Carr), polycarbonate (McMaster Carr), and membrane sheets (Sterlitech). After cutting, the plastic layers were cleaned by bath sonication for 15 minutes followed by a manual wipe down with isopropyl alcohol. Figure 11 displays a schematic of the device layers and a photo of a fabricated device. Figure 12 presents a unit of the multilayered cross-flow filtration device design, including a cross-section. Up to six units can fit onto the 90mm diameter disc.

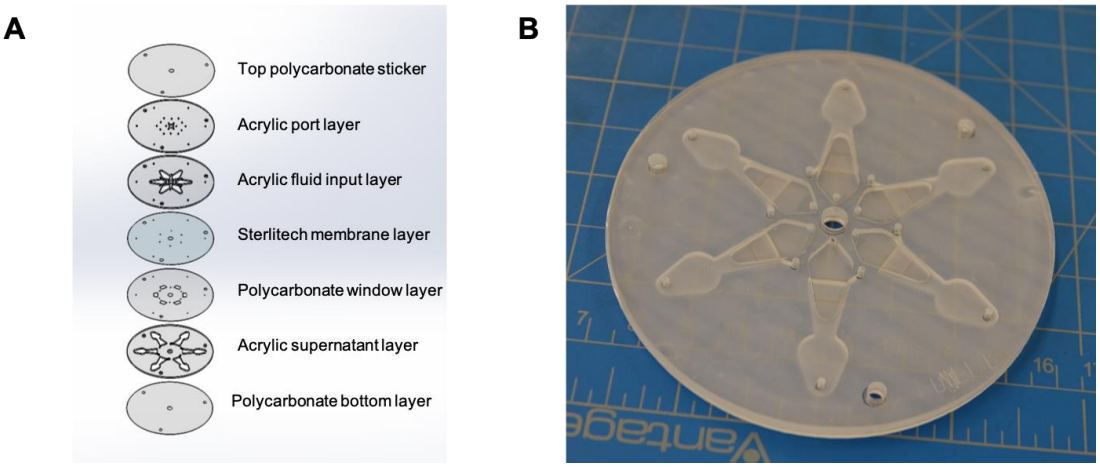


Figure 11: Original design for sample processing automation. A) Design layers consisting of acrylic, Sterlitech membrane, and polycarbonate, cut for alignment; B) A fully-fabricated device.

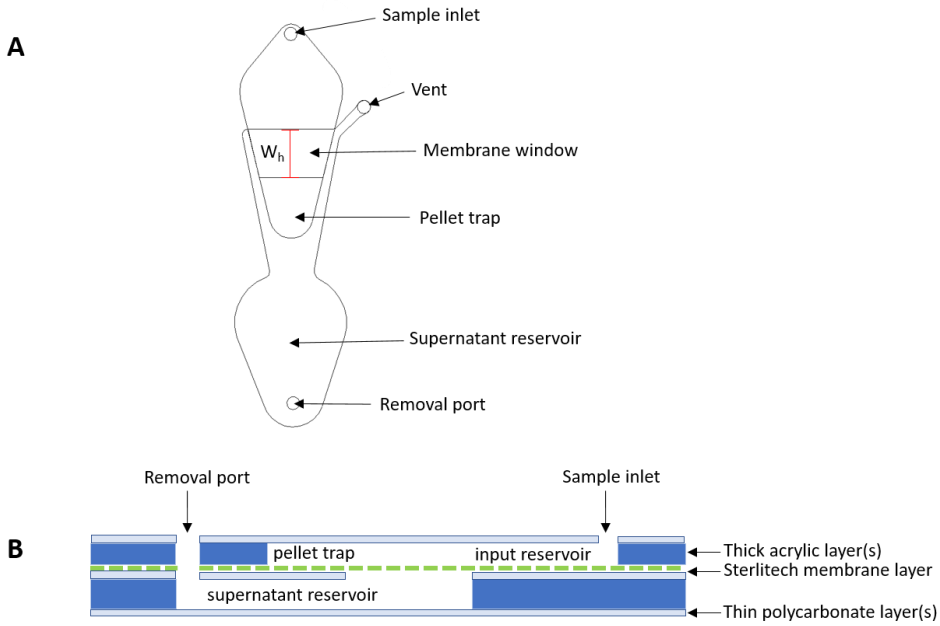


Figure 12: One unit of the multilayered cross-flow filtration device design. A) Design schematic drawn on SolidEdge 2D drafting software, where W_h is window height; B) Cross-sectional schematic of one of the experimental chambers.

To achieve phase separation, the membrane integrated in the device acts as a selective barrier to particles of certain sizes, while the supernatant can flow through to the next chamber. Depending on the application, different membranes can be integrated, or multiple filtration steps can be designed. Figure 13 uses the example of blood/serum separation to display how the platform works for phase separations. Blood is added to the inlet hole, which was cut to the diameter of a standard 1000µL pipette. When the disc is rotated at a given RPM, the centrifugal force moves the blood across the integrated membrane, which allows serum to flow through to the next chamber and prevents blood cells from flowing through.

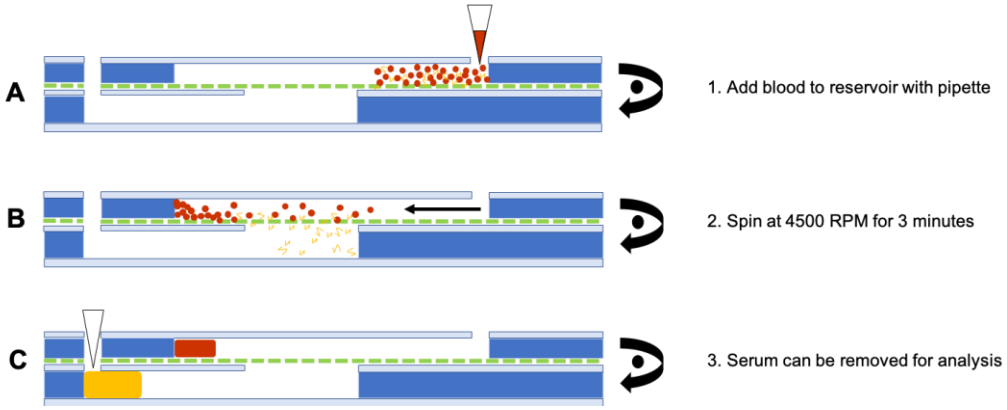


Figure 13: A phase separation (blood/serum) on the centrifugal microfluidic disc via cross-flow filtration. A) Blood is added to the inlet with a pipette; B) The disc spins at 4500RPM for 3 minutes, which provides the centrifugal force to cause tangential flow of fluid; C) Blood cells are prevented from flowing through the membrane window, while serum is able to, separating the two phases.

The membrane layers are made of Sterlitech hydrophilic polycarbonate and polyester membranes with 5µm diameter pores. The polycarbonate membrane is coated with polyvinylpyrrolidone to ensure hydrophilicity and sterilized with gamma irradiation, ethanol, and autoclaved.[59] The polyester membrane is sterilized in the same manner and displays greater resistance to organic solvents, an important feature for biomarker extraction, which utilizes chloroform.[60] The membrane sheets are reported by the manufacturer to be between 3-24µm thick, making them delicate to work with.[59,60] We developed a novel method for membrane integration that consistently produces a surface free from visible indentations or imperfections. The smoother membrane surface increases phase separation reproducibility.

To incorporate the membrane layer, 4-5 drops of distilled water were pushed through a syringe filter onto the base of the jig, which is made of acrylic. Using the jig holes for alignment, the membrane with its protective top layer were placed gently down. Since the

membrane is hydrophilic, it is pulled onto the base of the jig by the water through adhesion. The protective layer was able to be removed without disturbing the membrane underneath. The next acrylic layer of the device was then placed on top of the membrane, glue-side down, bonding the two layers with a pressure-sensitive adhesive. The now-sealed layers were removed from the jig and allowed to dry membrane-side up for 20 to 30 minutes at room temperature. The rest of the acrylic and polycarbonate layers were added in a similar fashion and fully sealed with a hand roller as necessary. Figure 14 displays the fabrication process for membrane integration.

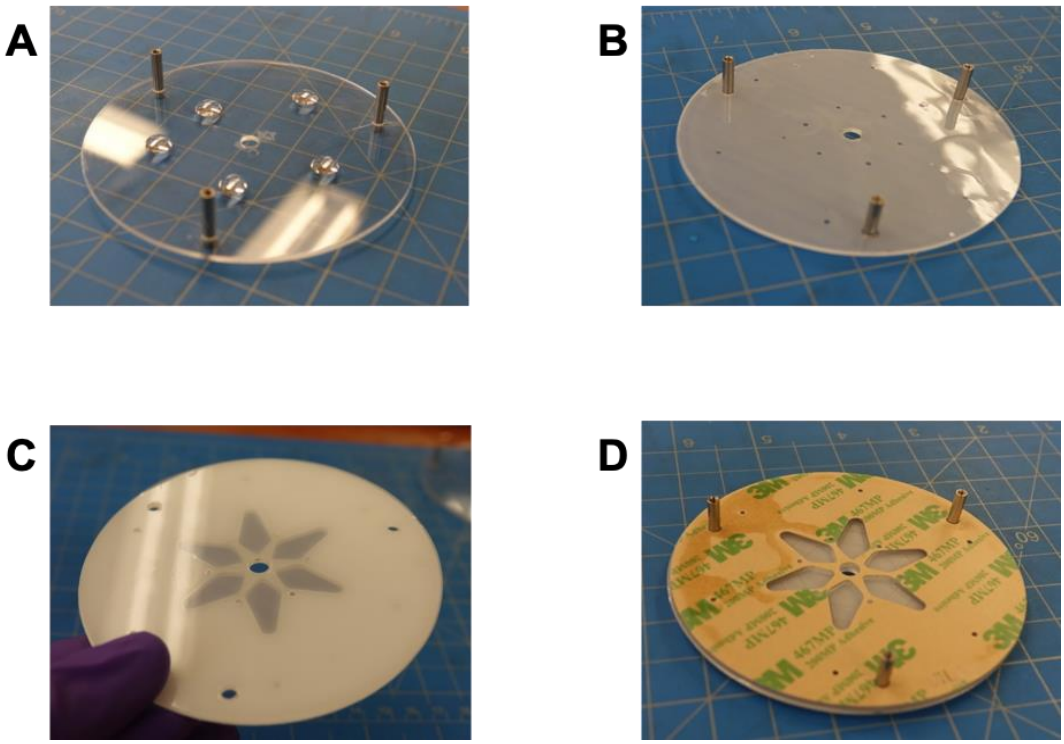


Figure 14: Fabrication process for consistent membrane incorporation; A) The jig was prepared with 5 drops of distilled water pushed through a syringe filter; B) The hydrophilic nature of the membrane caused it to lie flat on the jig; C) The next acrylic layer was bonded with a resulting smooth membrane surface; D) Using the jig for alignment, additional layers were added in a similar fashion.

3.3: Device Functionality Testing

Device functionality was verified in a series of experiments that determined ideal RPM, time, membrane type, and geometric design parameters for phase separations, as summarized in Table 3 and elaborated on in Appendix 2. The "window height" refers to the size of the exposed membrane area (Figure 12A- W_h). In order to test different conditions, the disc was placed on the jig, and 90 µL of whole sheep's blood was pipetted into each inlet hole. The inlets were designed to be the same diameter as the pipette tip in order to create a seal and prevent leakage. A one-sided adhesive layer was aligned on the top of the disc to seal all ports and prevent the escape of fluids during processing. A microcentrifuge (Scilogex) was used to test different RPM and time profiles. A central hole was cut into the microfluidic disc to fit over the rotor, and the cap from the microcentrifuge was securely fastened over the disc.

Table 3: Experimental Parameters to Optimize Device Functionality

Membrane Material	**Membrane Pore Size**	**Time (min)**	**RPM**	**Window height- W_h (mm)**
Polycarbonate & Polyester	2, 3, & 5 µm	2 – 5 Tested in 1 min increments	3500 – 5000 Tested in 500 RPM increments	3.5 – 5.5 Tested in .5mm increments

3.4: Blood/Serum Separation Efficiency

Serum purity, defined as the percentage of cells removed from whole blood, was determined by using the TC20 Automated Cell Counter (BioRad). Cell counts from whole sheep's blood were compared to counts on serum from the microfluidic device, and extraction efficiency was calculated using the following formula:

$$\textbf{Serum purity (\%)} = \frac{(\text{\# of cells in whole blood} - \text{\# of cells in serum})}{(\text{\# of cells in whole blood})} \times 100$$

Cell counts were also performed on commercially-available sheep serum (ThermoFisher) and serum separated from blood by traditional benchtop methods, for comparison.

The automated cell counter has a maximum cell count of up to $1x10^7$ cells/mL[61], so whole blood was diluted at 1:1000 in 1XPBS (ThermoFisher) in order to perform the count (blood usually has 10^8 - 10^9 cells/mL).[62] After dilution, 10μL of the blood in PBS was pipetted onto slides compatible with BioRad's TC20 Automated Cell Counter. Whole blood cell counts were multiplied by 1000 to account for dilution. Serum was not diluted in 1XPBS before pipetting 10μL onto compatible slides.

3.5: Biomarker Retention

3.5.1: Enzyme-Linked Immunosorbent Assays

Since the sample processing platform will eventually be integrated with our amphiphile-detection assays, it was necessary to validate the detection of biomarkers from samples processed on the microfluidic device as compared to those processed via traditional benchtop methods. Because the bacterial biomarkers of interest are amphiphilic in nature, this step also serves to validate device materials and surface chemistry, by ensuring that the biomarkers were retained in the sample and not adsorbed to the device.

Initial biomarker retention experiments were performed via enzyme-linked immunosorbent assays (ELISAs) as a rapid way to confirm the microfluidic materials

chosen were applicable for our sample processing. Lipopolysaccharide from *Salmonella typhimurium* was purchased from List Biological Laboratories, Inc., spiked in 1XPBS and horse serum at 50 µg/mL, and incubated at room temperature for one hour. After incubation, the serum was mixed with chloroform and methanol as per the Bligh & Dyer ratio. Half of the processed serum was used on the microfluidic device for lipid pellet precipitation, while the other half was used to complete benchtop processing. In each case, the serum/chloroform/methanol mixture was spun at 5500RPM for 1 minute (either on the microfluidic device or in a microcentrifuge tube). The pellet was re-suspended in 100 µL 1XPBS (again, either on the microfluidic device or the microcentrifuge tube) and 50 µL of the resulting solutions were pipetted into the wells of a 96-well polystyrene plate (ThermoFisher Scientific) and incubated overnight at 4^{o}C.

After overnight incubation, the plate was washed three times with wash buffer (1XPBS + 0.5% Tween-20, Sigma-Aldrich), 200 µL of blocking buffer (1XPBS + 0.05% Tween-20 + 0.5% BSA) was pipetted into each experimental well, and the plate was incubated at room temperature for 1 hour. The blocking buffer was discarded and 100 µL of anti-*Salmonella* monoclonal primary antibody (PAI-7244, ThermoFisher Scientific) at a dilution of 1:1000 in 1XPBS was added to each well. After another 1 hour incubation, the plate was washed three times using wash buffer. One-hundred µL of anti-rabbit polyclonal secondary antibody (SouthernBiotech) conjugated to horseradish peroxidase (HRP) was added to each well and incubated for 45 minutes. The plate again washed three times, 100 µL of 1-step TMB ELISA substrate (ThermoFisher Scientific) was added to each well, and the color developed within 20-30 minutes. Two molar sulfuric

acid was added to each well to stop the reaction, and absorbance was measured on the Versa max plate reader (Molecular Devices, LLC) at 450nm.

3.5.2: Waveguide-Based Optical Biosensor Assays

Continued experiments were performed on Los Alamos National Laboratory's waveguide-based optical biosensor, which has been previously shown to detect biomarkers from Gram-negative, -positive, and -indeterminate bacteria at low, physiologically-relevant concentrations.[7,10,11,38] Planar optical waveguides with a silicon oxynitride ($SiON_x$) film were prepared by nGimat (Atlanta, GA). Validation was performed using a model biomarker, lipoarabinomannan (LAM), the virulence factor associated with *Mycobacterium tuberculosis*. Stock LAM (19kDa) was procured through BEI Resources. Two LAM primary antibodies (171 and 24) were purchased from FIND and prepared in a 15nM cocktail in wash buffer (.5% BSA/1XPBS) for use on the waveguide. Dioloeyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-sn-glycero-3-phasphoethanolamine-N-(cap biotinyl) (cap-biotinyl-PE) were purchased from Avanti Polar Lipids, Inc. Whole sheep's blood in Alsever's anti-coagulant solution (2.05% dextrose, 0.8% sodium citrate, 0.0055% citric acid, 0.42% sodium chloride) was purchased from ThermoFisher Scientific.

Waveguide surfaces were cleaned and functionalized with a lipid bilayer as described in previously-published works.[35,37,38] To do so, waveguides and coverslips were cleaned by bath sonication for 5 minutes each in chloroform, ethanol, and distilled water. They were then dried with argon gas and cleaned by UV-ozone (UVOCS Inc.) for 40 minutes. Flow cells were assembled by bonding a silicone gasket in between clean

waveguides and coverslips. The lipid bilayer was prepared from 5mM stock DOPC and cap biotinyl-PE (both in $CHCl_3$). Sixty µL 5mM DOPC and 0.6 µL cap biotinyl-PE were added to a glass test tube using a syringe needle (cleaned with chloroform/ethanol 3 times each). Chloroform was evaporated under argon gas, and the lipids were rehydrated in 600 µL 1XPBS for 30 minutes on a shaker plate. Lipids were then exposed to ten freeze-thaw cycles by freezing in liquid nitrogen and thawing in warm water. The fluid was then sonicated with a probe tip sonicator (Branson, 50% duty cycle) for 6 minutes to ensure vesicle uniformity. Seventy µL of prepared lipids were pipetted into the assembled flow cell and incubated overnight at 4^{o}C to encourage bilayer stabilization. Whole sheep's blood was spiked with LAM to a concentration of .05 µM and incubated overnight at 4^{o}C.

Each assay began by clipping the flow cell onto a custom holder and aligning the laser for coupling of light. Two-hundred µL wash buffer (.5% BSA/1XPBS) was injected through the flow cell and the background signal was measured. Two-hundred µL of 15nM FIND antibody cocktail was injected and incubated at room temperature for 20 minutes. The flow cell was washed, and the non-specific signal was measured. Whole sheep's blood containing LAM was separated into serum/blood using the microfluidic device or by traditional methods, depending on the assay. For the microfluidic device, 90 µL serum was pipetted into each inlet hole, and the disc was spun at 4500 RPM for 3 minutes. For traditional methods, 500 µL whole blood was pipetted into a microcentrifuge tube and spun at 4500 RPM for 3 minutes. The serum from each method of separation was used for cell counting, and sample processing was finished by benchtop methods for the first round of experiments. One-hundred and twenty µL of serum was

mixed by pipetting with 150 µL chloroform and 300 µL methanol in low-retention microcentrifuge tubes. The mixture was spun at 5500 RPM for 1 minute, and the supernatant was discarded. The pellet containing amphiphiles of interest was re-suspended in 120 µL of 1XPBS, which was injected into the flow cell of the waveguide and incubated for 45 minutes at room temperature. After incubation, the flow cell was washed, and the specific signal was measured. Biomarker retention experiments were performed in the same manner for each major step of the sample processing procedure performed on the microfluidic chip and compared to the benchtop sample processing method.

For the second round of experiments, the second major step of sample processing (amphiphile extraction), was performed on the microfluidic device. Whole sheep's blood containing a concentration of .05 µM LAM was incubated overnight at 4^{o}C to use on the microfluidic chip and for traditional methods. Blood/serum separation was performed in the same way for microfluidic and traditional methods (4500RPM for 3 minutes, as above). Serum from the microfluidic chip was removed with a pipette and mixed with the correct ratio of chloroform:methanol in a microcentrifuge tube. The mixture was then pipetted onto a second chip designed for amphiphile extraction, which was spun at 5500 RPM for 1 minute to obtain the lipid pellet. The pellets were re-suspended in 100 µL of 1XPBS each and removed from the chip for analysis on the waveguide. Serum from traditionally-separated blood was removed from the microcentrifuge tube with a pipette, and sample processing was completed via the benchtop (modified Bligh & Dyer) method. Amphiphiles were also re-suspended in 150 µL 1XPBS and used for comparative

analysis on our waveguide-based optical biosensor. A schematic of the experimental conditions is presented in Figure 15.

After each waveguide experiment, relative signal intensity was calculated by factoring out the background and non-specific binding signals. The following formula was used:

$$\textbf{Signal intensity} = \frac{\text{(specific signal – background signal)}}{\text{non-specific signal – background signal}}$$

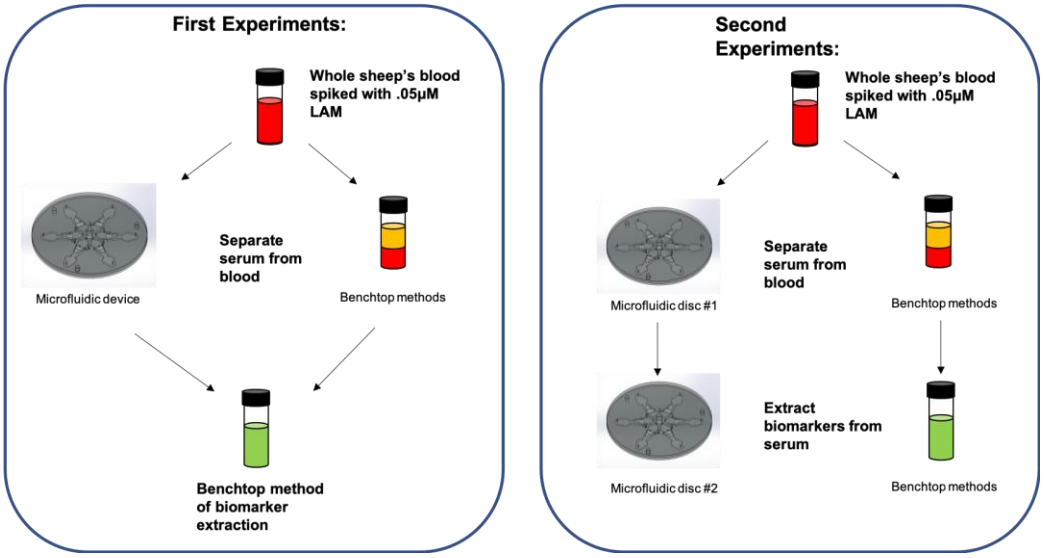


Figure 15: Experimental set up for two conditions of sample processing. In the first round of experiments (left), serum was separated from blood via benchtop methods and on a microfluidic chip. Serum was removed from the tube or dics and sample processing was finished on the benchtop. In the second round of experiments, serum was again separated from blood via benchtop methods and on a microfluidic chip. Lipid pellet separation then occurred on a second microfluidic chip or by traditional benchtop methods. All biomarker solutions were detected on LANL's waveguide-based optical biosensor.

Chapter 4: Results and Discussion

4.1: Device Functionality

Successful separation of serum from blood was achieved by spinning the disc at 4500 RPM for 3 minutes with a window height of 4.0mm. A two-step filtration design was determined to be more effective at separating serum from blood when compared to a one-step method (Figure 16**)**. An amphiphilic pellet was successfully obtained by spinning the disc at 5500 RPM for 1 minute, after the addition of serum:chloroform:methanol in a 0.8:1:2 volumetric ratio. The pellet was re-suspended in 1XPBS on the chip, and then removed for analysis. A one-step separation method was sufficient for amphiphile extraction.

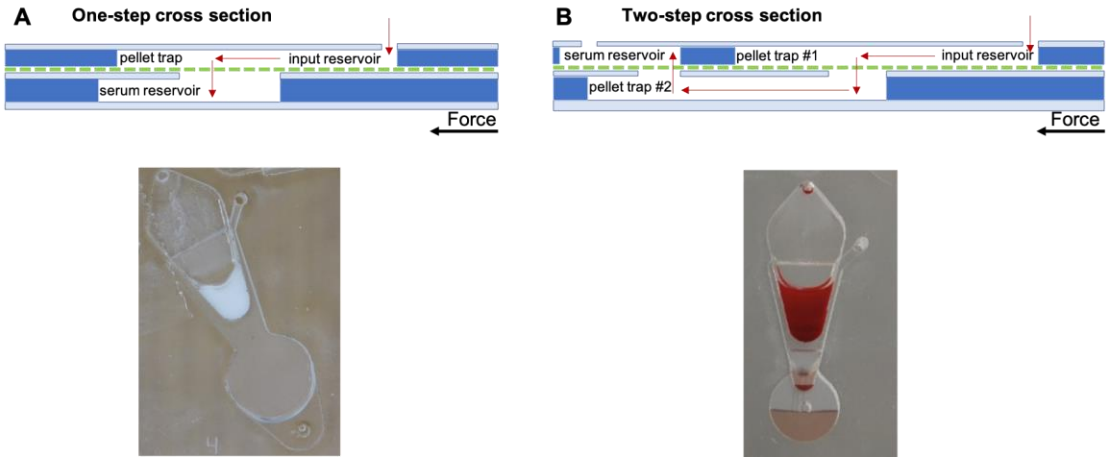


Figure 16: Schematics of one-step (A) vs. two-step (B) cross-flow filtration, and photos of phase separations; A) The one-step filtration method was suitable for amphiphilic pellet separation from chloroform/methanol supernatant; B) The two-step filtration method was successful at separating serum from blood.

4.2: Serum Purity

The first step of our sample processing procedure calls for serum to be separated from blood. When compared to traditional centrifugation and to commercially-available serum, the serum processed on our microfluidic platform had a significantly lower cell count ($p < 0.0179$ for microfluidics vs. benchtop; $p < 0.0128$ for microfluidics vs. commercial serum, Figure 17). Serum purity was calculated for benchtop methods of separation and for microfluidic separation. Both methods yielded a high percentage of cells removed from whole blood, greater than 99.99%, as shown in Table 4.

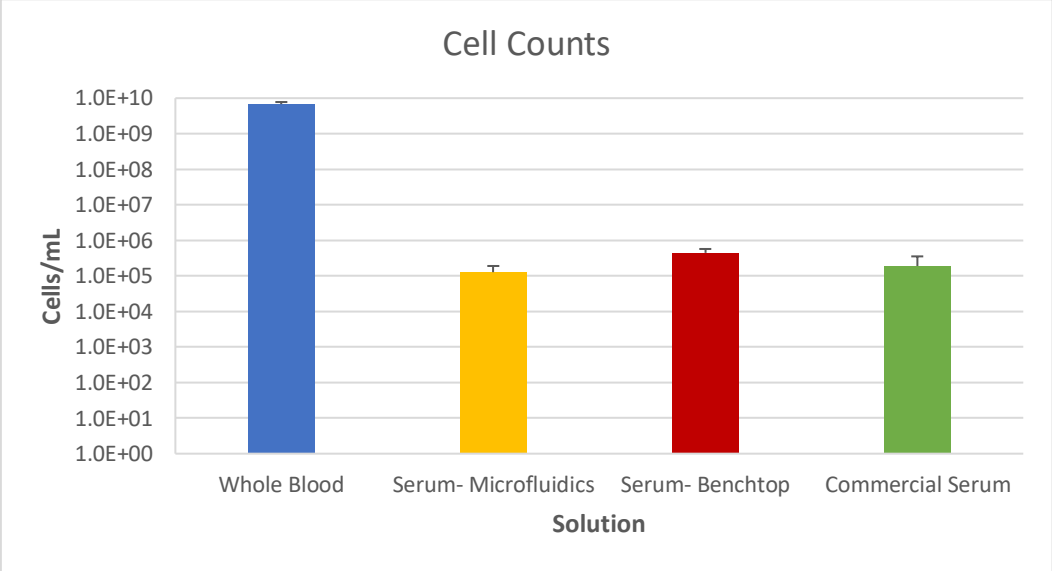


Figure 17: Cell count averages (n=3) on whole sheep's blood ($6.8x10^9$ cells/mL), serum processed on the microfluidic device ($1.27x10^5$ cells/mL), serum processed by benchtop methods ($4.45x10^5$ cells/mL), and commercially-available serum ($1.27x10^5$ cells/mL). All solutions were sourced from ThermoFisher.

Table 4: Serum Purity

Sample	Serum Purity (%)
Serum- Microfluidics	99.99813
Serum- Benchtop	99.99611

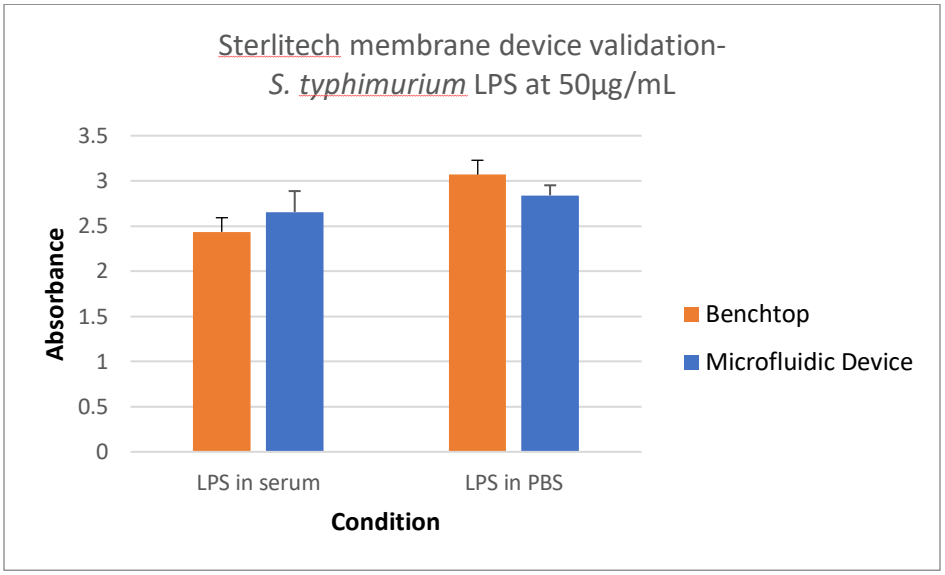
We determined that our device is suitable for blood/serum separation, a common first step of many sample processing methods, including amphiphile extraction from whole blood. The serum processed on the microfluidic device had a lower cell count than serum processed on the benchtop and commercially-available serum, as well as higher serum purity.

4.3: Biomarker Retention

4.3.1: Enzyme-Linked Immunosorbent Assays

The preliminary biomarker retention experiments, performed via ELISA, indicated that the device materials and surface chemistry are suitable for our application. When compared to benchtop methods of biomarker extraction, serum and 1XPBS containing 50 μg/mL of *Salmonella typhimurium* LPS processed on the microfluidic device had a similar or higher absorbance. This implies that the device materials including acrylic, polycarbonate, and the Sterlitech membrane did not adsorb a biomarker of interest, LPS. This information helped to guide future designs using the same materials. This experiment was performed on two different days using two different microfluidic chips. Interassay variability was low for both benchtop and microfluidic methods, indicating that both methods produce consistent signals. Figure 18 shows the compiled absorptions for the microfluidic device versus benchtop method performed on two different days, as well as interassay variability. The higher the absorption, as read on a plate reader at 450nm, the greater amount of analyte present in the sample.

A



B

Interassay Variability (%)	
Microfluidics	**Benchtop**
LPS in serum – 8.76%	LPS in serum – 6.42%
LPS in PBS – 3.94%	LPS in PBS – 5.16%

Figure 18: A) Absorbance at 450nm on LPS spiked in serum and 1XPBS on the microfluidic device vs. benchtop methods. Higher absorbance indicates higher concentration of biomarker retained in the serum sample; B) Interassay variability for conditions tested.

4.3.2: Waveguide-Based Optical Biosensor Assays

Biomarker retention was further validated for each step of the sample processing procedure performed on the microfluidic chip and compared to results from the benchtop method as measured on LANL's waveguide-based optical biosensor.

After serum from the microfluidic device was determined to be purer than serum from the benchtop or commercially-available serum (Figure 17), and device materials were considered suitable (Figure 18), we further validated the microfluidic chip by testing for biomarker retention in the sample under different conditions.

Lipoarabinomannan (LAM) was spiked in whole blood at .05µM before separating serum from blood on the microfluidic device. The same blood was used for benchtop blood/serum separation in microcentrifuge tubes. After separation, the serum was processed by benchtop methods and analyzed on our waveguide-based optical biosensor, as described in the Methods Section. There was no statistically significant difference between LAM levels in serum processed on the microfluidic device vs. by benchtop methods ($p < .9392$, Figure 19), indicating that the device's materials and surface chemistry are suitable for our application.

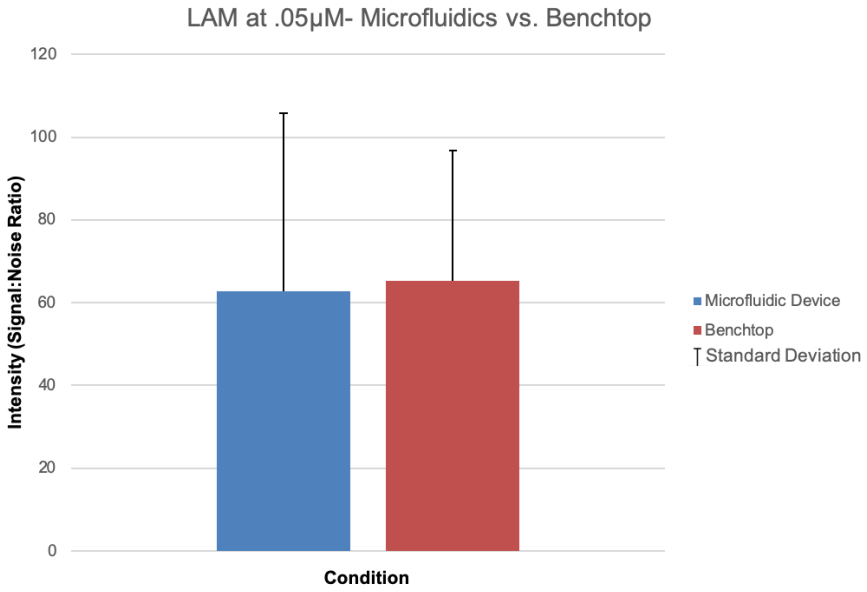


Figure 19: Signal intensity (n=3) on whole sheep's blood separated on the microfluidic device vs. benchtop methods. Higher intensity indicates higher concentration of biomarker retained in the serum sample.

To validate the second step of sample processing, amphiphile extraction, LAM was again spiked in whole sheep's blood at a concentration of .05µM for use on the microfluidic device and for benchtop extraction. Blood/serum separation was performed

on both the chip and by traditional methods. Serum from the chip was removed and a mixture of chloroform/methanol was added to the serum. The mixture was then pipetted onto a second chip designed for amphiphile pellet precipitation, and the disc was spun at 5500RPM for 1 minute. While the microfluidic chip was able to successfully separate the lipid pellet from the solvent mixture (Figure 16A), it was difficult to re-suspend the pellet on-chip with 1XPBS. For this reason, waveguide-based biosensor assays that accurately compare the benchtop methods to the microfluidic chip were not able to be performed. The microfluidic chip was able to function as predicted, but a new design that incorporates an easier way to re-suspend the pellet is needed.

Chapter 5: Conclusions and Future Work

The goal of this research was to design and demonstrate proof of concept for the automation of blood sample processing. In the benchtop method of sample processing, amphiphilic biomarkers of interest are extracted from whole blood for detection of bacterial infections via membrane insertion assays. For the automation of this process, two specific goals were established: 1) automate and validate blood/serum separation on a centrifugal microfluidic disc; and 2) automate and validate amphiphilic biomarker extraction from serum for use with our membrane insertion assays.

In this work, a semi-automated sample processing platform was designed and shown to extract amphiphilic biomarkers of interest from whole blood. The platform requires only 90μL of whole blood to account for potentially dehydrated patients. It is simple to manufacture, modular, disposable, pump-free, and does not require the dilution of blood. The platform is a promising design for complete automation of sample processing at the point-of-care. The microfluidic device also meets certain criteria for deployable applications: it is disposable, inexpensive, user-friendly, compact, and requires low amounts of power (and could potentially be solar-powered).[46,47]

The first aim of this work was to separate serum from blood on a microfluidic system. An original design based on the principles of cross-flow filtration was developed to achieve this goal. Experimental parameters including RPM, time, geometric designs, and membrane materials were tested (Table 3). Successful blood/serum separation was achieved by spinning the disc at 4500RPM for 3 minutes with a window height (Figure 12A) of 4mm using Sterlitech's 5μm pore polycarbonate membrane. A two-step filtration system was superior in performance when compared to a one-step filtration (Figure 16).

In terms of serum purity, serum from the cross-flow filtration platform produced serum with a lower cell count than serum separated traditionally and commercially-available serum (Figure 17). Biomarker retention in the blood sample processed on the microfluidic device was performed first by enzyme-linked immunoassays (ELISAs) and showed a similar signal to serum processed via the benchtop method. This step served to validate the materials and surface chemistry of the device. Secondary biomarker retention experiments were performed on the waveguide instrument, and again the signal was similar between serum samples processed on the microfluidic device versus benchtop methods (Figure 19).

The second aim of this work was to extract amphiphilic biomarkers of interest from serum on a microfluidic system. The same cross-flow filtration design that was developed for blood/serum separation was used for biomarker extraction, since both steps involve a phase separation. Successful amphiphilic pellet precipitation and separation from supernatant was achieved by spinning the disc at 5500RPM for 1 minute with a window height of 4mm using Sterlitech's 5μm pore polyester membrane. A one-step filtration process was sufficient for this step. Validation still needs to be performed on LANL's waveguide-based optical biosensor, which is more sensitive than ELISAs, since the pellet was unable to be re-suspended and removed from the microfluidic chip.[8]

Future work must be done on this platform in order to move towards a fully automated system. Having two chips to perform two steps increases the possibility of mistakes being made, so we have been working on designs that will incorporate both major steps of sample processing onto one microfluidic chip. On our current chip for amphiphile extraction, the pellet was difficult to re-suspend and remove for analysis.

Better mixing schemes and aspiration techniques will need to be incorporated and tested in future designs. Another future direction of this work will be to investigate reagent storage on-chip in order to decrease user interaction with chloroform and methanol. This could possibly be achieved by embedding blister packs into specific areas of the microfluidic chip. A long-term goal of this work is to develop a fully integrated microfluidic sample processing platform with our deployable waveguide-based optical biosensor. A fully integrated system would eliminate any need for user intervention at the point-of-care.

Certain aspects of the cross-flow filtration microfluidic platform make it easily adaptable for other phase separations or sample processing methods. Throughout the design process, a straightforward fabrication method that results in consistent device layers was developed. The design itself can be modified to include one, two, three, or more filtration steps, depending on the needs of the user. Chambers and reservoirs can be adjusted to hold different volumes of liquid. In this way, our system can be described as modular.

The separation of serum from blood is a common laboratory procedure that needs to be performed. Our platform offers users a safer way to separate serum from blood, especially when compared to using micro-hematocrit tubes, which are typically made of glass and have the potential to shatter. A standard pipette can be used to remove serum from our chip, making it easier to remove than from a hematocrit tube. This is useful for any protocol that requires further processing of serum, including standard laboratory blood tests such as total serum cholesterol, HDL, LDL, and triglycerides.

In summary, the microfluidic platform presented in this research is a promising design for automated bacterial biomarker extraction from blood, and could be applied to other sample processing procedures. The microfluidics-based cross-flow filtration platform provides the framework for future studies that could achieve a fully automated system of extraction. This would be a safe, user-friendly, and rapid approach for removal of bacterial biomarkers from patient samples at the point-of-care, which in turn would provide invaluable information to guide treatment decisions.

References

1. Stromberg, L. R., Mendez, H. M. & Mukundan, H. Detection methods for lipopolysaccharides: past and present. *Intech* 141–168 (2012). doi:10.5772/51895

2. Kubicek-Sutherland, J. Z., Vu, D. M., Mendez, H. M., Jakhar, S. & Mukundan, H. Detection of lipid and amphiphilic biomarkers for disease diagnostics. *Biosensors* **7**, 1–25 (2017).

3. Caliendo, A. M. *et al.* Better Tests, Better Care: Improved Diagnostics for Infectious Diseases. *Clin. Infect. Dis.* **57**, S139–S170 (2013).

4. Kozel, T. R. & Burnham-marusich, A. R. Point-of-Care Testing for Infectious Diseases: Past, Present, and Future. *J. Clin. Microbiol.* **55**, 2313–2320 (2017).

5. Boyles, T. H. & Wasserman, S. Diagnosis of bacterial infection. *South African Med. J.* **105**, 419–421 (2015).

6. Mohammadi, S. *et al.* Sensitive, Real-time and Non-Intrusive Detection of Concentration and Growth of Pathogenic Bacteria using Microfluidic-Microwave Ring Resonator Biosensor. *Sci. Rep.* **8**, 1–10 (2018).

7. Noormohamed, A. *et al.* Detection of lipopolysaccharides in serum using a waveguide-based optical biosensor. in (ed. Coté, G. L.) **10072**, 100720A (International Society for Optics and Photonics, 2017).

8. Mukundan, H. *et al.* Waveguide-based biosensors for pathogen detection. *Sensors* **9**, 5783–5809 (2009).

9. Beveridge, T. J. Structure of Gram-negative cell walls and their derived mebrane vesicles. *J. Bacteriol.* **181**, 4725–4733 (1999).

10. Mukundan, H. *et al.* Rapid detection of Mycobacterium tuberculosis biomarkers in a sandwich immunoassay format using a waveguide-based optical biosensor. *Tuberculosis* **92**, 407–416 (2012).

11. Stromberg, L. R. *et al.* Membrane insertion for the detection of lipopolysaccharides: Exploring the dynamics of amphiphile-in-lipid assays. *PLoS One* **11**, 1–21 (2016).

12. Park, B. S. & Lee, J. O. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp. Mol. Med.* **45**, e66-9 (2013).

13. Erridge, C., Bennett-Guerrero, E. & Poxton, I. R. Structure and function of lipopolysaccharides. *Microbes Infect.* **4**, 837–851 (2002).

14. Feingold, K. R. & Grunfeld, C. The role of HDL in innate immunity. *J. Lipid Res.* **52**, 1–3 (2011).

15. Lu, Y. C., Yeh, W. C. & Ohashi, P. S. LPS/TLR4 signal transduction pathway. *Cytokine* **42**, 145–151 (2008).

16. Murray, P. R. & Masur, H. Current Approaches to the Diagnosis of Bacterial and Fungal Bloodstream Infections for the ICU. *Crit. Care Med.* **40**, 3277–3282 (2012).

17. Lagier, J. C. *et al.* Current and past strategies for bacterial culture in clinical microbiology. *Clin. Microbiol. Rev.* **28**, 208–236 (2015).

18. Pfyffer, G. E. & Wittwer, F. Incubation time of mycobacterial cultures: How long is long enough to issue a final negative report to the clinician? *J. Clin. Microbiol.* **50**, 4188–4189 (2012).

19. Ogwang, S. *et al.* Incubation time of Mycobacterium tuberculosis complex sputum cultures in BACTEC MGIT 960: 4weeks of negative culture is enough for physicians to consider alternative diagnoses. *Diagn. Microbiol. Infect. Dis.* **83**, 162–4 (2015).

20. Holtz, T. H. *et al.* Time to Sputum Culture Conversion in Multidrug-Resistant Tuberculosis: Predictors and Relationship to Treatment Outcome. *Ann. Intern. Med.* **144**, 650 (2006).

21. Stewart, E. J. Growing unculturable bacteria. *J. Bacteriol.* **194**, 4151–4160 (2012).

22. Davies, J. & Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* **74**, 417–433 (2010).

23. Eades, C., Hughes, S., Heard, K. & Moore, L. Antimicrobial therapies for Gram-

positive infections. *Diagn. Microbiol. Infect. Dis.* **77**, 273–277 (2013).

24. Calandra, T. & Cometta, A. Antibiotic therapy for gram-negative bacteremia. *Infect. Dis. Clin. North Am.* **5**, 817–34 (1991).

25. Salton, M. R. The relationship between the nature of the cell wall and the Gram stain. *J. Gen. Microbiol.* **30**, 223–235 (1963).

26. Beveridge, T. J. Mechanism of gram variability in select bacteria. *J. Bacteriol.* **172**, 1609–1620 (1990).

27. Engvall, E. & Perlmann, P. Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109**, 129–35 (1972).

28. The enzyme-linked immunosorbent assay (ELISA). *Bull. World Health Organ.* **54**, 129–39 (1976).

29. Reither, K. *et al.* Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis. *BMC Infect. Dis.* **9**, 141 (2009).

30. Martínez-Sernández, V., Orbegozo-Medina, R. A., Romarís, F., Paniagua, E. & Ubeira, F. M. Usefulness of ELISA Methods for Assessing LPS Interactions with Proteins and Peptides. *PLoS One* **11**, e0156530 (2016).

31. White, B. A. *PCR Protocols*. **15**, (Humana Press, 1993).

32. Lorenz, T. C. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *J. Vis. Exp.* 1–15 (2012). doi:10.3791/3998

33. Garibyan, L. & Avashia, N. Polymerase chain reaction. *J. Invest. Dermatol.* **133**, 1–4 (2013).

34. Taitt, C. R., Anderson, G. P. & Ligler, F. S. Evanescent wave fluorescence biosensors: Advances of the last decade. *Biosens. Bioelectron.* **76**, 103–112 (2016).

35. Mukundan, H. *et al.* Optimizing a waveguide-based sandwich immunoassay for

tumor biomarkers: Evaluating fluorescent labels and functional surfaces. *Bioconjug. Chem.* **20**, 222–230 (2009).

36. Sakamuri, R. M. *et al.* Detection of stealthy small amphiphilic biomarkers. *J. Microbiol. Methods* **103**, 112–117 (2014).

37. Mukundan, H. *et al.* Quantitative multiplex detection of pathogen biomarkers on multichannel waveguides. *Anal. Chem.* **82**, 136–144 (2010).

38. Kubicek-sutherland, J. Z., Hengartner, A. C. & Mukundan, H. Membrane Insertion for Direct Detection of Lipoteichoic Acid. **42**, 101–105 (2017).

39. Bligh, E. G. & Dyer, W. J. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Can. J. Biochem. Physiol.* **37**, 911–917 (1959).

40. W.J.Dyer, E. G. B. and. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917 (1959).

41. Gorkin, R. *et al.* Centrifugal microfluidics for biomedical applications. *Lab Chip* **10**, 1758 (2010).

42. Tang, M., Wang, G., Kong, S. K. & Ho, H. P. A review of biomedical centrifugal microfluidic platforms. *Micromachines* **7**, (2016).

43. Michael, I. J., Kim, T. H., Sunkara, V. & Cho, Y. K. Challenges and opportunities of centrifugal microfluidics for extreme point-of-care testing. *Micromachines* **7**, 1–14 (2016).

44. Strohmeier, O. *et al.* Centrifugal microfluidic platforms: advanced unit operations and applications. *Chem. Soc. Rev.* **44**, 6187–6229 (2015).

45. Gilmore, J., Islam, M. & Martinez-Duarte, R. Challenges in the use of compact disc-based centrifugal microfluidics for healthcare diagnostics at the extreme point of care. *Micromachines* **7**, (2016).

46. Lee, W. G., Kim, Y.-G., Chuang, B. G., Demirci, U. & Khademhosseini, A. Nano/microfluidics for diagnosis of infectious diseases in developing countries.

Adv. Drug Deliv. **62**, 449–457 (2010).

47. Peeling, R. W. & Mabey, D. Point-of-care tests for diagnosing infections in the developing world. *Clin. Microbiol. Infect.* **16**, 1062–1069 (2010).

48. IBRD/World Bank. *Making Services Work for Poor People*. (2003). doi:10.1596/0-8213-5468-X

49. Ayukekbong, J. A., Ntemgwa, M. & Atabe, A. N. The threat of antimicrobial resistance in developing countries: Causes and control strategies. *Antimicrob. Resist. Infect. Control* **6**, 1–8 (2017).

50. Tacconelli, E. *et al.* Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* **18**, 318–327 (2018).

51. Hin, S. *et al.* Membrane-based sample inlet for centrifugal microfluidic cartridges. *Microelectron. Eng.* **187**–**188**, 78–83 (2018).

52. Amasia, M. & Madou, M. J. Large-volume centrifugal microfluidic device for blood plasma separation. *Bioanalysis* **2**, 1701–10 (2010).

53. Corporation, P. Membrane Asymmetric polysulfone Vivid ™ Plasma Separation Membrane.

54. V, E. S. B. Theoretical descriptions of membrane filtration of colloids and fine particles: An assessment and review. **56**, 141–200 (1995).

55. Iritani, E., Katagiri, N., Takenaka, T. & Yamashita, Y. Membrane pore blocking during cake formation in constant pressure and constant flux dead-end microfiltration of very dilute colloids. *Chem. Eng. Sci.* **122**, 465–473 (2015).

56. Iritani, E., Mukai, Y., Tanaka, Y. & Murase, T. Flux decline behavior in dead-end microfiltration of protein solutions. *J. Memb. Sci.* **103**, 181–191 (1995).

57. Porex Filtration Group. Cross Flow Microfiltration, Ultrafiltration, & Cross Filtration. Available at: http://www.porexfiltration.com/learning-center/technology/what-is-cross-flow-filtration/. (Accessed: 21st January 2019)

58. A., S. & Duc, N. Organic–Inorganic Mesoporous Silica Nanotube Hybrid Anodic Alumina Membranes for Ultrafine Filtration of Noble Metal Nanoparticles. *Noble Met.* (2012). doi:10.5772/33768

59. Sterlitech, C. Polycarbonate Track Etch (Pcte) Membrane Filters Data Sheet. 50

60. Sterlitech, C. Polyester membrane filters data sheet.

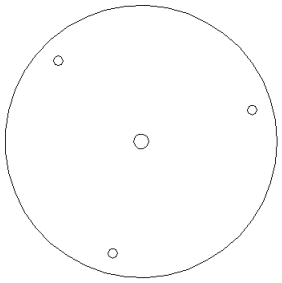
61. BIO-RAD. TC20 Automated Cell Counter. 1 (2008). doi:10.1016/j.solener.2004.06.020

62. Alomari, Y. M., Sheikh Abdullah, S. N. H., Zaharatul Azma, R. & Omar, K. Automatic detection and quantification of WBCs and RBCs using iterative structured circle detection algorithm. *Comput. Math. Methods Med.* **2014**, (2014).

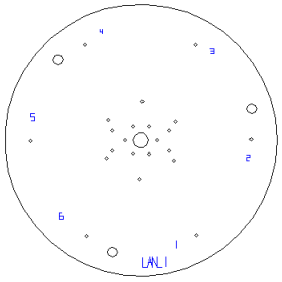
Appendix

Appendix 1: Microfluidic Device Layers (SolidEdge 2D)

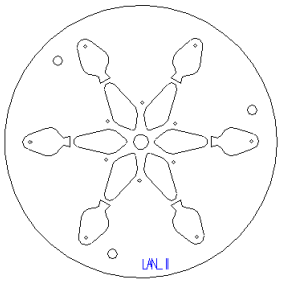
Layers were drawn on SolidEdge 2D drafting software. Materials and laser specifications were included for each layer to ensure consistent device layers.



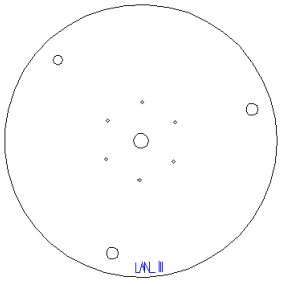
stickers
.125 PC
one-sided glue



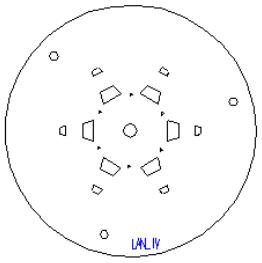
Top layer
1.5 mm acrylic
no glue
1 pass @80% power



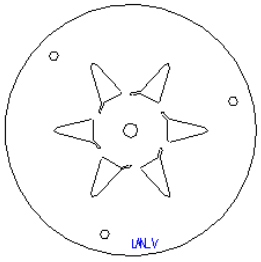
Blood layer
1.5 mm acrylic
both side glue
2 passes @ 70% power



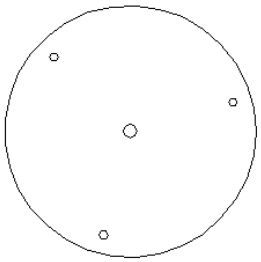
Membrane layer
2 micron sterlitech- polycarbonate
10%- one pass



Plastic blood layer
0.125mm PC
top sided glue
1 pass @ 30%



Serum Layer
1.5 mm acrylic
both side glue
2 passes @ 70%



bottom layer
0.125 mm PC
1 pass @ 28%

Appendix 2: Experimental Parameters

We took a systematic approach for optimizing experimental parameters. For example, when comparing window height and time, all experimental combinations were tested three times and photographs were taken. Cell counts were performed on each design and averaged in order to determine the best combination of parameters.

	Time (minutes)				
Window Height (mm)		2	3	4	5
	3.5				
	4.0				
	4.5				
	5.0				

	Time (minutes)			
Window Height (mm)	2	3	4	5
3.5	1.74×10^7 cells/mL	2.91×10^6 cells/mL	Too high to count	Too high to count
4.0	1.47×10^7 cells/mL	3.24×10^6 cells/mL	3.57×10^6 cells/mL	2.05×10^6 cells/mL
4.5	Too high to count	Too high to count	8.74×10^6 cells/mL	1.86×10^7 cells/mL
5.0	Too high to count	Too high to count	Too high to count	Too high to count