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Low Cost Malaria Detection

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A Major Qualifying Project Report submitted to the faculty of WORCESTER POLYTECHNIC INSTITUTE in partial fulfillment of the requirements for the degree of Bachelor of Science

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Abstract

Malaria is a parasitic disease that infects over 200 million people and kills around half a million people per year. Lack of proper diagnosis is a major barrier to effective treatment and maintenance of the disease. Malaria causes many nonspecific symptoms including fever, nausea, and fatigue making clinical diagnosis difficult. Current diagnostic tools are often too expensive to be accessible to many endemic regions or are inaccurate in correctly diagnosing a patient. The goal of this project was to create a malarial diagnostic tool that was to be used in low resource regions with a high prevalence of malaria. A microfluidics device that utilizes magnetic cell separation to identify the presence of malaria infected cells was created. The device was designed to be accurate, low cost, durable, easy to use, fast, and minimally invasive.

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Chapter 1: Introduction

Malaria is a protozoan blood based disease transmitted to humans through mosquito bites caused by the plasmodium parasite family. Infected individuals present a multitude of nonspecific symptoms, including fever, nausea, and fatigue, making clinical diagnosis based on symptoms difficult and inaccurate. Under-diagnosing of malaria results in poor patient management, causing higher rates of mortality and morbidity due to lack of treatment allowing the severity of the disease to progress and spread to other individuals. Over-diagnosing of patients occurs in areas of high infection rates, rates of over 30%, where diagnosis is often based on clinical symptoms and this can lead to patients with other infections or diseases being diagnosed with malaria as a safety measure [1]. This use of treatment on uninfected, misdiagnosed patients is an economic burden, limits treatment access due to low resources, and increases the rate of resistance development in highly endemic locations. Malaria is prevalent in many tropical and subtropical areas where the climate is conducive for mosquito survival allowing disease transmission to occur year-round. There is need for an accurate diagnosis technique that can differentiate between malarial infections and other tropical diseases that present with similar symptoms, such as fever and nausea, to ensure proper treatment and patient management. While there are many international efforts to reduce the rate of malarial infection, the infection rate is still high. In 2015, there were 214 million cases of malaria resulting in 438,000 deaths, mostly children and the elderly, from malarial infections [2]. Accurate diagnosis techniques can reduce the mortality and incidence of severe malaria by allowing for a faster and effective distribution of treatments.

Many areas where there is a high infection rate of malaria correspond to low resource areas without access to sophisticated technologies for diagnostics. The gold standard for malaria treatment is microscopy, in which a differential Giemsa stain of a blood smear is observed and analyzed under a microscope. The stain allows for visual differentiation of parasites in blood to be used as a highly accurate diagnosis method [3]. This method requires an individual well trained in identifying parasites and access to a light microscope, which costs at least several hundred dollars. Each test slip costs up to two dollars and several tests are sometimes needed per patient to confirm the diagnosis. This method can take anywhere from thirty minutes to several hours depending on how many slides need to be observed to confirm diagnosis. Clinics that do not have a microscope will send off the blood samples to another lab to get a definitive diagnosis, which can take days. This has led to the necessity for alternative diagnostic tests such as rapid diagnostic tests (RDTs) due to their portability, ease of use, and lower initial cost. Most RDTs are dependent on a two-stage antigen-antibody binding to detect malarial antigens in a patient's blood. Color labeled antibodies complementary malaria antigens are added to the blood sample and then run across a strip containing fixed antibodies that present a pigmented strip if the antigen-antibody complex is formed. Presence of this strip is indicative of malaria antigens in a small blood sample and is commonly used for diagnosis. Degradation of the antibodies happens rapidly in tropical environments, therefore limiting the effectiveness of these tests to a short period of time when the antibodies are stable. RDTs become less reliable and can even lose viability as this degradation occurs. These deficiencies in current diagnostic technology reveal areas where improvements could be made to create a

device or technique that would be more accurate and cost effective in areas with high malaria infection rates.

Based upon the discovered needs and restrictions, the objective of this project was to design, test and manufacture a highly accurate and reliable detection device for malaria at a price range and technical simplicity level that would make it easy to be used in high risk areas of the world. Accuracy was placed as the most important objective of the device, as proper diagnosis is essential for maintenance of the disease. Cost was placed as the second most important objective as lack of resources limits many areas from providing adequate diagnostic services. The drawbacks of current rapid detection methods; reliability, training, and stability, were all targets by the project for areas of improvement in final design. A detection method that addressed these issues would increase the number of clinics that would have access to reliable malarial detection that is low cost and help reduce the number of misdiagnosed cases in high risk areas.

Various physiological changes in the body of individuals infected with malaria were investigated as potential biomarker targets for diagnosis. Conceptual designs were developed to detect these various malaria biomarkers within the body. A final design focused on utilizing the change in magnetic properties of infected red blood cells was decided upon based on the objectives of the project including economic feasibility and accuracy. The device has in preliminary testing proven to be very accurate in detecting the presence of malaria. While malaria is still a global issue that affects millions each year, this device has the potential to reduce the number of severe cases by providing earlier diagnosis and allows health care workers to administer treatment faster than they would be able to normally to improve recovery rates and save lives.

Chapter 2: Literature Review

2.1 Malaria

In 2015, there were approximately 214 million cases of malaria worldwide, 438,000 of them resulted in death [4]. Malaria is a parasite that is transmitted to humans through a mosquito bite and multiplies within the person's blood and liver. Today, 3.2 billion people are still at risk of contracting this disease, consisting mostly of children and elderly in southeastern Africa and Asia [1]. Infection and mortality rates have dropped 37% over the past 15 years due to improved treatments and dissemination of insecticide treated nets and repellants [5]. However, new hurdles of resistance to medicines and insecticides are emerging and slowing efforts. While the rates of infection and death from malaria have dropped significantly over the past decade, there are still a significant number of people at risk of malaria. The cost associated with diagnosis and treatment is a major obstacle to those affected by the disease and leads to many people forgoing diagnosis or treatment [6].

2.1.1 History of Malaria

The first recorded treatments for malaria were a compound in a Chinese medicine later called artemisinin and a compound found in the cinchona tree called quinine, both of which are still used in antimalarial medicine today. The mechanisms of action of both compounds are still not completely understood but are hypothesized to involve maintaining toxic waste products that kill the parasite [7]. Following these discoveries, in 340 CE and early 17th century respectively, malaria was then discovered to be a parasite in 1880, and the discovery of different species of malaria began in 1889 by Camillo Golgi. Following this Ronald Ross determined malaria spread through mosquito bites in 1897 and in later years it was confirmed [8].

During the time of the World Wars, malaria became a focus in both domestic and military functions for the United States. After World War 2, dichlorodiphenyltrichloroethane (DDT), an insecticide used to prevent typhus during the war, was used in the United States and in the countries their military bases occupied as a means of eradicating mosquitoes carrying the malaria parasite. Through the 1950's and 60's the Center for Disease Control and Prevention, (CDC), had overseen the eradication of malaria in the US and abroad using DDT, water control, and proper treatment of those infected [8]. However, DDT was found to be harmful to both the environment and people who encountered it and the malaria parasite was becoming resistant to the traditional treatment methods. Eventually this eradication program was abandoned resulting in an increase in incidence of malaria leading to current methods such as insecticide treated tents.

2.1.2 Why is Malaria Still a Problem

Malaria remains prevalent in tropical and subtropical areas where mosquitoes can survive year-round, allowing for continuous transmission of disease. Countries affected by malaria often have programs in place to both treat infections and prevent them in the first place. However, healthcare in the countries affected by malaria is often below the standard of what is needed due to a lack in funding and poor accessibility to health clinics and medicines. The cost

of treatment and the time required by families to travel to the clinics, even if they are supported by outside funds, makes it difficult to receive treatment or purchase preventative measures such as mosquito nets for sleeping. Many of the deaths related to malaria are caused by it going undiagnosed or by the patients not receiving treatment because it is too expensive or the clinic where the treatment is offered is too far away [9]. According the World Health Organization (WHO) more than 10% of cases of malaria are not treated properly either due to misdiagnosis or unavailability of treatment [10].

Treatment and control of malaria are still significant problems in areas where malaria is still prevalent. In many countries with high infection rates, the average citizen is impoverished, without the financial resources for proper medical care. The tests required for diagnosis and the medicine used in treatment are too expensive for the average person to afford [2]. Traveling to the clinic can be time intensive due to the large geographical distance between health clinics. Additionally, some clinics do not have the tools required to make a diagnosis, requiring a sample of the patient's blood to be sent to a separate lab for testing adding days until results are returned. Since the patient would need to return to the clinic later, it means they would have to miss more time away from work and home losing income, making the second trip to the health clinic not likely to happen. Clinics are often so spread out that many people must be travel for hours to days in one direction to reach a clinic, making it difficult to make even one trip to the clinic let alone several for follow up tests and treatments.

Antimalarial drug resistance has been climbing in the past years and leading to the introduction of new treatments using a combination of different therapies rather than just one [9], [11]. While this is important in being able to continue treatment, these new therapies and treatments come at an added cost. Additionally, there is a need for universal testing procedure for all those infected with malaria [11]. Many patients go without diagnosis either due to inaccurate results or lack of access to diagnostic tools. With proper diagnostic methods, cost of malaria treatment would be lower for the clinics since they would only treat those confirmed for having malaria rather than beginning a treatment before the disease is confirmed.

2.2 Pathogenesis and Biology of Malarial Parasites in Humans

While there are five distinct species of parasites that cause malaria disease in humans, the pathogenesis of *Plasmodium* is very similar across these five species. The species that causes malaria are *P. falciparum, P. vivax, P. malariae P. ovale* and *P. knowlesi.* The life cycle of these parasites is a cyclic process between humans and mosquitoes that allows the parasites to continually reproduce and spread from individual to individual [12]. There are many changes in physiology in response to a malaria infection that provide a variety of targets for treatments and diagnosis.

2.2.1 Malarial Life Cycle

The malaria parasite survives by replicating both in humans and in *Anopheles* mosquitoes in a cyclic method of continuously transferring between the two species. Infection starts when a mosquito infected with a malarial parasite bites a human victim and unknowingly injects the parasites in the form of sporozoites into the human bloodstream. Sporozoites first invade liver cells where they grow and divide until they reach a critical density of tens of

thousands haploid parasites, called merozoites, per liver cell. The parasitic merozoites then exit the liver and enter the bloodstream, where they invade red blood cells (RBCs). This movement of parasites initiates the period of disease where the infected individual begins to experience noticeable disease symptoms. Merozoites invade RBCs, consuming most of the RBCs' contents, including hemoglobin, and undergo asexual reproduction to become mature schizonts. These schizonts rupture the RBC releasing 6-30 daughter merozoites into the bloodstream, where they invade more RBCs, continuing a process of invasion and rupture of RBCs. Some of the merozoites exit this cyclic asexual reproduction process to develop into gametocytes, the sexual form of the parasite, within the bloodstream. These gametocytes get taken up by mosquitoes that bite an infected human, and within the mosquito, the gametocytes develop from gametes to zygotes and then to sporozoites, which are then injected back into a human and restart the cycle [13], [14]. A diagram of the malaria parasite life cycle is shown in Figure 1.

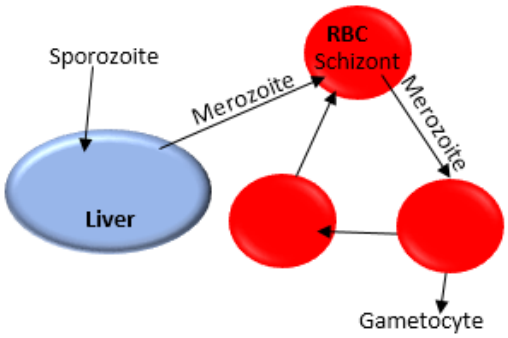

Figure 1: Diagram of malaria parasite life cycle [15]

2.2.2 Biology and Symptoms

The invasion and rupturing of RBCs as the parasites continually undergo asexual reproduction manifests in a variety of physical symptoms in the host. The physical characteristics of infected red blood cells (iRBCs) are altered by the parasite making them more rigid as well as creating protuberances with adhesive proteins on the outside of the cells. Resulting from this, iRBCs will adhere to the endothelial surfaces of capillaries and veins, and leads to occlusions in blood vessels [16]. This adherence damages the endothelial cells allowing the iRBCs to leak into surrounding tissues; the sequestering of iRBCs in vital organs such as the brain, heart, and lungs, deprives these organs of sufficient oxygen supply causing tissue hypoxia and lactic acid buildup through anaerobic glycolysis. Occlusions block regular blood flow inhibiting the ability of the host immune system to reach the parasites and allow the parasites to develop further. The rigidity of iRBCs allows the parasite protection against removal as the lack of deformability prevents these cells from fitting through the interendothelial slits in the spleen [14]. As an immune response to an infection in the blood, the spleen increases rate of removal of RBCs from the blood stream. Since the infected cells are too rigid to enter the spleen allowing them to escape this removal, this response removes only the already lysed erythrocytes while the iRBCs continue to circulate. Splenomegaly is common in malaria patients

due to a rapid removal of the lysed erythrocytes damaged after infection and rupture by the parasite causing an enlargement of the spleen [17].

The rapid destruction of RBCs and occlusions in blood vessels contribute to anemia experienced by many patients due to inefficiencies in delivering oxygen to the entire body. When the malarial parasite invades RBCs, it occupies a hemoglobin spot on the cell and digests the hemoglobin within the cell resulting in poor oxygen transportation. Digestion of hemoglobin results in a waste product, heme, which is toxic to the parasite. Therefore, the parasite polymerizes the toxic waste product into a microcrystalline structure called hemozoin. This hemozoin creates a brown pigmentation and is taken up by monocytes and macrophages, inducing an inflammatory response [18]. The infection in the bloodstream induces proinflammatory cytokines, including tumor necrosis factor (TNF)-alpha and interleukin (IL)-1, that can cause severe fever, inflammation, and promote tissue destruction [19] . The deficiency of the blood circulatory system caused by a malarial infection induces a variety of biological changes within the infected individual's body that can manifest in mild to severe symptoms.

2.2.3 Biomarkers

These biological changes induced by a *Plasmodium* infection, create a large market of biomarkers that can be targeted for diagnosis. Biomarkers are an important aspect of pathology that allow for diagnosis and prognosis of disease due to the presence and concentration of a particular biomarker. Most malarial biomarkers occur within the bloodstream, particularly erythrocytes, as that is where parasites are concentrated making blood the biggest target for current detection methods. In areas with high infection risk, a large percent of the infected population presents asymptomatic due to build up immunity making any clinical diagnosis impossible. Biomarkers allow for the diagnosis of asymptomatic individuals to combat the continuing source of malaria parasites in human blood [20]. Diagnosis and subsequent treatment of individuals with malaria, even if they do not present symptoms, is important in decreasing the number of individuals who act as hosts for the malaria parasite allowing for it to continue its life cycle.

Histidine Rich Proteins

Plasmodium synthesizes a set of histidine rich proteins (HRPs) unique from any of the host proteins. HRPI creates the protuberances or knobs on the outside of RBCs and aids in the adhesion to endothelial walls. HRPII is a protein that can bind up to fifteen heme molecules at once to aid in heme polymerization. Heme is the waste product produced after the parasite digests hemoglobin. HRPII is released outside of the RBCs into the blood plasma, cerebrospinal fluid, and urine [18]. This biomarker is often the target for rapid diagnostic tests (RDTs) and ELISAs, which use a HRP antibody to diagnose malaria by identifying the presence of HRP in a patient's blood [21].

Hemozoin

Once heme is polymerized into the insoluble, microcrystalline hemozoin, it creates a brown pigmentation. Hemozoin decolorizes internal organs to a brown pigment where iRBCs are sequestered due to its pigmentation [20]. Due to its unique structure and color, hemozoin can be detected in blood to allow for definitive diagnosis of malaria. It is most often detected

using flow cytometry, which utilizes a depolarized laser that is targeted at cells flowing through a chamber. The pigmentation of hemozoin causes it to reflect the depolarized light differently than healthy blood allowing for the diagnosis of malaria based on light deflection [3], [22].

Glycolytic Enzymes and Products

Infected erythrocytes consume glucose at a rate 100 times the normal to sustain the rapid growth of parasites. As a result, all glycolytic enzymes to be upregulated within iRBCs including lactate dehydrogenase, aldolase, and glutamate dehydrogenase. Between sixty to seventy percent of the glucose used is converted to lactic acid and excreted from the erythrocyte. Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate for anaerobic respiration; the parasite's primary method for ATP generation. LDH levels increase during the intraerythrocytic part of the infection cycle and decline to zero during schizont stage. Aldolase is an important enzyme used for cleavage fructose during glycolysis and may also play a role the invasion mechanism of the parasite. Glutamate dehydrogenase (GDHs) serves in nitrogen-carbon metabolism. RBCs do not contain GDHs normally making them a biomarker for malaria as well as a species-specific biomarker as different species of malaria parasite produce different GDH isomers [23], [24]. These products are also the target of some RDTs as well as other tests that utilize antibody: antigen binding. Antibodies for GDH and species specific GDH are used to identify the presence of this enzyme as an affirmative diagnosis of malaria. LDH antibodies are also utilized, although these are more variable during different stages of the malaria parasite's life cycle and since LDH is present in healthy individuals, this method is only accurate during the intraerythrocytic stage of the parasite to identify the increased levels of LDH.

Physical Attributes of iRBCs

Plasmodium infection of RBCs, alters the physical characteristics of these cells as the parasite manipulates RBCs to fit its need for growth. Knobs appear on the outside of the RBCs to promote adherence to endothelial cells. Crosslinking of parasitic proteins with the cytoskeleton and the presence of the parasite itself reduces deformability and increases rigidity of iRBCs [25]. The adherence and rigidity of iRBCs has been used to separate healthy and infected blood cells using microfluidics devices that contain a chamber that narrows in size pushing more rigid cells to the outside [26]. Optical tweezers also have been used to separate more rigid cells by using a highly focused laser causing cells to spin at different rates. Since infected cells are more rigid, they spin at a different speed than healthy cells and this difference in speed can be measured to identify the presence of rigid cells in a sample [27].

Electrical Conductivity

The electrical conductivity and capacitance of these cells also increases significantly compared to healthy erythrocytes, due to the electron dense knobs on the surface of iRBCs and increased membrane permeability that alters ionic exchanges across the membrane. Dielectrophoresis utilizes this change in electrical conductivity to separate infected and healthy red blood cells. Cells with different electric properties move to different positions when exposed to an inhomogeneous electric field. Therefore, exposing a sample of red blood cells to an alternating electric field will cause a separation of healthy from infected cells due to differences in the cells' electrical conductivity [28].

Magnetism

Hemozoin in iRBCs contains iron crystals that make the cell paramagnetic, or realign in response to a magnetic field, while healthy RBCs are diamagnetic when oxygenated and repel magnetic fields [29]. Removal of malaria infected cells from healthy cells can be achieved with magnetic cell separators. Blood samples are passed by a magnet that causes the iRBCs to move closer to the magnet and farther away from healthy cells. This technique has been used to flow the blood sample onto a microscope slide so that the infected cells are concentrated together and easier to identify on the slide [30]. Figure 2 below is a schematic of magnetic separation where a magnetic field is applied on the left side of a vertical chamber causing attraction of the infected red blood cells and repulsion of healthy cells. Repulsion of healthy cells would only occur with fully oxygenated cells as oxygen is diamagnetic. Otherwise slightly oxygenated or deoxygenated red blood cells stay in the center of the streamline. Infected cells can still be separated from deoxygenated red blood cells by separating the infected red blood cells using a magnet to pull the infected cells away from the streamline flow.

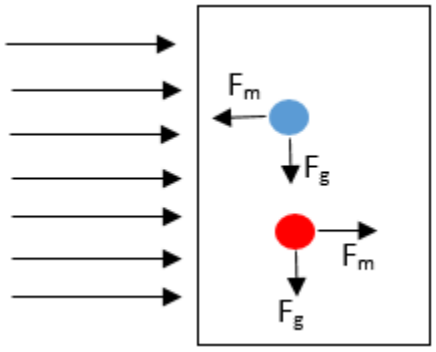

Figure 2: Schematic of the forces acting on infected and healthy red blood cells flowing by gravity down a chamber. Arrows on left represent a magnetic field [31].

Volatile Organic Compounds

Analysis of individuals infected with malaria, have identified increased levels of over ten volatile compounds (VOCs). Four thioethers (allyl methyl sulfide, 1-methylthio-propane, (*Z*)-1-methylthio-1-propene, and (*E*)-1-methylthio-1-propene) are released in a malaria patient's breath and are not present in healthy individuals or individuals with other parasitic infections. While the mechanism to produce these thioethers is unknown, their concentrations in an individual's breath correspond positively to the level of parasitemia in the blood [32]. One of these thioethers, allyl methyl sulfide, is also released when consuming garlic, and therefore, can be present in the breath based on what the patient has eaten in the past several hours and can provide a false positive for malaria diagnosis [33].These compounds are typically detected through gas chromatography and mass spectrophotometry due to the trace amounts present in the breath.

There currently are existing methodologies to detect all these biomarkers such as gas chromatography to detect thioethers and antibody tests for glycolytic enzymes but not all are efficient and cost effective. Many methods still require microscopy, antibodies that easily degrade, or other expensive equipment, such as a mass spectrophotometer. Deficiencies in

current detection methods make these biomarkers targets for new detection methods that allow a more economic and effective diagnosis.

2.3 Current Standards of Malaria Detection

There are three main methodologies for detecting malaria including clinical diagnosis, microscopy, and rapid diagnostic test. Clinical diagnosis is the most widely used but the least reliable method for detection. Microscopy is considered the gold standard detection method in terms of accuracy but is high in cost making it inaccessible in many regions. Rapid diagnostic tests have become more widely used in recent years due to their low cost, but have accuracy issues that leave a gap in the market for new diagnostic techniques.

2.3.1 Clinical Diagnosis

Diagnosis of malaria based on clinical observation of symptoms is the preliminary method of diagnosis, used before others when determining the likelihood of a malaria infection. In areas where no other resources are available, clinical diagnosis is the only method used. Clinical diagnosis consists of a patient history and physical examination that are used by a healthcare clinic worker to determine the cause of the patient's symptoms. One of the difficulties with successful detection of malaria infections is that the symptoms experienced by malaria patients are unspecific and can often be misdiagnosed as a bacterial infection or flu because of this. Symptoms, especially early in the diseased stage, include fever, nausea, headaches, and fatigue. The overlap of malaria symptoms with other common febrile or infectious diseases reduces the specificity of clinical diagnosis. Over diagnosis hurts many clinics in endemic regions, resulting in an unnecessary expenditure of time and resources and is a major block in producing effective diagnosis plans in impoverished areas. In high risk areas, the number of false positive results is ranges and can be from 40-80% patients diagnosed through presumptive clinical diagnosis are not infected with malaria [34], [35].

2.3.2 Microscopy

Due to the unspecific nature of its symptoms, malaria has been historically misdiagnosed as a fever or bacterial infection since before the discovery of its parasitic nature. The first person to observe the malaria parasite was Alphonse Laveran in 1880, a military doctor inspecting the blood of soldiers in Algeria, who had come down what was then known as "Marsh Fever" [36]. By analyzing blood samples, Laveran found that the only consistent feature from case to case was dark pigmented granules, observed with a light microscope, on and around the red blood cells in blood samples. While highly controversial at the time, this discovery found him awarded the Nobel Prize in 1907 for his discoveries and contribution to protozoa pathology.

While understanding of the nature of malaria infections has advanced dramatically since then, microscopy is still the golden standard when it comes to verification of malaria infections around the world. Advanced microscopy and staining techniques have made detection of parasites a standard procedure and one relied on for accurate detection of malaria. The World Health Organization recommends the Giemsa stain above all others, as the most reliable method for detecting malaria [37]. This staining utilizes two dying agents; Eosin, which stains

the parasite nucleus red, and methylene blue that stains the cytoplasm blue. Since red blood cells do not have nuclei, this differential stain is helpful in identifying the presence of foreign bacteria or parasites in the cells. In outpatient clinics and laboratories, a rapid method using 10% Giemsa working solution is the most popular for getting expedient results when running fewer than fifteen slides at a time. Blood samples can be analyzed as thick or thin films, depending on desired information: thin smearing allows for identification of malaria species and qualitative and quantitative analysis of the state of infection, whereas thick smearing can get up to forty times the sensitivity for detecting malaria parasites [38]. After blood is harvested from suspected patients and the chosen smears placed onto the slide, they are fixed with methanol and dried before applying the stain working solution. Staining solution is left in contact with the blood film for eight to ten minutes, before being washed away with pH 7.2 controlled water and being left to dry completely again before microscopy can be performed. The time estimated to run and analyze a basic test of this type is between 20 and 60 minutes depending on the quality of the equipment and reagents, the type and quality of the blood smear, the stage of infection, and the skill of the analyzing technician and how thorough the technician wants to be with a slide. The monetary investment in one slide is estimated to be between $0.60 to $1.00 per test, with one diagnosis using around four slides or more to confirm infection, excluding the cost of the microscope [39], [40]. Performed under optimal conditions, microscopy techniques are highly accurate and capable of detecting presence at the level of 50 parasites per microliter or 0.001% parasitemia [41]. However, the level of detection ranges from 50 to 500 parasites per microliter depending on the skill of the microscopists and time spent at the microscope. On average the level of detection is closer to 100 to 200 parasites per microliter [3].

Despite being the gold standard for detection, microscopy is not without its downsides when used in endemic countries. Many local clinics do not have a fully equipped lab or reliable access to the reagents or slides that keep the cost of this method of detection low. The reliability of this method is also heavily reliant on the skill, knowledge and motivation of the administering technician with decisions such as when to run additional slides of how long to spend looking each slide are left to their discretion. This insertion of human error and bias is undesirable for a fast-paced clinical environment when lives are on the line and an accurate diagnosis is needed quickly. Due to the lack of access to proper lab equipment, trained microscopists, or electricity to power the microscope, diagnosis by microscopy is not commonly used in areas with high infection rates.

2.3.3 Rapid Diagnostics Tests

With the growing need for diagnostic techniques that can be used by volunteers and technicians with limited training and provide a clear diagnosis without room for obscured results, over two hundred separate rapid diagnostic tests (RDTs) have become available on the market. With their ‘ready out of the box’ nature and clear results, RDTs have become an accessible alternative to microscopy for the diagnosis of malaria in the field [42]. Most of these products use malaria-targeted antigens targeting the common proteins that are produced by malaria parasites during their lifecycle in the blood stream. Histidine-Rich Protein-II (HRP-2) is produced by the parasite while in the asexual merozoite stage and is expressed on iRBCs membranes during invasion. This was of the first antigens to be targeted by RDTs [41], [43]. Plasmodium

lactate dehydrogenase (pLDH) is an enzyme produced by both merozoite and gametocyte stages, and is a target for RDTs used for determining the specific *Plasmodium* species, as it is known to have different isomeric structures based on the producing parasite, which is useful in determining which treatments to administer based on the varying efficacy of each treatment to different species [41]. The tests utilize a two antibody-stage process. The first is where the blood sample is exposed to targeting monoclonal antibodies that are conjoined to a detectable dye or particle. Presence of the antigen in the sample leads to the formation of antibody-antigen complexes within the blood sample. The sample is then run across a nitrocellulose membrane on which one or more fixed regions of antibody-complex targeting antibodies are placed, that will fix the dye conjoined complexes within a location if they are present. This results in a visual line at a location on the strip that indicates to the technician the presence of the antigen in the sample. Figure 3 shows a schematic of how RDTs work. Parasitized blood is flushed down the strip containing labeled antibodies. If a specific parasite antigen is present in the blood, then it will bind to the antibody and two visual lines from the labeled antibody will be visible indicating the presence of malaria.

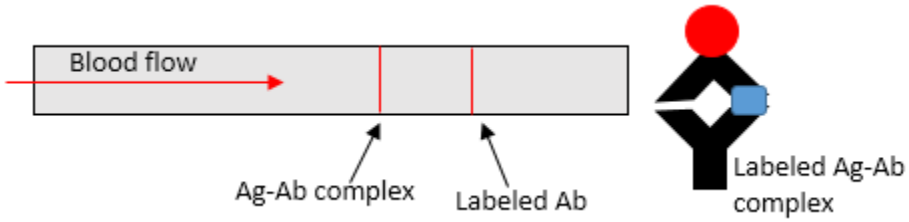

Figure 3: How RDTs work, parasitized blood is flushed across the strip, parasite antigens bind to antibody and are labeled and captured [44].

By using different antibodies at different position lines, it is also possible to create a test that can narrow down what type of malarial infection the patient is experiencing. This all helps to make RDTs a highly versatile and expedient option for field usage. Antigen capture tests have a high rate of accuracy, detecting up to 100 parasites per microliter or 0.002% parasitemia and given rapidly determined results in 15 to 20 minutes after sample harvesting [41]. RDTs range from an accuracy of detection of 100 to 500 parasites per microliter with an average detection of 300 parasites or higher per microliter [41].

The World Health Organization currently sets the most common standards for evaluating, using and procuring RDTs by volunteers, local clinic, non-governmental organizations or other groups that seek to operate in endemic areas. For validation of the accuracy of an RDT, WHO has a product testing evaluation panel consisting of a series of controlled low density parasite samples, from which a device must be able to accurately detect infection in 75% from these samples. Devices should also a false positive rate of less than 10% and fewer than 5% invalid tests [45]. Despite there being over two hundred RDTs on the market currently, there are only twelve that are currently qualified and eligible for WHO procurement [46].

While an attractive and promising alternative to laboratory microscopy, antibody-based RDTs are not without their downsides. The regions endemic with malaria are often tropical or

subtropical in climate, as these areas are the most supportive to the mosquitos that help facilitate the life cycle of the malaria parasite. The levels of heat and humidity found in these regions can take a large toll on the shelf life of antibody based RDTs by denaturing or detaching the sensitive antibodies or through degradation to the nitrocellulose membrane. Manufacturers recommend keeping tests in refrigerated conditions during the entirety of storage, but for many locations this is not possible due to lacking infrastructure. A study in Brazil found that storage at ambient temperatures could lower the sensitivity of tests by 20% after fifteen months, putting them well below WHO recommended standards [42]. RDTs also only function optimally in cases with high parasite density, meaning that they are much less useful for preventative or early detection.

2.3.4 Treatment After Diagnosis

Once a patient is diagnosed with or without malaria based on one of these methods a treatment plan is decided upon based on the patient's diagnosis and available resources. Malaria treatment often are in the form of some artemisinin-based combination for *P. falciparum* and chloroquine for *P. vivax [47]*, [48]. In areas with high infection rates of malaria, diagnosis with malaria is not necessary for a treatment to be prescribed. In a study in Zambia, even after diagnosis through microscopy, 35% without any parasitemia were prescribed an antimalarial drug [49]. In a rural region in Burkina Faso, 79.8% of patients with negative RDTs tests were treated for malaria during the dry season and 85% of negative patients were treated during the rainy season [50]. Confirmation bias and distrust of the accuracy of current diagnostic methods drives clinical decisions and treatment of patients with malaria-like symptoms leading to an ineffective distribution of antimalarial treatments. In addition, WHO requires treatment based on clinical diagnosis if other diagnostic results are not available within two hours of patient's arrival at the clinic. This is to help prevent any further complications that could occur from waiting for treatment. Therefore, a fast and accurate method of diagnosis is necessary for effective management and treatment of malaria.

This project's aim was to address the problems with current detection methods by creating a method to diagnose malaria that is both economically feasible and accurate.

Chapter 3: Project Strategy

3.1 Initial Client Statement

The initial client statement stated: “Develop a low-cost method or test that will enable clinicians in countries with high malaria infection rates to determine if a patient is infected with malaria.” The target demographic of our product is to be NGOs and local clinics located in endemic areas of the world that were struggling malaria based fatalities. The only restriction given by the client was to limit the amount of blood or other material needed to be collected from the patient as they are mostly children that need attention. Emphasis was put on making it cost effective, simple to use and read the result, and not to require a microscope to use.

3.2 Design Requirements: Technical

The client statement and research of current methods were used to determine the technical objectives for this project. These objectives were divided into primary objectives, which served as the main focuses of the project, and secondary objectives that served as additional goals that were of a lower priority. Primary objectives consisted of accuracy and cost of the device while secondary objectives were the durability, ease of use, time, and sampling method.

3.2.1 Objectives

To distinguish between primary and secondary objectives, a pairwise comparison chart was created to rank each of the objectives of the design against each other. Objectives listed on the left were given a 1 if the objective was considered more important than the objective in the column, a 0 was assigned if it was considered less important, and a 0.5 was assigned if the objectives were considered equally important. Objectives above a 3 were determined to primary objectives as they ranked greater than at least half of the objectives, while the others were determined to be secondary objectives.

Table 1: Pairwise Comparison of Objectives

	Accurate	Cost Effective	Durable	Ease of Use	Fast	Minimally Invasive	Total
Accurate	x	0.5	1	1	1	1	4.5
Cost Effective	0.5	x	1	1	1	1	4.5
Durable	0	0	x	1	0.5	1	2.5
Ease of Use	0	0	0	x	1	1	2
Fast	0	0	0.5	0	x	0.5	1
Minimally Invasive	0	0	0	0	0.5	x	0.5

Primary Objectives

The primary objectives for this project were centered on making a device that was both accurate in detecting malaria and was economical and relatively low cost as these ranked the highest on the pairwise comparison chart.

Accuracy

Accuracy requirements were based around the World Health Organization's standards for malaria detection tests. These requirements were that the test must detect at least 75% of low parasite densities (~200 parasites per microliter of blood), have a false positive rate of less than 10%, and fewer than 5% invalid tests [51]. Accuracy was determined to be one of the most important objectives as misdiagnosis of patients with malaria-like symptoms contributes to the ineffective distribution of treatment. Creating a more accurate method to diagnose malaria will allow for health clinics to make more informed decisions when treating patients suspected of having malaria.

Cost

Cost requirements were determined based on the current standards for detection to aim to develop a test that costs less than these methods. Microscopy costs at least $200 initial investment in a microscope, often up to several thousand dollars for higher quality microscopes, and approximately $0.60 to $1.00 per test, with around four tests being performed for confirmation of diagnosis, plus additional cost for the time it takes to observe one slide, which can be around 30 minutes. Rapid diagnostic tests (RDTs) cost approximately two and five dollars per test. Based on these costs, the client statement, and the average GDP and per capita income in areas highly affected by malaria, the objective of this project was to develop a test that costs on average fifty cents per test [40]. Reducing the cost of a test to diagnose malaria will help increase the number of patients that are tested for malaria allowing for improved management and treatment of the disease.

Secondary Objectives

Secondary objectives were determined to be making a device that was durable, easy to use, fast, and had a minimally invasive sampling collection method. These were determined to be important characteristics that an ideal device would have but are not necessary for the device to achieve the project goals and therefore, were given less priority than the primary objectives.

Durability

Areas that have a high prevalence of malaria are typically tropical areas; therefore, this device should be able to withstand such temperatures up to 35°C and 60% humidity without infringing on the integrity of the test results. Current RDTs become ineffective at such temperatures and humidities due to denaturing of the antibodies in the tests. Therefore, this device aims to be more durable than current devices mimicking the conditions that the device will be used in an incubator at 37°C.

Ease of Use

The device must also be easy to use, both in sample collection and analysis of results. The results of the test must be easy to understand and the sample for diagnosis must be able to be collected by a health clinic worker without extensive training to ensure that this diagnostic tool can be fully utilized.

Speed of Diagnosis

Rapid diagnostic tests typically take 20-30 minutes to complete and microscopy usually takes 30 minutes per slide. However, due to the inaccuracies of the tests and need to observe several microscope slides sometimes several tests are done for one diagnosis. Therefore, this test should not take more than two hours to report results to reduce time before treatment if positively diagnosed and if possible should take less than 30 minutes.

Minimally Invasive Testing

This device was intended to be minimally invasive to minimize additional risks associated with surgeries and avoid stigmas around blood collection. There are cultural and personal taboos against blood collection, which is utilized in the current detection methods for malaria. Therefore, ideally this device can provide a diagnosis through a less invasive manner [52]. However, this was not prioritized as highly since malaria parasites are present in blood, noninvasive measures of diagnosis are difficult without expensive equipment such as the mass spectrophotometers used for breath detection. Since this device is intended to be used in areas without advanced lab equipment, less invasive measures are ideal so that the procedure does not have to be performed in a sterile environment.

3.2.2 Functions

Based on the objectives of this project, a list of functions was developed. The functions of the device allow for accurate positive or negative diagnosis of malaria. This includes taking a sample from a patient suspected of having malaria and running it through a diagnostic test. This test must be able to identify some biomarker in the sample that is indicative of a malarial infection and report this information in a way that the user of the device can read.

3.2.3 Constraints

This project was constrained by time, cost, and resources. A final device and report had to be completed within eight months, limiting the amount of time the project team could dedicate to research, prototyping, and testing. The number of iterations of designs and tests run were limited by the team's budget, which was 600 dollars after lab fees. The low-cost objective of the project also limited the materials and methods that could be used for a detection method. Resources used for testing were another constraint as there was limited access to patients with malaria due to standards, ethical, and geographic reasons. In addition, malaria may cause unknown effects *in vivo* that are not predicted and modeled with *in vitro* models. This may cause the device to perform different in testing than in actual application. The device must reduce the financial burden on health clinics by costing less than current standards of microscopy and RDTs.

3.3 Design Requirements: Standards

Since this device will be built to test for malarial markers in a patient's blood, sweat, breath or any other bodily substance it must adhere to certain standards related to safety relating to the patient, the health provider administering the test, and the sample taken itself. The most comprehensive organization of these engineering standards is the International Organization for Standardization (ISO). Many standards from this organization are related to medical devices that will have direct contact with the patient to ensure biocompatibility and safety such as ISO 10993: Biological Evaluation and Biocompatibility Testing of Medical Devices. ISO 15198:2004 references *in vitro* diagnostic medical devices based on validation of user quality and procedures by manufacturer. Therefore, this device must be able to be validated for its performance so that when health clinics or others use it, it is consistent. ISO 16142 is for the safety and performance that apply to all medical devices. These standard references manufacturing principles that apply to the creation of medical devices, therefore it depends on how the final design will be manufactured. It also requires conformity of the medical device so that when multiple devices are manufactured it is ensured that each device will perform with the same level of quality and safety. ISO 23640:2011 is for *in vitro* medical devices and evaluating the stability of *in vitro* diagnostic reagents. Since the final design utilizes a luminol solution, this standard is important in ensuring the stability and shelf life of reagents used in diagnostic tests to ensure proper results of testing. Toward the manufacturing process of the device itself, the World Health Organization (WHO) states that it should adhere to the standards as stated in ISO 13485:2015. This ensures that the organization and safety of the manufacturing process is consistent and that the quality between multiple devices does not vary significantly. Regarding the sterility of the device, ISO 11737-2:2009 ensures the methods of sterilization are appropriate for removing all cell presence in the device as well as leaving it safe for repeated use.

WHO is one of the largest organizations that studies malaria and malarial diagnostic tests. WHO has standards for the accuracy and sensitivity of RDTs. To make this device competitive, it must also meet those standards. The malaria diagnostic tests must have at least 75% accuracy when detecting low parasite densities as defined as 200 parasites per microliter

of blood. It must also have a false positive rate of less than 10% and fewer than 5% invalid tests [53].

3.4 Revised Client Statement

Through exploration of current state of the art methods of detection, research on conditions in endemic regions of the world, and conversations with the MQP project advisor the client requirements for the project were revised to be more specific: “Develop a method or test that will enable clinicians in countries with high malaria infection rates to determine if a patient is infected with malaria for under $0.50 per test, that is at least 75% accurate at a low parasite density of 200 parasites per microliter, easy to use, durable, and takes less than 30 minutes to complete.”

Accuracy was given the highest priority to ensure correct diagnosis and low cost was also prioritized to improve access to device. The device would have to meet or exceed the standards for RDTs as set by the World Health Organization to be competitive and provide accurate diagnosis to patients. In addition to being accurate, the device needed to be low cost to provide diagnosis to low resource regions. The price point was set at $0.50/test for disposable tests or $15 for a reusable testing device to ensure affordability. Durability was also important in maintaining accuracy of the device in the hot and humid environments where malaria prevalent. Because of this specific targeted audience for the device, considerations were also given as to make sure this device would be culturally considerate of the people that lived in these regions.

3.5 Management Approach

3.5.1 General Project Phases

To complete the objectives, set by the client statement, a general approach was developed to determine what kind of device would be the most successful at maintaining an acceptable level of accuracy for detecting malaria while improving on the cost of the device and the speed at which diagnosis could be reached.

Idea Generation and Evaluation

Known biomarkers of malaria were investigated and used to generate initial conceptual designs that could meet the needs for this project. Devices that detected similar markers were investigated and evaluated for applicability for malaria. Designs brought in ideas from many different branches; biofluidics, magnetism, and organic chemistry. From these devices, the realistic options for this device were narrowed in on based on which designs would best meet the objectives of the project and could be completed with available resources.

Prototyping and Design Finalization

The final design was decided upon based on evaluation of the conceptual designs generated. Designs were evaluated using a decision matrix to weight the objectives of the project in order of importance. The design best suited to meet these objectives was decided upon as the final conceptual design to pursue for further prototyping and development.

Initially, methods of manufacture for the device were considered and one was chosen based on its availability and ability to create the features needed in the device. Following the manufacture, testing was conducted to determine the abilities of the initial prototypes and improvements were made to the initial design to improve the device's functionality.

3.5.2 Financial Management

The budget provided an economic constraint for this project that was considered during each project phase. An expense tracking sheet was maintained to monitor spending and available funding. A sample of the expense tracking sheet is located in Appendix A. Each purchase was approved by all team members to ensure consensus on budgeting. Initially, prototypes were created using the least amount of the budget as possible to determine its proof of concept. Once the prototypes showed signs of success, more of the budget was applied to further their development.

Chapter 4: Design Process

4.1 Needs Analysis and Functions

4.1.1 Needs Criteria

The need for this project was to develop a device that effectively diagnoses malaria at low cost. There is a significant need for such a device due to the high prevalence of malaria and the lack of affordable diagnostic resources to detect the disease. Almost half a million-people died from malarial disease in 2015. Treatment for malaria is effective when administered before an individual reaches a severe disease state above 2% parasitemia [53]. Due to the cost of treatment, it is often not administered until a definitive diagnosis is known, which is difficult due to lack of resources and effective tests [54]. The investment cost of a microscope, which can be several hundred dollars at least, and the unreliability of rapid diagnostic tests (RDTs), which decrease in sensitivity by 20% at room temperatures over a 15-month period, with only a 71% specificity [55]. This creates a need for diagnostic test that does not create a financial burden on health clinics or patients and provides accurate results. A device that meets these needs would decrease the mortality rate in individuals suffering from malaria by creating the opportunity for a more rapid and targeted administration of treatment methods.

4.1.2 Functions and Specifications

The function of the low-cost malaria detection device is to have an option for diagnosing a patient's infection without having to rely on microscopy for a confirmation of infection. The device should meet a specific set of specifications for it to be considered a success. These are rapid production of results, accurate as current RDTs, cost approximately fifty cents per test, and can be stored for long periods of time without losing accuracy. It is important that these specifications be met to create a device that is competitive in the market as well as a successful way of diagnosing a patient's malarial infection.

Speed of diagnosis is a major factor determining whether a patient will receive treatment for an infection. A major problem with being able to treat those infected with malaria is that microscopy results take a couple of days to get to the lab, be interpreted, and then send the results back to the clinic making the patient take two trips and using up extra time just for a diagnosis. Current RDTs return a diagnosis rapidly but are inaccurate [56]. This device should provide a diagnosis in relatively the same amount of time as a RDT or less. It should not require a patient to wait multiple hours for a diagnosis thus making the visit last all day.

The accuracy of the device will determine if it is usable as a way to diagnose an infection. RDTs are regulated by the WHO to be able to diagnose infection in 75% of samples when the parasite density in the patient is 200 parasites per microliter, which usually occurs within 48 hours of the parasites being released into the bloodstream [57]. RDTs increase in accuracy with increasing parasite densities [7]. This project needs to create a device to be at least as accurate as current tests to meet the World Health Organizations standards for field use.

Initial cost for microscopy testing is incredibly high, ranging from two hundred dollars to

several thousand dollars, and following tests can cost one dollar per test, the cost for RDTs is similar, which are typically two to three dollars per test. This device must be at a cost that patients in areas of high prevalence of malaria can afford. For example, in Uganda, a country that has over nine million cases a year, the average income per day is 1.25 USD. On average, patients in Uganda spend 62 minutes travelling to and 87 minutes waiting in a government hospital to be treated, adding additional costs in time lost. Three to six days of work are lost due to malaria related illnesses per person infected with the disease and there is a slightly higher prevalence of malaria (2% increase) in people who are considered below the poverty line [58]. From this and knowing that the cost of testing is one of the largest hurdle for the patient, it was decided test should cost between fifteen and fifty cents per use. This was determined by consulting with the client and comparing the current methods costs to each other. With setting the cost low, this will allow those that may not have been able to afford the procedure to be able to afford and receive treatment, if needed, easier.

The last function is the durability of the device, so that it does not lose accuracy while being stored in tropical environments. While RDTs are good at detecting malaria, they lose accuracy the longer they are exposed to warm and humid environments, which is where malaria is normally found [59]. Therefore, the device should be able to be stored easily for months at a time without it having to be replaced due to it becoming unusable. The sensitivity of RDTs degrades over 15% over fifteen months from 95% to 79% [60]. The function of the device in this project would be to exceed the current durability of RDT. This would lead to lower costs as well since the clinics would not have to continuously buy new tests to make sure the results they received were still accurate. These functions and specifications are outlined in Table 2 below:

Table 2: Functions and Specifications of Device Design

Function	Specification
Low financial burden	<$0.50 per test
Accurate diagnosis of malaria	>75% detection rate at low parasite densities <10% false positives <5% false negatives <5% invalid tests
Provide quick results	<30 minutes
Withstand adverse environments without compromising test results	15 months of storage in tropical climates (35 C and 60% humidity) without degradation

4.2 Conceptual Designs, Design Concept Prototyping, Feasibility Studies

Based on research of the pathological changes induced by malarial parasites, conceptual designs were developed to target these changes to detect malaria. These designs were all ruled out due to preliminary research and calculations indicating infeasibility of successful detection and/or low cost.

4.2.1 Liver Biopsy

The liver serves a key role in the initial propagation of the malaria parasite after the infection initiated by the mosquito bite. After the parasites initially enter the bloodstream, they are gathered in the liver where they begin asexual growth until a critical mass is achieved and they spread out through the bloodstream and start attacking red blood cells. This very early role in the life cycle of the malaria parasite means that options such as liver biopsy could be used to detect a malarial infection before symptoms could be shown. Some parasites stay in the liver dormant during the disease state so this method would cover all stages of infection for diagnosis. Genetic analysis methods such as PCR provide a way to analyze a taken sample of tissue for the presence of early stage parasites. This could be used in conjugate with a dying agent to produce a test that could produce clear and unambiguous results [61]. The tissue can also be observed under a microscope or by eye for hemozoin pigmentation and endothelial cell proliferation [62].

This method of testing however fails most of the other criteria set by this project. While minimally invasive biopsy techniques have been standardized for other fields, these procedures require a very skilled and undistracted surgeon in a sterile environment to perform which is not easily accessible in low resource regions where this device is intended to be used. PCR machines are a nonstandard piece of lab equipment and their cost in inhibitive to the objectives of this project. Even if minimally invasive procedures are used, the primary recipients of this project are going to be young children, and biopsy is likely to cause more emotional distress compared to other conceptual designs developed. There is also concerns with infection that comes from improper sterilization of the tools used, and inadequate internal healing from the biopsy post procedure. For these reasons, this idea was abandoned early in the explorative process.

4.2.2 Retinopathy

The ability to detect an infection through the state of the patient’s retina was another conceptual design developed. It has been shown that prolonged infection with the malaria parasite leads to macular degeneration as well as whitening of the retina and retinal hemorrhages. If there was a lens that could be attached to a camera or cellphone it may be able to provide photos similar to that of a fundus camera, a camera focused through a low power microscope lens to take pictures of the retina, and allow a clinician or worker to make a diagnosis.

While this method would provide a definitive answer on some cases, it was not a good fit for the project’s criteria. While the retinopathy does present due to malaria, it is not a symptom that appears in all patients as it is a rare complication in severe cases, and when it does appear

it is associated with high counts of parasitemia. These severe cases include cerebral malaria, when the parasites invade the blood brain barrier, and in significantly late stages of infection; both occurrences when diagnosis and treatment should occur before appearance of retinopathy to achieve effective treatment [63]. Additionally, taking a picture of a patient's retina using just a lens and a normal camera is possible but will most likely be impossible to be done due to the complexity of focusing the lens to capture the retina. Even with the pictures, there would need to be a trained worker, most likely an ophthalmologist, to interpret the pictures to give a definitive diagnosis [64]. Due to these main reasons, this idea was ruled out due to its complexity and the fact that it does not present in all instances of infections.

4.2.3 Ultrasound

One alternative design was to develop a portable and cost effective ultrasound for minimally invasive detection of malaria. Two methodologies were discussed utilizing an ultrasound, one was to identify parasites and the other was to detect an enlargement of the spleen as a diagnosis of malaria. The first design was to create an ultrasound that could produce an image of the liver that would allow a technician to identify if parasites had sequestered there in a patient with malaria. Limitations of this idea lay in the resolution of current ultrasound devices and the lack of technical experience in the team to develop an ultrasonic device that could produce a high enough resolution picture to distinguish malarial parasite in liver cells.

Ultrasound can provide images of organs, which is useful in the diagnosis of many diseases. It was conceptualized that the enlargement of the spleen that occurs in malarial patients could be identified in an ultrasound images. Enlargement would occur once parasites enter the bloodstream and begin to cause clinical symptoms. However, detection of malaria through ultrasound of spleen is not sensitive enough to accurately detect malaria, with a sensitivity of only 55% [65]. This methodology has a high specificity of 95% and therefore, could be used for ruling out false positives from other diagnostic tests, but could not be used as a standalone method for detection of malaria [66]. Interpretation of ultrasound images requires only trained individuals to make a diagnosis based off an ultrasound, which provided another limitation from pursuing this alternative design. Due to the insufficient resolution of current ultrasound machines and the cost of current machines being over $10,000, this methodology was ruled out to the lack of probability of producing a cost effective and accurate product that could be used by a minimally trained health care worker.

4.3 Alternative Designs

Based on background research and defined functions and needs for this project, several alternative designs were created based on the biological and biochemical effects of malarial infections. These designs were more thoroughly researched and developed compared to conceptual designs as the alternative designs offered more feasible options to pursue. Alternative designs were all considered as potential final designs as each offered a potentially accurate or low cost method to detect malaria.

4.3.1 Rigidity

The mechanical properties of infected red blood cells (iRBCs) can be utilized to isolate these cells from healthy red blood cells (RBCs). iRBCs are less deformable than healthy RBCs, which could be utilized in a microfluidics type device. The elastic modulus of iRBCs can increase up to ten times that of healthy RBCs as the parasite develops going from ~ 4-8 μN/m to ~ 40 μN/m, creating markedly different mechanical properties [67]. This device would intake a blood sample and have an output channel for both healthy RBCs and iRBCs. As they pass through the chamber there are small ridges on its bottom positioned at a 45-degree angle to the direction of fluid flow. As the cells pass over these ridges the pressure exerted on the cell body increases causing stiffer cells (iRBCs) to be pushed up to a collection chamber due to the resulting force, while softer cells (healthy RBCs) would be pushed towards the bottom of the chamber to a different collection area [68]. This would allow a separation of cells in the flow chamber. However, this method would only show a difference between the numbers of rigid cells compared to deformable cells, which is not conclusive evidence of malaria. Other diseases can cause mechanical deformations; sickle cell anemia also results in rigid RBCs, which would lead to false positives if detection was based solely on rigidity [69]. Figure 4 below demonstrates the conceptual design of a device utilizing the rigidity of iRBCs to separate healthy and infected cells.

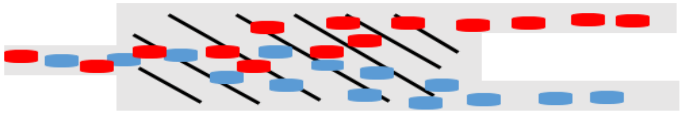

Figure 4: Conceptual Design based on rigidity. Blue circles to indicate healthy red blood cells that deform from the increased pressure. Red circles indicate infected red blood cells that deflect downward due to their stiffness.

4.3.2 Volatile Organic Compounds

Another alternative design investigate was to utilize the thioethers found to be in high concentrations in the breath of infected test subjects after the first lifecycle of the parasite. The volatile organic compounds of allyl methyl sulfide, 1-methylthio-propane, and (E)- and (Z)-1-methylion-1-propene were found to be expressed up to 100 times higher in patients that were known to be infected with malaria [70]. Inspired by chemical reactions shown with other sulfur containing organic compounds, a device was designed that would bubble the breath of a patient through a solution containing a reacting compound, resulting in a hypothesized release of pure sulfur and oxidized byproducts [71]. If enough of a reaction could take place, a visible amount of sulfur could form within the vessel. If this proved to be too little of a reaction to produce visible sulfur particles, a staining agent could be used to detect the oxidable groups that would also result from the reaction. The specifics of this hypothesized reaction were not fully explored.

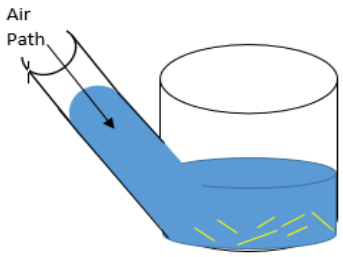

Figure 5: Concept design based on the presence of elevated levels of thioethers in the breath of malaria patients. Yellow lines indicate sulfide precipitates that would form when breath reacts with chemicals in the solution.

This device would then be tested with samples of breath taken from malarial patients that could be run through the device to test its reactive potential. Variables such as number of breaths, time spent bubbling or amount of reacting agent could be tested with this malarial air and used to determine a proper method of test verification afterwards. The test would then also need to be controlled against a healthy sample of breath to check for any unexpected reactions with the naturally forming VOCs in healthy breath.

The thioether allyl methyl sulfide is released when garlic is broken down and therefore, can appear in healthy individuals who have consumed garlic within the few hours prior to testing [33]. This could cause a false positive result if allyl methyl sulfide was detected using this method. A history of the patient's food consumption prior to the test must be investigated, and the test would have to be delayed if the patient ate garlic to ensure that the garlic is not affecting the results of such test.

4.3.3 Oximetry

Oximetry was also considered as a way of determining infection due to the way the parasite behaves once it has entered a RBC. Once inside the cell, it digests a portion of the hemoglobin and takes up one of the spots for oxygen to bind to thus lowering the ability for the RBC to transport oxygen throughout the body and to the mitochondria within the cell itself [72]. Due to this the cells ability to transport and utilize oxygen is lowered leading to hypoxia and eventually cell death, therefore if the levels of blood O2 are lowered enough it would be a way of determining infection.

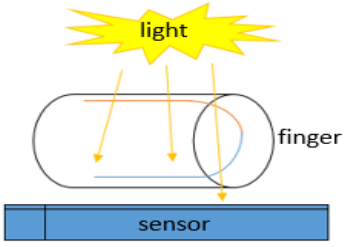

Figure 6: Light diode shining through a patient's finger to measure oxygen levels

While the effect of parasitic infection of the RBC's does affect the heme group it does not pose a significant change until the infection becomes severe, parasitemia approaching 5% [73]. Therefore, while it is usable for determining infection, it would not become useful until the infection was close to irreversible or had already cause irreparable damage to the patient. In addition, low levels of oxygen can occur for a multitude of reasons including lung damage or heart problems. Such a device could lead to a significant number of false positives due to the lack of specificity similar to clinical diagnosis.

These methods would require an electrical input to run the light and sensor receptor that would pose a problem in areas without consistent access to electricity and batteries would cause a significant waste problem.

4.3.4 Polarization

In addition, light can be utilized to detect the presence of hemozoin, a unique malaria waste product. Using a microscope this makes the hemozoin, the portion of the heme that was digested by the parasite which becomes paramagnetic, easily distinguishable because of its unique color and formation. Hemozoin is birefringent, or doubly refractive, so that when exposed to polarized light waves perpendicular to each other, will refract in two separate light waves [74]. Cells containing hemozoin are easily distinguishable under a microscope as the hemozoin appears a dark brown and black color. If white light is exposed to a sample, hemozoin will refract the light in polarized waves that can be detected through a light sensor. The amount of polarization can be related back the level of parasitemia to give an indication of how severe the disease is at time of diagnosis [75]. Figure 7 below shows a schematic of how such a device could work, white light is passed through a polarizer. The polarized light is then shown through a thin blood sample and the light refracted is captured by an analyzer and quantified.

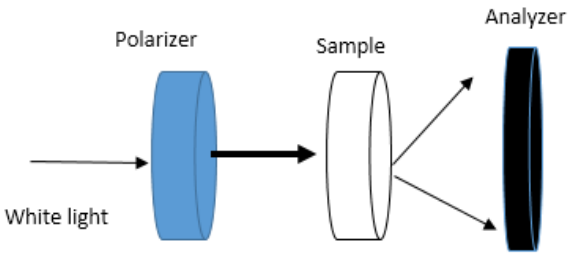

Figure 7: Detection of hemozoin through polarization light.

This device was not pursued as it is often used in conjunction with microscopy to make hemozoin more easily visible rather than replacing microscopy. Additionally, building an analyzer to quantify the light refracted requires advanced technical skills and can be very costly. This device is not a feasible option for this project due to the potential for high costs and the lack of technical skills need to assemble an analyzer to quantify light waves in an easily and cost effective way.

4.3.5 Antigen Markers

Antigen markers are what is being used currently in the rapid diagnostic tests offered today. While they make up the entirety of the options for field tests they are still liable to give a false result as they only should be 75% accurate with a parasite density of 200 parasites per microliter of blood [76]. These tests work by having a receptor antibody to one of the Histidine Rich Proteins (HRP- 1,2, or 3) released by the malaria parasite when it has infected the red blood cells. A blood sample is taken and passed through a cassette containing the antibody and if the HRP is present in the blood a mark will appear in the window to confirm that the blood contains parasites. A potential design approach for utilizing current this current technology due to its relatively low cost compared to microscopy while improving accuracy, is to improve storage of antibodies therefore, improving the durability of such tests. Freezing antibodies in sucrose and then lyophilizing, allows for storage of antibodies that can then be reactivated with hydration in sucrose [77]. This would require a methodology to create a vacuum chamber for the tests to be lyophilized but would create a longer lasting test. Current RDTs available degrade within fifteen months, with a 20% decrease in sensitivity [60]. One study found lyophilization can stabilize antibodies for over thirty-three months at 40°C without degradation, making it a promising method for improving existing diagnostic products [78]. Lyophilizers, however, generally cost greater than 7,000 dollars, making it even more expensive than some microscopes.

4.4 Final Design Selection

4.4.1 Design Concept

The final design utilized the magnetic properties of iRBCs, which are paramagnetic, compared to healthy, oxygenated RBCs, which are slightly diamagnetic. This causes infected cells to align with a magnetic field and healthy cells to align perpendicular to a magnetic field based on oxygenation. Infected cells have altered magnetic properties because of the digestion of hemoglobin by the parasite and the subsequent crystallization of the waste. This crystallization of the waste product heme, changes the orientation of the iron resulting in its paramagnetic properties [79]. The design consists of a microfluidics chamber with two output channels and a magnet that lays across the length of the flow chamber. The iRBCs are attracted to the side of the channel with the magnet and flow to the output channel closest to the magnet while healthy RBCs are either repelled or unaffected depending on oxygenation levels and, therefore, stay in the center of the flow and enter the other output channel. A magnetic field of 7,000 Gauss or higher is sufficient for separating the blood cells and at a flow rate of 0.14 μL/min the healthy and infected RBCs can be separated with an accuracy of 99.9% [30]. A faster flow rate can be used with a longer magnet and flow chamber to ensure the cells have prolonged exposure to the magnetic field as iRBCs in early stages of infection have weaker paramagnetic properties as not all hemoglobin has been metabolized.

4.4.2 Preliminary Evaluations of Design Concepts

To determine the final design, a selection matrix was developed using the design objectives. Each objective was assigned a weight from 1 to 5, with 5 being most important, and 1 being least important. Designs described above were compared to a baseline; the gold standard for malaria diagnosis, microscopy. A +1 was assigned to each design that was determined to be an improvement upon microscopy diagnosis for that objective, a -1 if the design fell behind the current gold standard, and a 0 if the design was similar to the baseline. Based on this matrix, the concept of utilizing separation by magnetism to diagnose malaria, showed to have the largest improvement upon the current gold standard of microscopy. This method was chosen due to its superior accuracy compared to other methods as well as the feasibility of making a low-cost design.

Table 3: Selection Matrix for Design Concepts

Objective	Accurate	Cost Effective	Durable	Ease of Use	Fast	Minimally Invasive	Final Rank
Weight	**5**	**5**	**4**	**3**	**2**	**1**	
Microscopy	0	0	0	0	0	0	0
RDTs	-1	1	-1	1	1	0	1
Liver Biopsy	0	-1	1	-1	-1	-1	-7
Oximetry	-1	1	0	0	1	1	3
VOCs	1	-1	-1	-1	1	1	-4
Rigidity	-1	1	0	0	1	0	2
Ultrasound	-1	-1	0	0	1	1	-7
Retinopathy	-1	1	0	-1	0	1	-2
Magnetism	1	1	1	0	0	0	14

4.4.3 Final Design and Chamber

Based on the decision to utilize magnetism as a detection technique, a design was developed to separate healthy and infected cells in a microfluidics chamber. Separation would allow for diagnosis through identification of infected cells in a sample as no separation would

indicate a healthy sample. A micrometer sized chamber was needed to allow for separation of individual red blood cells.

Feasibility of this design was heavily based on the ability to create a microfluidics chamber at a low cost. While the materials used are economically feasible, many methods for machining chambers with a micrometer scale resolution have significant costs. Figure 8 below is a computer-generated drawing of a negative mold of the chamber. The chamber was designed to be 100 mm in total length, with two output channels separated at a 45-degree angle for collection of red blood cells. The chamber was designed to have a width of 50μm and equivalent height, and therefore, is difficult to view in the figure below.

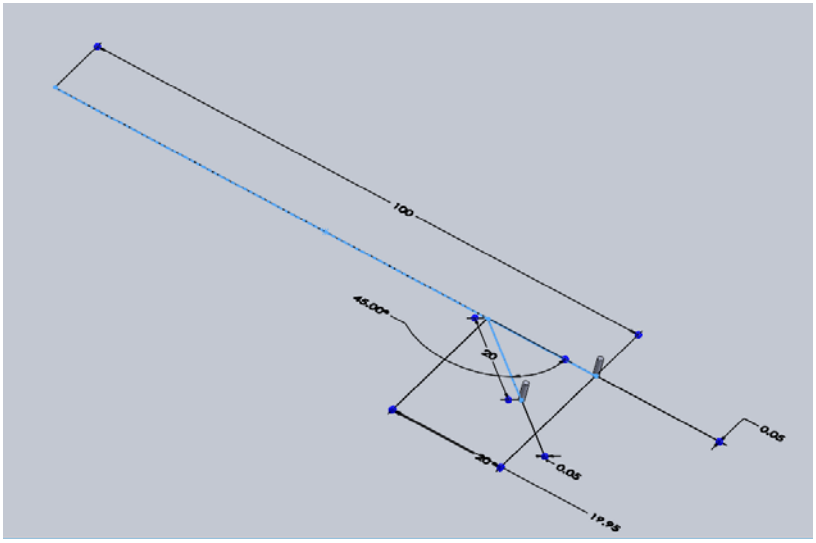

Figure 8: Dimensional drawing of overview flow chamber negative in mm.

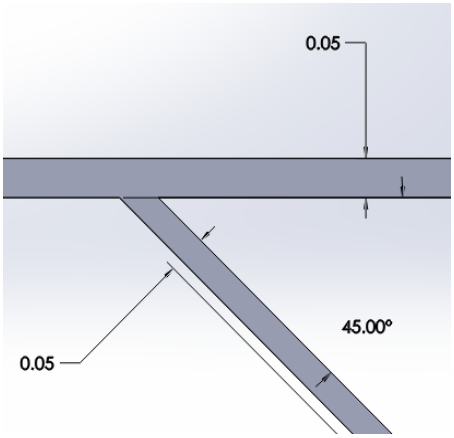

Figure 9: Drawing of the branching point of design. Each chamber has a width of 0.05 mm, and the angle between the chambers is 45 degrees.

The chamber was designed to have a small width so that even weak magnetic forces would have a noticeable impact on red blood cell movement. Therefore, a chamber width of 50μm was decided upon [80]. This helped minimize the distance infected red blood cells must

travel so that these cells will travel the full width of the chamber due to the pull of the magnet before they travel the length of the chamber due to the fluid flow allowing the cells to exit into the lower chamber [81]. The angle between the chambers was chosen as 45 degrees so that cells traveling along the streamline would not encounter the second exit channel to allow for continuous flow of the healthy cells.

Chamber composition was decided upon based on limited manufacturing processes that can obtain sizes in that are in the order of micrometers at low cost. After consultation with WPI's Professor Albrecht over various methodologies for microfluidics manufacturing and James Loiselle in WPI's manufacturing shops over the capabilities of various machines, a milling procedure to create a negative mold of the chamber was decided upon. Originally, 0.002-inch end mill (50.8μm) was planned to be used to mill into an acrylic sheet, however, due to the size of the end mill and the availability of milling equipment, breakage of the end mills was a concern as one mill purchased broke before use. Therefore, the chamber was machined by milling a piece of aluminum in various strokes to remove the material on either side leaving a 50-100μm ridge in the center that a polymer could then be casted over to create the chamber. Image of the mold can be seen in Figure 10 with two collection chambers indicated by red arrows and length of chamber traced with a red line as well as a drawing of the outline of the chamber.

Figure 10: Negative mold (below), ends of collection chambers indicated by red arrows. Above drawing is a schematic of the outline of the chamber

Figures 11 and 12 below show images of the collection chamber and branching point taken with a dissection microscope. Figure 11 is of the output chamber mold which is raised so when casted creates a well to collected liquid and Figure 12 is of the flow chamber where it splits towards the two separate output chambers.

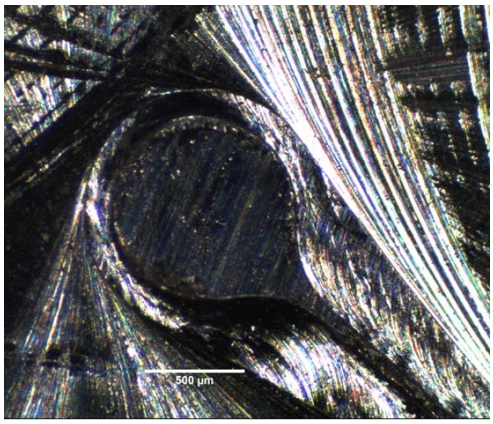

Figure 11: Output chamber, negative mold, machined in aluminum

The branching point of the negative mold was larger than the two chambers due to manufacturing restrictions. The milling process used to create the mold did not have the accuracy or size end mills to create a branch of desired size without damaging the surrounding channel mold.

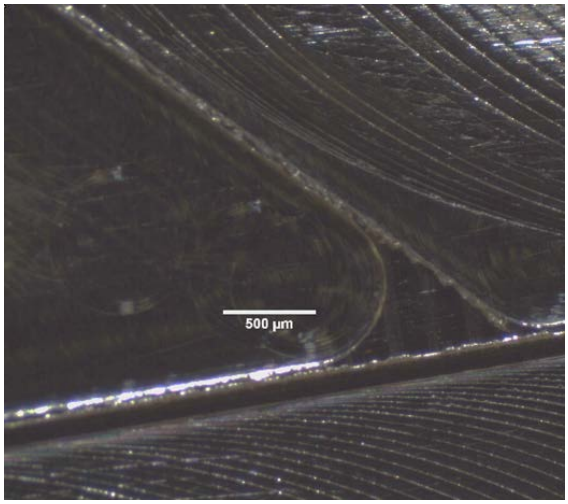

Figure 12: Flow chamber at split, negative mold, machined in aluminum

The final selection of the chamber material was based on a desire to create the chamber with the intention of be single use, but with the possibility of being sterilized. This decision was made as many areas where this device is intended to be used have limited laboratory resources and therefore, often reuse items that are intended to be single use to save money [82]. Testing for the device was done with a silicone derivative, polydimethylsiloxane (PDMS), as it was readily accessible and easy to create a chamber out of using a negative mold. PDMS is an ideal microfluidics chamber material as it has poor cell adhesion preventing cells from clotting and causing them to flow the entire length of the chamber. Due to the lack of cell binding sites, PDMS can be sterilized through several washes and soaking in boiling water [83]. Therefore, it was chosen as the chamber material as it is low cost and therefore, can be disposed of after

one use but is also easily sterilized for reusability.

The mechanism of action of the device depends on healthy red blood cells aligning with the streamline as occurs in the body once laminar flow is achieved in the smaller blood vessels. To ensure that the device would allow for laminar flow to occur, the Reynold's number (Re) and entrance length (Le) required for fully developed flow was calculated using the density of diluted blood (ρ), the velocity of the fluid flow (v), the width of the chamber (D), and the viscosity of diluted blood (μ).

$$R_e = \frac{\rho v D}{\mu} \quad (1)$$

$$\frac{L_e}{D} = 0.06 R_e \quad (2)$$

The calculated Reynold's number was 0.0012 indicating fully laminar flow in the chamber and the entrance length required for fully developed flow was 0.005μm indicating the fluid would be fully developed laminar relatively close to the entrance to the chamber since the chamber is greater than 100mm in length total. This ensures that the fluid will be laminar and fully developed for a majority of the time through the chamber ensuring the predicted fluid flow characteristics will occur.

4.4.4 Identification of Red Blood Cells

This design was based on the concept that all healthy cells will flow into one output chamber, and all infected red blood cells into the other output chamber. Therefore, if there are red blood cells in the infected chamber, a positive diagnosis of malaria can be determined. Due to the low volumes of blood that are passed through the microfluidics chamber, unaided visual inspection of the chamber for blood cannot be made. Even in a healthy sample, fluid will flow down each chamber, however, in the lower chamber just plasma with no cells will be present. Therefore, it is impossible to determine if there is cell presence just by visual inspection without a microscope. Table 4 outlines the calculation used to determine that visual determination of blood was improbable as less than two hundred picoliters would be present if infected cells were separated. These preliminary calculations were based on the assumptions that all iRBCs would be separated, the volume of healthy red blood cells is the same as infected, and that 10 μL of blood would be flown through the chamber. 10 μL was chosen as the minimum that would go through the chamber while keeping the time of the test relatively low, at 10 minutes of flow time.

Table 4: Sample Volume Calculations

Detection Level	200 parasites/μL
Volume of Blood Tested	10 μL
Total Infected iRBCs in Sample	2000 cells
Volume of Red Blood Cell	0.083 pL
Volume of Infected iRBCs in Sample	170 pL

Due to these small volumes, a method to determine presence of red blood cells was needed. A luminol test was determined to be the most effective way to detect the presence of blood cells. Luminol testing can identify trace amounts of blood up to dilutions of one part per million and therefore, was determined to be the most accurate way to decide if red blood cells are present in a sample [84]. Luminol can detect less than 0.01 picoliters of blood in a sample, and therefore, will react with the volumes of blood used in this test.

4.4.5 Magnet

The magnet used in the design was determined based on previous literature that used magnetic separation, a desired magnetic field of at least 7,000 Gauss, and total cost. Based on these criteria a rare earth, neodymium magnet, grade N45, that was four inches (101.6mm) in length was purchased as it was long enough to run along the 100mm length of the flow chamber and could produce magnetic fields up to 13,000 Gauss at the center of the magnet aligned with the center of the flow chamber [85]. A pure nickel wire with a diameter of 0.254 mm was used to run alongside the width of the channel. Nickel is highly susceptible to magnetization and in the presence of a magnet, will generate a magnetic gradient. This magnetic gradient is the driving force that pulls infected red blood cells down the width of channel. Chamber length, width, and flow rate were based previous literature to determine a channel size that would allow for sufficient time for cells to separate before reaching the branch in the channel [30], [86].

4.4.6 Syringe Pump

Maintenance of a slow flow rate was pertinent for allowing sufficient time for the magnetic field to pull infected red blood cells far enough across the width of the chamber to allow for separation. Electric syringe pumps are expensive, often costing more than a microscope, and therefore the development of a low cost syringe pump was important in order to meet the objectives of this project. Many designs were iterated through to create a device that could achieve the needed flow rates at a low cost.

Alternative designs included a multi-spring driven syringe drive. In this design, a spring would be used to push a rail-guided force plate along a fixed track like those found on electric syringe pumps. The force of the spring needed to be high enough to overcome frictional forces within the syringe, but the rate of displacement needed to be slow enough that the fluid was expelled at the required flow rate of 1μL/min. The standard 1 mL syringes used in the lab were

measured, and the rate of displacement needed to achieve this flow rate was calculated. The equation below shows the calculation used to find how far the syringe needs to be depressed to achieve a change in volume of 1µl.

$$\frac{1ul}{\pi r^2} = d \quad (3)$$

For a standard 1ml syringe, the distance the plunger must be depressed is 0.055 mm each minute to create the needed flow rate. To accomplish this, opposing springs would to be used to control the rate it displaced the plate while still allowing the force of the spring to overcome friction in the syringe. Initial positions of the spring would be determined based upon the amount of fluid desired to be dispensed for any given test. This design was ruled out due to the high spring constant required to have a spring depress at a slow enough rate and the cost and inaccessibility of springs with highly accurate spring constants.

Another alternative design was a gear system to use on a simple screw driven force plate syringe pump. A designed series of gears would translate an initial rotational velocity such as one that could be created by a small hand crank into smaller rotational velocities based on gear teeth width and gear diameter. This would allow for a rotational rate that would be easy for a technician to generate to be translated into the much smaller rotational rate that would be needed for the screw drive. A basic screw drive identical to the one that is described in the final design section was designed to be used with this device, but this method of powering the device was not chosen. This design was not developed further due to the complexity gear mechanism that would be needed and the opportunity for user error if the system was dependent of constant turning of the gears by an individual. An automated method of generating screw rotation was ideal for maintain the accuracy of the testing method.

The final design selected was a gravity powered method of rotating a screw-driven force plate to depress the syringe to drive fluid flow. The design incorporated two suspended weights that were held in equilibrium through a pulley system while being connected to the screw through a wire to a bar attachment. To start the device, one side of the weight suspension would be offset by an initial force to accelerate it. The force would be removed once the desired velocity is achieved. These weights in equilibrium would then continue to move at the achieved velocity, rotating the screw and driving the force plate. This rotation of the screw can be translated to a linear motion of the screw that will push the force plate into the syringe at the needed 0.055mm/min velocity and driving the flow rate.

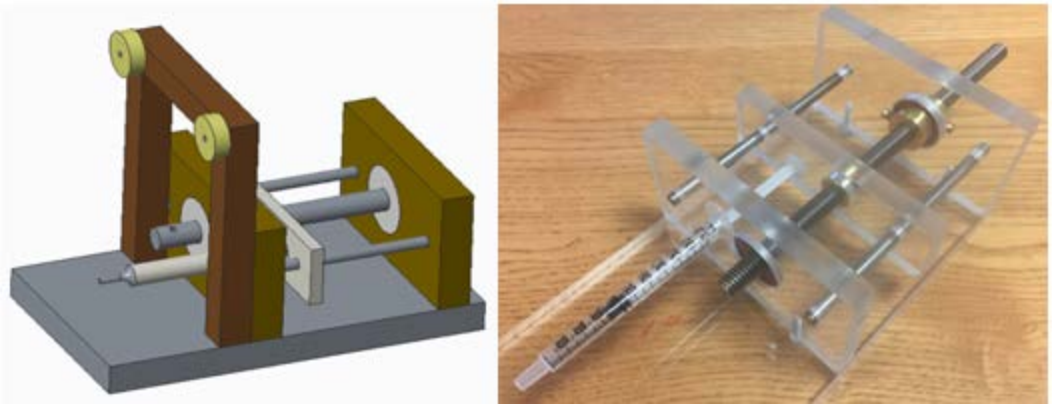

Figure 13: Preliminary design for screw driven mechanical syringe pump without pulley attachment.

The base syringe pump mechanism was modeled off the electrical screw driven pumps being used in the lab for testing. It was fabricated with help from Thomas Partington in the Goddard machine shop at Worcester Polytechnic Institute. The material chosen for the plate, base and walls of the devices was a plexiglass acrylic sheet that was cut to specification from the computer generate model shown in Figure 13 above. Stainless steel rods were used as the guiding supports on the left and right sides of the device to hold the pushing force plate in alignment while in motion. To reduce the friction in the system, skateboard bearings were chosen to support the drive screw due to ease of rotation. The inner dimensions of the selected bearings supported a 5/16th sided standard screw-threaded rod, which was then used to displace the force plate using a matching threaded cuff set into the force plate. This basic model proved to be functional for displacing the syringe.

The amount a screw would have to rotate depends on its pitch/threading and diameter of the screw. The number of rotations per minute can be calculated by dividing the distance needed to travel (d in above equation) by the pitch of the screw. The distance the weights need to travel per minute was calculated based on the diameter of the screw and the number of rotations per minute by multiplying the number of revolutions per minute by the circumference of the screw to get the linear distance needed per revolution. In this equation, V is velocity, r is the radius of the screw, and rpm is the number of revolutions per minute required to achieve desired linear displacement.

$$V = 2\pi r * rpm \quad (4)$$

The screw used for the syringe drive was a 5/16th standard threading screw rod and had 18 threads per inch (TPI) resulting in 1.4mm displacement per rotation. With the required displacement of the syringe at 0.055mm/min, the screw would need to rotate at 0.04rpm to achieve this speed. With a major diameter of 7.95mm, 0.04rpm is equivalent to 2mm/min. Using fine adjustment screws with a higher thread density would increase the degrees of rotation, however, due to time constraints all testing was performed with the 18 TPI screw. Therefore, if achieving the correct velocity is dependent on an initial gravitational force, the force must be applied for 3.3e-6 seconds at an acceleration of 9.81m/s^2 to achieve this speed. Alternatively, forces can be applied to both sides of the pulley to offset each other and achieve a net

acceleration that is smaller, making the desired rotational speed more easily obtained. Additionally higher thread screws would require a faster rotational speed to achieve the same linear distance due to their higher pitch. With further development, the syringe pump can be altered to better suit the needs of this project.

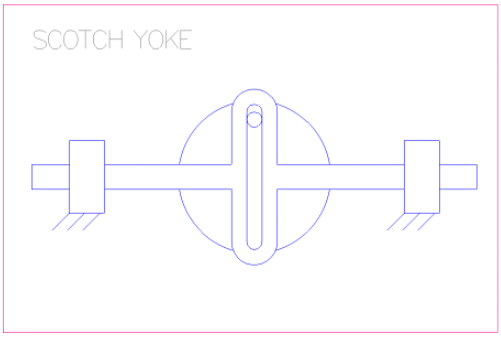

Figure 14: A basic scotch yoke mechanism that translates linear motion into rotational motion.

An alternative design for translating linear velocity into rotational velocity was a scotch yoke mechanism that could be used to rotate a pin attached to the screw of the drive with a band. This mechanism would allow for smoother translation of the velocity from the weights, as it allows the wire to be kept horizontal while the weights move. However the difficulty overcoming friction within the system was the major restriction to the functionality of the syringe pump device, and the band mechanism added more of these forces and as such was not pursued.

Due to constraints in developing a functioning syringe pump for testing, an electric syringe pump was used during prototyping of the microfluidics chamber to get proof of concept of cell separation in the chamber.

Chapter 5: Final Design Verification

This project's main objective was to design and create a device that could accurately detect malaria at low cost to reduce the financial burden of diagnostics in areas where malaria is prevalent. The device was intended to withstand the tropical conditions where malaria is widespread without compromising accuracy and to cost less than the golden standard of diagnosis, microscopy. The altered magnetic properties of infected red blood cells (iRBCs) provide a feasible mechanism for separating infected and healthy cells. A microfluidics device was cast from polydimethylsiloxane (PDMS) using an aluminum milled negative mold to fabricate the flow chamber with two chambers for cell collection. A rare-earth magnet was used to provide the local magnetic field needed for separation and was focused through a nickel wire for directionality. To verify the functionality of this design, the device was tested with water, healthy blood, and malaria-infected blood to verify continuous flow and separation of healthy and infected cells. After a sample was processed through the channel, luminol was used to test for cell presence in each chamber, and the results were used to determine a diagnosis.

Figure 15: Schematic of chamber with magnet aligned along the length

5.1 Preliminary Chamber Design

The negative mold was milled using a HAAS milling machine at Worcester Polytechnic Institute. An aluminum block was milled on the surface to remove a surface layer of approximately 50-100µm. The middle region of aluminum was not milled to leave a center ridge of 50-100µm in width and height in the center to act as channel mold. Milling the branch mold involved milling in repetitive circular motions in between where the two channels would be. Due to constraints with size of end mills available and accuracy of machines, the area around the branch was much larger than that of the surrounding chamber. The milled negative mold as shown in Figure 10 was used to cast two microfluidics chambers in PDMS. PDMS is a polymer silicone compound often used for microfluidics devices because of its ease of use and low cost. PDMS was used throughout the project as it was readily available, is low-cost, and can be easily sterilized. When cured upon a featured surface, PDMS maintains the features of the mold down to micrometer level making it a good choice for microfluidics applications with strict tolerances. The first chamber was fabricated with a 10:1 ratio of elastomer base to curing agent, put in a vacuum chamber for 45-60 minutes to remove bubbles, and then was placed in a curing oven set to 60C for 90 minutes. Later chambers were fabricated using the same protocol, but a 5:1 ratio of elastomer base to curing agent to make a stiffer mold for increased durability.

Pictures of the chambers were taken with a Leica EZ4D microscope at two different

magnifications (8x and 35x). Pictures of the grid on a 4-Chip Hemocytometer were also taken at these magnifications to use as scale references during processing. ImageJ was used to set a global scale with the hemocytometer pictures and five measure lines were drawn on each image to gather average dimensions. Lines were drawn in ImageJ using the line tool from across the chamber width. Lines were drawn on four different regions of the chamber, equally spaced apart down the chamber to check for consistent width. Each region was measured five times to validate consistency. The mean and standard deviation of each chamber were calculated as shown in Table 5 below. All measurements taken at each magnification were averaged together to get the mean and standard deviation width for the entire chamber.

Table 5: Negative Channel Widths at 35X Magnification

	Mean (mm)	SD (mm)
Region 1	0.087	0.004
Region 2	0.088	0.000
Region 3	0.092	0.003
Region 4	0.094	0.010
Total chamber	**0.090**	0.003

The average length along the negative shaft was an average of 90 +/- 3μm. Standard deviation of the total chamber was based on the standard deviation between the mean measurements at each region.

While the width of the chamber was around twice what was intended, the chamber still allows for the separation of infected cells as cells flowing along the center or lower portion of the streamline have time to move down the chamber. Infected cells at the top half of the streamline do not have sufficient time to move down the width of the chamber due to this wider channel. It still allows some of the infected cells to separate and does not affect the flow of healthy cells. Therefore, the correct diagnosis can still be obtained even with the wider channel. Figure 16 shows the aluminum milled mold and the resulting PDMS chamber.

Figure 16: Milled mold (top) and PDMS chamber (bottom), approximately 127mm in length. Red arrow indicates where the chamber splits on both the negative and casted chamber

5.2 Preliminary Fluid Flow Testing

After fabrication, a 1ml syringe with a 34-gauge needle attachment was inserted into chamber with water and pressed manually to watch fluid flow. Water flowed down the entire length of chamber. All water that could be seen under the microscope flowed straight into the main outlet, none was observed to branch off into lower outlet during first flow test. Repeated flow tests had observable fluid flow down both chambers.

The syringe was then fitted to a syringe pump, and the pump was set to push the fluid into the chamber at a rate of 1.0 µL/min. Flow was continued until water reached the end of each chamber and tests repeated five times to ensure reproducibility. Each test indicated uninhibited flow through the chamber and a lack of pressure buildup during flowing, allowing for further testing to be conducted.

5.2.1 Initial Blood Flow

For initial testing of blood flow in the chamber, blood was collected from laboratory rats after a small animal surgery laboratory had concluded. The blood was collected in vaccuvials and treated with 7.2 mg of ethylenediaminetetraacetic acid (EDTA) per milliliter of blood to prevent it from clotting. In preparation for flowing through the chamber the blood sample was mixed with Dulbecco's phosphate buffered saline (DPBS) containing calcium and magnesium as a flowing agent in a ratio of 1 mL of blood to every 4 mL of DPBS.

The sample was attached to a SPEC syringe pump and pushed into the chamber at the flow rate of 1.0 µL/min. Initial testing resulted in a backflow of blood, with none of the sample flowing the entire length of the chamber. A strip of polystyrene was place over the top of the chamber and pressed down to act as a seal to help with blood flow. The sample flow still indicated signs of backflow with the diluted blood sample leaking out the sides of the chamber and back out the needle entrance.

Due to the backflow, the change in pressure needed to achieve the desired flow rate of 1 µL/min was calculated using equation 8. Viscosity was taken to be 3.5 cP for blood. Water has a

viscosity of 0.890 cP, resulting in a much smaller pressure required when flowing water through the chamber and therefore, could flow down the entire length of the chamber without noticeable backflow. The change in length was 100mm and the height and width was 0.090mm based on measurements under the microscope at 35X.In the following equation ΔP is the change in pressure, μ is the viscosity, Q is the flow rate, Δx is the change in length, h is the height of chamber, and w is the width.

$$\Delta P = \frac{12\mu Q \Delta x}{h^3 w} \quad (5)$$

The change in pressure needed was found to be 1.07 kPa. The applied force to a 1ml syringe to achieve such pressure was calculated based on the syringe plunger's diameter of 1cm. The needed force was found to be 0.084 N as seen in equation 2. Although the functional losses due to friction in a syringe range up to 20%, which would then require a 0.104 N force to compensate for the frictional force. The syringe pump is capable of applying this force, however, to maintain this pressure differential, there must be a seal around the chamber to create such a pressure gradient.

$$F = PA \quad (6)$$

The hypothesized cause of the backflow was air bubbles created when the needle was inserted into the chamber. A 34-gauge needle has an outer diameter of 190μm and an inner diameter of 80μm. Therefore, the needle was larger than the flow chamber so when inserted pushed upwards on the cover of the chamber causing an air bubble to form. This air bubble caused a loss of pressure and for the pressure gradient to favor fluid reflection back out the entrance of the chamber rather than to flow down the chamber.

5.3 Final Chamber Design

Based on initial testing, a second negative mold was milled with a larger entry port for the needle to help reduce backflow. The dimensions of the chamber after the entry port were set to 50μm as with the initial design. Figure 17 and 18 show the new mold for the entrance chamber that leads into the flow chamber of approximately 74 +/- 4μm. This design was intended to allow the needle to rest in the entry port and push the blood into the chamber as with the initial design, the insertion of the needle into the flow chamber itself resulted in an air bubble due to the larger size of the needle compared to the chamber.

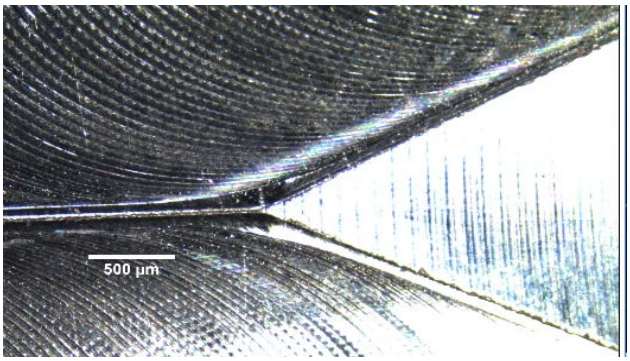

Figure 17: Negative mold of entrance chamber.

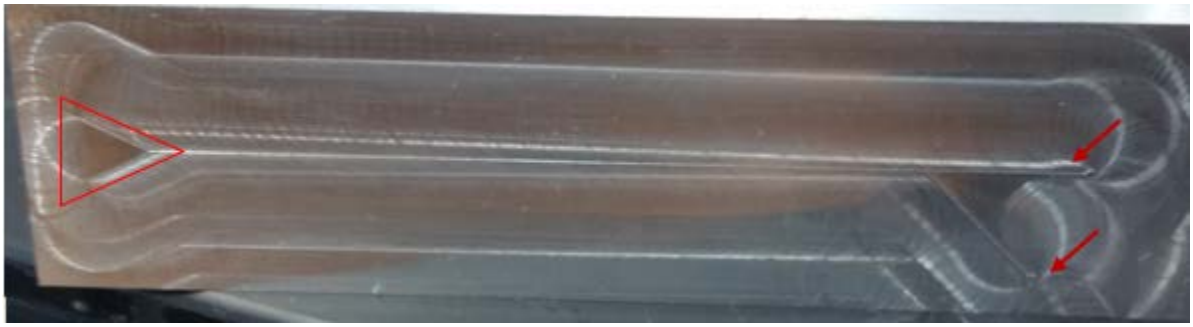

Figure 18: New chamber with larger entry port (red triangle), collection chambers indicated by red arrows.

A PDMS chamber was cast using this mold using a 5:1 ratio of elastomer base to curing agent. This ratio was used as it created a stiffer chamber compared to the 10:1 ratio creating a more durable and easy to use chamber. The chamber length was again validated using ImageJ. Images of the chamber were taken at various regions to measure the width across the entire length of the chamber. A hemocytometer with known widths was used as the standard to convert pixels to millimeter measurements. Measurements in each region were taken five times to ensure consistency. Using all measurements taken, the chamber was found to be 74µm with a standard deviation of 4µm. This chamber was closer to designed value of 50µm compared to the initial chamber due to refinement of the manufacturing process.

Table 6: Final Chamber Width at 35X Magnification

	Mean (mm)	SD (mm)
Region 1	0.076	0.003
Region 2	0.075	0.004
Region 3	0.077	0.003
Region 4	0.072	0.005
Total chamber	**0.074**	0.004

5.4 Blood Identification

Due to the small quantities of blood being passed through the device, unaided visual identification of red blood cells in the infected chamber is impossible; 1 microliters of infected blood yields only 60 picoliters of infected red blood cells. Initial testing included identification of infected red blood cells under microscopy to confirm proof of concept. Further testing was done using a luminol solution to detect the presence of red blood cells. Luminol is a chemical solution that releases a blue chemiluminescence glow when it reacts with an oxidizing agent, in the case of red blood cells, hemoglobin.

5.4.1 Luminol Testing

A premixed luminol set was obtained from Innovating Science. It contained a premixed luminol solution and 6% hydrogen peroxide to obtain chemiluminescence. The solutions were mixed in a 20:1 ratio and lights were turned off. No blue light was observed. With lights on, it was observed that the luminol solution (blue) and hydrogen peroxide (clear) had made a yellowish-brown solution and a few bubbles appeared. This solution was made six times before chemiluminescence was observed between the luminol and the hydrogen peroxide.

Luminol was tested against both rat blood, hydrogen peroxide, and a mixture of both to test its reactivity. The luminol was added to rat blood in a 20:1 ratio to allow for full dilution of the blood to increase chance of reactivity. No chemiluminescence was observed in dark conditions. The 6% hydrogen peroxide was added to solution to make it 20:1:1 with luminol solution, hydrogen peroxide, and blood. This was based on instructions in the provided luminol kit for mixture of hydrogen peroxide and luminol was well as ensure a large enough volume for visual observation. There were noticeably more bubbles in the blood-luminol-hydrogen peroxide mixture than in the just luminol and hydrogen peroxide mixture as can be seen in Figure 19. However, the hydrogen peroxide causes a reaction with the luminol with or without the presence of blood so solutions of just blood and luminol were mixed and observed under dark conditions. Various ratios of the solutions were attempted with up to a ratio of 50:1 luminol-blood down to 1:1 ratio in increments of 10 to maximize chances of reactivity. In addition, 50-100µl of blood was scraped onto a cell culture plates, luminol was added to one immediately and one after the blood was dried. There was no visible blue light under dark conditions for any attempted solutions.

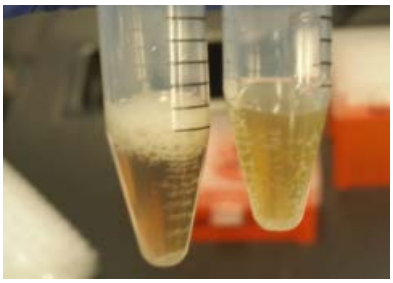

Figure 19: Images of the luminol/hydrogen peroxide mixture. Mixture with blood (left) and control solution (right).

The luminol kit obtained was intended for chemiluminescence demonstrations between the luminol solution and the hydrogen peroxide provided. A black light was shown on each of the solutions made under dark conditions and no luminescence was observed.

A new kit from Pioneer Forensics specifically for blood detection with luminol was obtained for additional trials. The new luminol kit contained two parts, one luminol solution and one of dry sodium perborate. Approximately 10 flakes of the sodium perborate were mixed until dissolved 1ml of the luminol solution and used immediately. 50µl of the luminol mixture was added to five wells that had previously had rat blood added to them and dried. The lights were turned off and chemiluminescence in each well containing the luminol solution and blood was observed. Figure 20 shows the results of the experiment. The bottom left well contains the luminol solution but no blood and the other five wells contain the dried rat's blood and the luminol mixture.

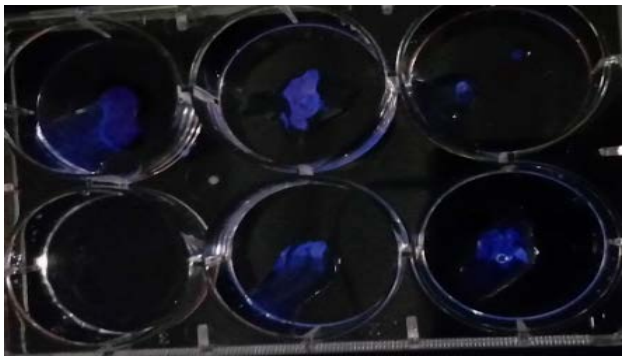

Figure 20: Luminol and blood in dark conditions. Bottom left well is control luminol solution. A blueish glow observed in each well with luminol and blood.

Sensitivity Testing

To identify the sensitivity of luminol testing, serial dilutions of blood samples was performed to determine the feasibility of using such method in this device. 1µl of blood was serially diluted in 1:10 dilution increments down to $1:10^8$ dilution. 10µl of luminol solution was added to each of the wells under dark conditions and chemiluminescence was observed in each well as shown in Figure 21 for week 3. Testing was repeated three times. The chemiluminescence was visible for an average of 132 seconds for dilutions $1:10^5$ through $1:10^8$ and 186 seconds for the less dilute samples (1:10 through $1:10^4$). While all wells showed chemiluminescence dilutions below $1:10^6$ were very faint and only visible when there was not external light on the wells. The smallest volume detected is equivalent to a parasitemia of 10 infected red blood cells per microliter of blood, which is far lower than symptoms usually present.

Figure 21: Blood samples with luminol (top row). Diluted from 1:10 (top left well) blood with DPBS down to 1:10^8 (top right well).

Reactivity Testing

To ensure that the luminol reacts with the hemoglobin in the red blood cells and not the plasma, the hematocrit was separated from the serum and both were tested with luminol. Blood samples were centrifuged at 2200 RPM for 10 minutes [87]. The top layer (plasma) and bottom pellet (red blood cells) were pipetted into separate wells of a twelve well plate.

Luminol solution was added to wells as per above protocol and observed under dark conditions. Luminescence was observed in all the wells containing red blood cell pellet and in four of the six wells of the plasma. Luminescence was observed in the supernatant of centrifuged blood samples approximately 82% of the time. In these samples, there was not a distinct plasma layer so centrifuging at higher speeds and longer times were completed to ensure separation.

Blood samples were centrifuged at 2200 RPM for 20 minutes. After the first centrifugation, the supernatant was aspirated into two new vials and centrifuged again to ensure full separation conditions. Luminol was added to samples of the supernatant and no chemiluminescence was observed in the supernatant of plasma.

Degradation Testing

The luminol is intended to have a shelf life of 2 years at room temperature. To test its shelf life at warmer temperatures, the luminol solution and sodium perborate were stored separately in an incubator set to 37°C. The reactants were taken out every 7 days and a 100µl solution was made each time, 10µl was added to blood serially diluted in 1:10 dilution increments down to $1:10^6$ dilution with DPBS to test if chemiluminescence could still be observed after storage in warmer conditions than intended. After eight weeks, the luminol mixture still showed reactivity in all dilutions of blood. The sodium perborate flakes melted to liquid within 24 hours of being in the incubator, but were still reactive. Sodium perborate was stored in individual wells with between 10 to 20 flakes per well intending that one well be used each time the luminol solution was made.

Device Validation

When validating the device, the collection chambers were observed under a microscope to check for cell presence. Luminol was then added to the chambers to ensure the chemiluminescence was observed in the correct chambers. Luminescence was only observed in the chambers with cell presence validating the detection method. Figure 22 shows the chamber without luminol and with luminol for both healthy and infected blood.

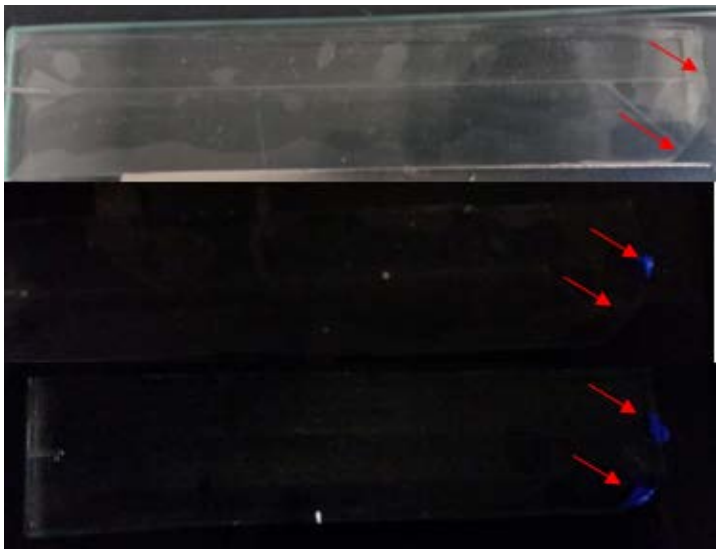

Figure 22: Chambers with luminol: healthy blood (middle) and infected blood (bottom), chamber at top used as reference for orientation. Red arrows indicated location of collection wells. Pictures taken with cell phone camera.

5.5 Final Fluid Flow Testing

5.5.1 Water Flow

After fabrication of a new chamber, tests were repeated with water initially to ensure proper manufacturing of channel. A 34-gauge needle attached to a 1 ml syringe was inserted into the entry port of the channel and manually depressed. Water flowed down the entire length of the chamber and into both collection outlets. The syringe was then attached to a syringe pump and set to 1 µL/min and water continued to flow down each chamber at the set flow rate ensuring that both chambers were cleared of blockages.

5.5.2 Blood Flow: Clotting

Blood was then used for further flow testing. Blood was treated as described in section 5.2.1. A syringe containing the blood sample was fitted with a 34-gauge needle, attached to the syringe pump, and inserted into the entry port of the chamber. The blood moved approximately three fourths down the length of the channel before a clot was encountered in the syringe preventing further blood flow. The slow flow rate increases the risk of clotting due to the extended time the blood is sitting in the syringe, however, this flow rate is required to achieve desired effects from the magnetic field. Additionally, the large step down in size from the 1ml syringe to the 34-gauge needle, which is over a 4.5 mm difference in diameter, also increases the risk of clotting. Therefore, a 20-gauge needle was used for continued testing to allow for a larger space for the blood to pass through to reduce the risk of clotting. The chamber was cast again with needle tip inserted into the PDMS before curing so that there was a mold for better fit of the needle into the entry port of the chamber to reduce the risk of air bubbles forming when the needle is inserted into the chamber.

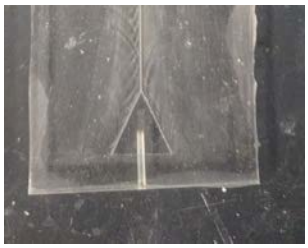

Figure 23: Image of casted chamber with entry port for needle

5.5.3 Chamber Seal

With the larger gauge needle, continued issues with the fluid leaking out of the chamber occurred due to the markings on the mold and subsequent chamber due to the manufacturing technique. Due to the small nature of the device and subsequent pressure build up, these markings from the machining acted as additional flow chambers extending off the preliminary chamber resulting in the fluid flowing out laterally from the chamber rather than flowing down the entire length of the chamber. This leakage was a result of the end mills used in machining the device that caused coarse surface finish that was seen on the PDMS chamber as well. To minimize this issue the mold smoothed by hand using 600 and 1200 grit sandpaper to reduce markings around the flow chamber where fluid could leak. With this smoother mold, the chamber sealed much better to the glass slide allowing for improved fluid flow. With this mold, at 1µL/min, blood diluted 1:4 with DPBS flowed through the chamber and into the correct collection chamber.

Issues with the fluid leaking outside the chamber still occurred in chamber after many repeated uses dues to cracks in the insertion point for the needle. Needles that were not inserted correctly into the port or were not completely straight would hit the PDMS and eventually caused it to crack for the fluid to leak out the sides. Therefore, chambers had to be repeatedly checked to ensure that there were no cracks that would cause issues with leakage. Chambers still could be repeatedly used ten to twenty times without noticeable issues.

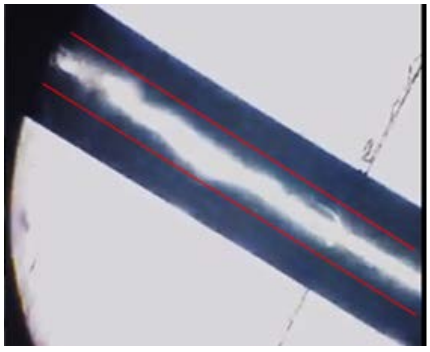

Figure 24: Microscope image of the needle entry port at 20X, red lines outline the crack down the length of the entry port.

5.5.4 Healthy Blood Flow

Rat Blood

Flow tests with the laboratory rat blood were repeated as before. The blood was diluted with DPBS at a ratio of 1ml of blood to 4ml of DPBS. Approximately 0.2ml of the diluted blood was loaded into a 1ml syringe with a 20-gauge blunt needle. The needle was inserted into the entrance port of the chamber and flowed at a rate of 1µl/min. Chambers were observed under a microscope for cell presence using a hematoxylin and eosin stain to stain the cytoplasm of the cells as well as luminol was added to each well to observe chemiluminescence indicating the presence of red blood cells. Cell presence was only detected in the main chamber and not in the lower chamber, indicating a negative result for malaria.

Human Blood

Negative control tests were repeated with healthy human blood obtained from Dr. Pamela Weathers and Brian Gruessner in the Biology/Biotechnology Department at Worcester Polytechnic Institute. A spun down human blood sample was resuspended in DPBS to achieve a 40% hematocrit fluid sample. 1ml of this sample was then added to 4ml of DPBS to make the flow solution. Tests were repeated as with the rat blood. The diluted blood was flowed at 1µl/min. Red blood cells were confirmed to only flow down the chamber that flowed straight and not into the lower chamber by adding luminol to each chamber and observing any luminescence. Fluid sample from each collection chamber was also checked under a microscope to ensure cell presence only in main chamber and not in the chamber for infected red blood cells. Due to time constraints, tests with healthy human blood were only conducted twice, both with the correct result. However, the assumption was made that the healthy human and rat blood behaved the same in the conditions of the tests as the hematocrit and oxygenation levels of samples were comparable. Total results including both human and rat healthy blood were combined for accuracy calculations. Out of seventeen tests conducted, fifteen showed the correct result with no cells in the lower chamber, and two tests had cells in the lower chamber resulting a false positive result. This resulted in an 88.2% accuracy rate for tests involving uninfected blood.

Table 7: Healthy blood testing results

Negative	False Positive	Accuracy (%)
15	2	88.2

5.5.5 Infected Blood Flow

Positive control tests were completed using the same blood as that in the negative human blood tests. A centrifuged pellet of cultured red blood cells infected with a 3D7 strain of malaria was used to model a sample of infected blood at both 0.2% and 0.028% parasitemia modeling both an average patient's parasitemia and one very soon after infection respectively. These samples were treated as with the healthy blood. Red blood cell pellets were resuspended

to achieve a 40% hematocrit sample and then diluted with DPBS in the same 1:4 ratio with one part blood and four parts DPBS. Flow speed through the channel was set at 1 µl/min using a syringe pump. Cell presence at the collection chambers was confirmed through luminol detection.

Table 8: Infected blood testing results

Parasitemia	Positive	False Negative	Null	Percent Accurate
0.2%	4	1	0	80
0.028%	8	0	1	88.8

Shown in Table 8 are the results of infected blood testing, which resulted in a success rate of 80% for 0.2% parasitemia and 88.8% for 0.028% parasitemia. From the preliminary results of the chamber, reliable separation of healthy and infected blood has been achieved for both parasitemias.

5.6 Sterilization

The chamber was designed with the intention that it could be single use to reduce need of sterilization, however, cost can be reduced through multiple uses of a single chamber. To maintain the integrity of the test, the chamber must be sterilized and completely cleaned of blood in between tests. A low cost and low resource method for sterilization is use of boiling water. To ensure a PDMS chamber could be sterilized through this method, chambers were repeatedly placed in boiling water and checked for adhesion and presence of blood cells.

5.6.1 Adhesion

Adhesion of the chamber was measured during the testing procedures following sterilization. A picture was taken of the chamber after fifteen uses to determine if there was any degradation in its ability to form a complete seal on the glass slide. After 15 uses of the device along with sterilization in boiling water for 15 minutes the area not adhered to the glass slide is 2.07 cm^2. There did not seem to be many detrimental effects from repeated sterilizations to the PDMS chamber, as it still allows for a solid seal around the portion surrounding the channel itself, as shown in Figure 25. Tests completed with chambers sterilized fifteen times still allowed from complete fluid flow down the chamber as losses in adhesion were not around the chamber and therefore, did not affect fluid flow. This meets the desired repeatability of ten uses, which was used when determining the cost of a single test.

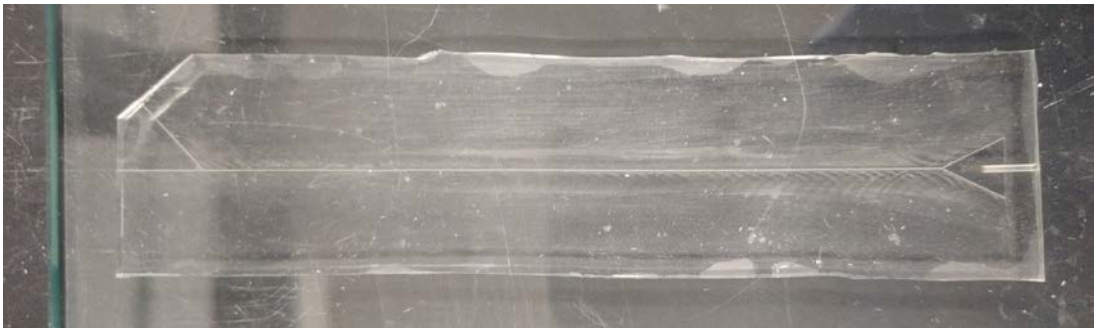

Figure 25: Chamber after 15 sterilizations and tests, lighter areas around the edges of the device indicate where it will not adhere to the glass slide.

5.6.2 Sterility

Blood was flowed through chambers for sterility testing. Luminol was added to the top of the chamber to ensure that luminol would have visible luminescence with the blood in the chamber. Luminescence was observed in all five chambers tested. Blood was passed through the chambers again and each chamber was put into a beaker of boiling distilled water for sterility testing. Chambers were removed after one minute dried with a paper towel. Luminol was added to each chamber along its length to check for chemiluminescence. There was no luminescence observed in these chambers, which was used as an indication that the chamber had been sterilized as there was no cells left on it. Chambers were then observed under a microscope and there was no noticeable presence of blood cells in the chambers.

Chapter 6: Discussion

The goal of this project was to create a device that could diagnose malaria at a low enough cost to be used in endemic countries where access to proper diagnostic and laboratory equipment is often limited. The primary objectives established for this project were that the device must be accurate and low cost. In addition, secondary objectives of durability, ease of use, speed of diagnosis, and minimally invasive were established. The device meets these objectives to provide a low-cost method to detect malaria.

6.1 Objectives

6.1.1 Accuracy

For the detection of blood, luminol is accurate at one part per million, which was seen in testing and verification (Figure 20). Luminol resulted in detection of blood in dilutions down to 1 picoliter of blood in 10µl of DPBS, equivalent to volumes of parasitemia at a level of two infected cells per microliter. This would allow for accurate detection of the presence of blood in the output chamber where only infected blood cells should flow.

Preliminary testing using healthy rat and human blood showed accuracy in testing healthy blood 88% of the time, indicating a method of diagnosis more accurate than many rapid diagnostic tests, but fell below the 10% standard for false positives. However, further tests are needed to test repeatability as the number of tests run to completion was limited due to fluid leakage from an improper seal. With more time, a larger sample size of tests could give a better indication of how accurate the device is. False positive rates are likely induced by the chamber split as manufacturing limitations created a much larger area where the two channels split off inducing some mixing in fluid flow, which would hinder the separation process. Previous studies have shown no healthy red blood cells going into the lower output chamber, therefore, if in future testing this design achieves similar results the device could reach near 100% accuracy in detecting malaria [30]. Magnetic separation of cells offers a far more accurate method to detect malaria than the 75% accuracy required by the World Health Organization for rapid diagnostic tests.

Testing with infected red blood cells has shown similar levels of accuracy with 85.7% of the tests showing the correct result, 7.14% false negative, and 7.14% null tests. Out of fourteen tests completed, one showed a false positive and one showed cell presence only in the infected chamber and therefore, was considered null/invalid. Five tests were completed at a parasitemia of 0.2% (8000 parasites per microliter) and nine tests were completed at 0.028% (1120 parasites per microliter). The lower limit of 0.001% parasitemia was not tested due to limitations in resources as the volume of infected blood needed was smaller than any pipettes available could accurately dispense. Therefore, more testing needs to be done on this device to determine its lower limits to test if it is more accurate than the levels set by the World Health Organization. Initial testing does show this device offers a promising accurate method to detect malaria.

6.1.2 Cost

The current gold standard for malaria diagnosis is microscopy, which given enough time and skill of the microscopists can be very accurate in detecting low levels of parasites in a patient's blood. However, many endemic regions do not have access to a working microscope or the ability to train health workers on how to identify and diagnose malaria under the microscope. Therefore, this device was intended to cost less than fifty cents per test with only one test used in most cases to diagnose a patient.

For the chamber, the cost will depend how the chamber is manufactured. Currently, the chamber is cured in polydimethylsiloxane (PDMS), which is relatively low cost. When bought in bulk, the chamber itself could cost fifty cents. A cost of which could be lowered if the PDMS was used for several tests. Through testing, PDMS chambers were sterilized in boiling water, which was determined to be sufficient for removing all cell presence from the chambers. Therefore, with repeated uses the cost of the chamber is under five cents if the chamber is used ten times. The magnet used in this device costs around 14 dollars, and neodymium magnets will last over 100 years with only losing 1% of their flux density. Therefore, the magnet could be used indefinitely and the more tests performed the lower the cost will be.

Luminol will cost less than a cent per test but depends on how small volumes are mixed at a time as the solution made at one time will go to waste if not used within a couple hours as it will lose its reactivity. The luminol mixture costs thirteen cents per milliliter of solution. Even if some of the solution goes to waste, making one milliliter batches of luminol will still make this device and test low cost compared to the alternative investment of a microscope and related equipment.

The biggest constraint in meeting the low-cost objective of this project was the syringe pump. An essential part of the mechanism of the device requires a controlled flow rate of around 1 µl/min. However, automated syringe pumps, such as the one used in this project for proof of concept testing, cost several thousand dollars. These pumps can cost more than a microscope and therefore, would not be affordable in places that do not own a microscope. The mechanical syringe pump designed to avoid these costs is still not validated in its ability to control such flow rates but is relatively low cost as it is built out of acrylic, and therefore, would help maintain the low-cost nature of the device at a materials cost of $36.65 per pump. Before accounting for the cost of the syringe pump, all the other parts in the device result in a cost of around fifty-three cents per test after several tests are performed. If a low-cost syringe pump can be validated, this device will have a significantly lower up front cost compared to a microscope and be comparable if not slightly lower in cost compared to rapid diagnostic tests. The total cost of materials used in the device excluding the aluminum mold was 119.21 USD, and the cost per test was 0.79 USD. The initial upfront cost of the device is significantly lower than that of a microscope and the cost per test is lower than that of rapid diagnostic tests.

6.1.3 Durability

One of the secondary objectives of this project was that the device was durable as a common problem were current rapid diagnostic tests (RDTs) is the loss of accuracy when stored for long periods of time especially at warm and humid locations.

The neodymium magnets last for over 100 years without losing more than 1% of its magnetic flux. Luminol lasts over two years at room temperature and through testing has shown no degradation in hot and humid conditions mimicked by the incubator over the course of two months. Continual testing would be needed to validate the degradation rate of luminol in hot temperatures. The intended PDMS flow chambers cannot be used continuously but may be used for several cycles of testing before being replaced. After ten to twenty uses, the PDMS flow chambers needed to be replaced due to tearing in the chamber. PDMS flow chambers not being used can last for years without adverse effects on the test. No aspect of this device has adverse effects to being stored in hot and humid conditions and therefore, offers a promising option compared to rapid diagnostic tests that degrade quickly.

6.1.4 Ease of Use

Another secondary objective was to make the device easy to use so that administrators of the diagnostic test would not require extensive training to operate the device or make a diagnosis as with training someone to use the microscope to observe parasites. The casting of the device is relatively straightforward to make and is repeatable allowing for mass production of the device once a mold is made. Setup includes attaching the syringe and needle into the syringe pump and inserting the needle into the entry port. This setup does not require any additional training and is relatively easy despite the small size of the chamber. Since the chamber has been cast with a needle in it, there is an obvious and easy entry port that the needle easily fits into.

The luminol detection is easy to use although it does require the mixture of the two components before use. These components do not have to be mixed at an exact amount for the luminol solution to still work allowing the user to visually approximate five to ten flakes of the sodium perborate per milliliter of luminol solution. The amount added to the blood solution is not exact as well, the more luminol the more intense the luminescence is but it will not affect whether the solution with glow at all. This allows for quick and easy identification of blood that does not require exact measurements or extensive training.

6.1.5 Speed of Diagnosis

The World Health Organization requires treatment of a patient in less than two hours after the patient arrives at the clinic with suspected malaria to combat negative side effects of waiting and allowing the disease to progress to more severe stages. A test would not be efficient in improving treatment methods if it took longer than two hours as the patient may be treated anyway without waiting for a positive or negative diagnosis. The volume of the channel is only 0.5625 microliters at a time and does not require large volumes of blood to be flowed through the chamber. With ten microliters pushed through the chamber at one microliter a minute, the test takes ten minutes plus additional time for the entry chamber to fill up resulting in a fifteen to twenty-minute test. With sampling time and luminol detection, the test is still be under thirty minutes. This allows for efficient use of time and for the patient to be treated quickly after diagnosis. Even smaller amounts of blood could be passed through the flow through the chamber depending on the parasitemia level before luminol can be added to the wells for

detection. Issues with fluid leakage would require a second test to be run to completion, which would double the time until diagnosis. These problems have been relatively mitigated but an improved seal could almost completely eliminate this issue.

6.1.6 Minimally invasive testing

Another objective was to minimize the invasiveness required to take samples for testing during diagnosis. This objective was to help reduce any potential cultural or personal objections to drawing blood or other invasive procedures. However, this objective was not prioritized as a primary objective since malaria is most prevalent effects in the blood stream specifically red blood cells. The decided design is just as invasive as a microscopic test sampling blood from a patient. The only conceptual designs that were not invasive were not feasible due to inaccuracy until very late stages (oximetry measurements) or expensive and dangerous chemicals (volatile organic compounds). Therefore, it was decided that accuracy and cost were more important objectives than creating a minimally invasive detection method for a blood based disease. However, since this device requires volumes in the microliter range, the amount of blood drawn is minimal, no more than one milliliter. Although this objective was not met in a way that makes this new device superior to microscopy or RDTs, it still allows for an accurate detection of malaria without invasive surgeries or biopsies.

6.2 Limitations

At this stage, further developments on the device are needed to improve a method of controlling flow rate to keep the device at a low cost. This design is dependent on a way to control fluid flow a minimal costs and current syringe pumps are too expensive to invest in this project. Further testing on infected blood at varying parasitemia levels is needed to determine the range of the device. However, due to limited resources low levels of parasitemia down to the 0.001% standard were not able to be tested. Further research and testing must be done before this device is ready for use to validate the accuracy and low cost of this method.

6.2.1 Manufacturing

While cost of the chamber itself is low, the manufacture of the negative mold needed to create the chamber poses a problem. The need for such a small chamber width and the consistency needed along its length has narrowed the possibility of other production methods down considerably. It has been made relying on the ability to access HAAS milling equipment as well as precise cutting tools. The ability to access such technologies in the future threatens the ability to produce adequate chambers for testing and alternative methods of fabrication need to be considered. However, once built, the negative mold can be used indefinitely allowing for a reduction in costs through repeated use.

Chapter 7: Final Design Validation

7.1 Final Design

The final design was a polydimethylsiloxane (PDMS) microfluidics chamber with attached neodymium magnet and nickel wire. The chamber has a height and width of approximately 75 µm and 100mm length. A piece of pure nickel wire lay next to the flow chamber along its length place between the chamber and the magnet. The final design was determined by assessing how several alternative designs would meet the project's objectives, primarily for accuracy and low cost. Figure 26 shows the general setup of the flow chamber.

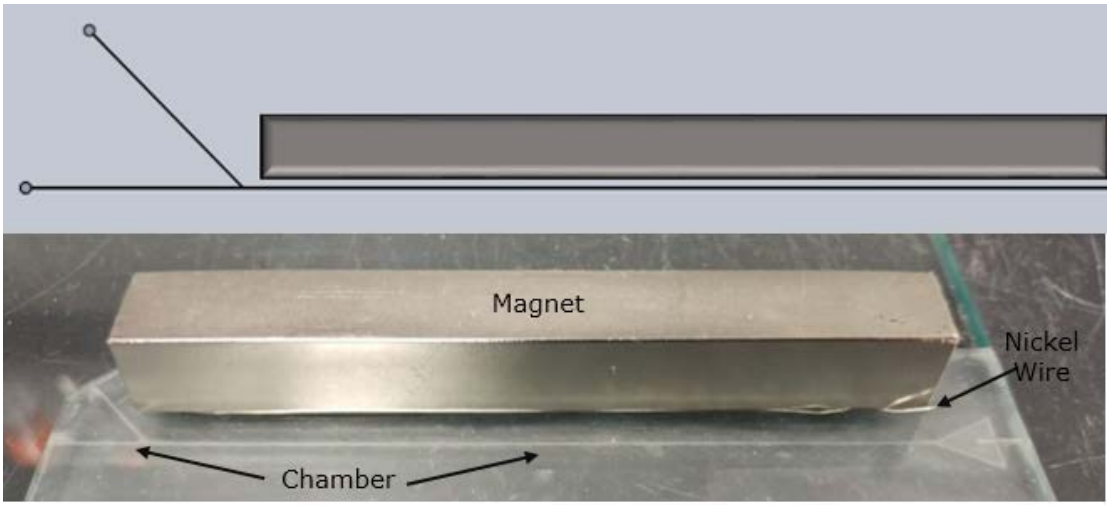

Figure 26: Flow chamber with nickel wire and magnet across the flow chamber length.

The negative mold for the chamber was machined out of aluminum due to ease of manufacturing and accessible materials. PDMS was chosen for the chamber material due to its low cost, ease of casting, and ability to be sterilized using low cost methods. There materials helped meet the project goal of keeping the diagnostic design low cost while still providing a highly accurate test method.

The design for the syringe pump was to utilize a screw and pulley system. Two weights of equivalent masses were strung through the threading of a screw. An initial force was applied then removed to start the movement of the masses, which translated to the rotation and linear motion of the screw attached to the syringe to push the plunger down. This design is still in initial stages of prototyping but was included in cost calculations as a method to control flow rate is necessary for the final design to function.

The estimated cost of the device was calculated. Assumptions about the number of times each item could be used were made based on literature as not complete repeatability testing can be completed due to time restrictions. More testing is necessary to ensure the reusability of the device as the low cost is dependent on repeated uses of many components of the device. Calculations were based on the number of uses for each material before it could be

replaced and the cost of each material. Several materials including the nickel wire, neodymium magnet, screws, and ball bearings theoretically could be used indefinitely; an estimate of 100 uses was used for calculations with the assumption this device would be used in areas with frequent testing for malaria and account for loss, breakage, or other reasons for replacement. The costs for the syringe pump is also rough estimates as a final design and testing has not been validated so costs were estimated based on current preliminary designs. The cost of manufacturing the negative mold was not included as that was created at no cost to the team due to accessible milling machines on campus. These machines have initial high cost, so to make this device low cost, the negative mold should be manufactured at a facility that already has such machines. Once the mold is made it can be used indefinitely to create many flow chambers and therefore, the cost of the mold will decrease over time.

Table 9: Cost estimates for the device

	Item	**Initial cost**	**Cost (per unit)**	**Units used per use**	**Estimated Number of Uses**	**Cost per Use**
Flow chamber	Aluminum mold	$12.99	$12.99/block	1	100	$0.13
	PDMS	$35.00	$0.01/gram	25	10	$0.03
	Neodymium Magnet	$11.49	$11.49/magnet	1	100	$0.11
	Pure Nickel Wire	$6.39	$0.21/meter	0.100	100	$0.0002
	Luminol	$30.68	$0.13/ml	1	1	$0.13
Syringe pump	Syringe +needle	$2.60	$0.13/syringe	1	1	$0.13
	Screw	$4.39	$4.39/screw	1	100	$0.04
	Ball bearings	$12.78	$6.39/bearing	2	100	$0.12
	Weights	$3.24	$1.62/weight	2	100	$0.02
	String	$4.59	$0.04/meter	1	1	$0.04
	Acrylic	$8.05	$8.05/ft^2	0.5	100	$0.04
Total	Materials	$132.20	Per test			$0.79

The cost per device and test does not fall within the original objective of fifty cents per test but does fall within the dollar ranges comparable to rapid diagnostic tests (RDTs) while providing potentially significantly better results in terms of accuracy making it a more cost-effective method. Before the syringe pump, the cost of one test can be lower than forty cents a test. Manufacturing of the mold was also not included in cost calculations as manufacturing would require use of a facility that already had access to the automated milling machines used for manufacturing. However, once manufactured the negative mold could be used indefinitely, significantly reducing the cost the more chambers are made. With the syringe pump, the cost per test would be around eighty cents with repeated use. The initial cost to the syringe pump would be around thirty dollars, which is higher than was set by the objectives of this project but is still significantly lower than the upfront costs of a microscope.

7.2 Experimental Methods Summary

The negative mold for the chamber was created using a milling process of an aluminum block in the Worcester Polytechnic Institute's machine shops. The flow chamber was cast using Sylgard 184 PDMS curing kit using a 5:1 ratio of elastomer to curing agent, placed in a vacuum chamber for 45 minutes, and an oven at 60C for 90 minutes to cure. Blood was collected from laboratory rats and stored with 7.2mg/ml of EDTA. Blood was diluted with DPBS before blood flow testing. Human blood infected with 3D7 strain of malaria was obtained from Dr. Pamela Weather's laboratory at Worcester Polytechnic Institute and tested at the same dilutions and flow rate as the rat blood. Flow testing was performed using a 1ml syringe, 20-gauge needle, and syringe pump set to a 1µl per minute flow rate. Luminol was tested for reactivity to blood, sensitivity to plasma, and degradation in humid conditions.

7.3 Industry Standards

The main standards that had to be met in this device were the World Health Organization's (WHO) standards for accuracy of malaria detection tests. The device must be able to identify malaria in 75% of cases with concentrations of 200 parasites per microliter. Since tests have not been performed with this level of parasitemia, it cannot be determined if the device meets the WHO standards, however based on literature and initial designs, this device has promising potential to exceed the 75% accuracy rating required for low parasite densities.

There are many ISO standards that relate to requirements of medical laboratories for *in vitro* diagnostic tools but not of the diagnostic tools themselves. ISO15198:2004 references *in vitro* diagnostic medical devices based on validation of user quality and procedures by manufacturer and applies to all *in vitro* diagnostic medical devices. ISO 16142 is for the safety and performance that apply to all medical devices. Once fully developed, this device should meet these standards, however, further development is needed in solidifying a manufacturing plan and validate its performance further. Additionally, as the device can be sterilized and reused, ISO 11737-2:2009 specifies the methods of sterilization of medical devices that must be met before use. Overall, there are extensive standards on *in vivo* drugs and therapies, but there

is a lack of standards for diagnostic tests [88]. The World Health Organization provides guidelines on drawing blood that must be followed for the collection of samples for this device. In addition, this device is intended to be used in various countries where malaria is highly prevalent, each of these countries have different standards that must be met and varying between locations depending on where the device is used.

7.4 Impact of Device

If this device is to be sold and used in areas with high malaria prevalence, there are many potential positive and negative impacts and implications of this. Such a diagnostic device could affect the economy, environment, the society, global markets, ethical concerns, health and safety, manufacturing, and sustainability based on its use.

7.4.1 Economics

This device has the potential to reduce the economic burden associated with diagnosing malaria. Often patients with fever like symptoms in areas with a high prevalence of malaria are given antimalarial drugs without a definitive diagnosis resulting in high treatment costs. An effective method to diagnose malaria would lead to more efficient distribution of medicine. This device would have an initial investment cost lower than that of a microscope, currently used for malarial detection. Its cost per test around the cost of current RDTs, but at higher accuracy. The durability of the device is higher than that of RDTs, which degrade quickly, making this device a more economical investment. Reducing the financial burden associated with malaria diagnosis would encourage more testing and definitive diagnoses before pursuing treatment. More accurate diagnosis would help for more effective treatment, therefore, saving money that is currently being spent on treating patients without a definitive diagnosis. The device does have an initial investment cost that while lower than a microscope could still pose an economic burden as the device is not completely reusable. This could create an economic barrier in delivering the device to the regions that could benefit from it. Overall, while the device has a higher than expected initial investment, it provides an economical method for detecting malaria.

7.4.2 Environmental Impact

This device would not have a larger negative environmental impact than current diagnostic methods as all involve collection of a blood sample, which would for safety and sterility, require disposable parts. This device uses non-reusable plastics; the syringe and needle for holding and dispensing the blood sample, which would be the main source of waste from this device. If not disposed of properly, over time these syringes could generate significant plastic waste, which would be damaging to the natural environment. However, all other materials used in the device are reusable so this device does not generate significant amounts of waste products. No electricity or other external resources are needed to run the device reducing the effect this device would have on the natural environment.

In the case of inappropriate disposal of the reusable components of the device, it should continue to have a low environmental impact. While the microfluidics chamber is made of a non-

biodegradable PDMS, Siloxanes have been found to have a very minimal effect on the environment and organisms when introduced into the environment [89]. The other reusable parts of the device are made of metals that similarly should not have a large effect on the environment if disposed.

7.4.3 Societal Influence

Since the device lasts longer than current RDTs and has better durability and reusability, the public can have better access to diagnostic testing. Due to the better access, more people in need of treatment will receive it thus lowering the incidence of malaria in the area. The fast speed of diagnosis and minimally invasive nature of blood collection allows patients to return to work and daily activities faster. This along with lowering infection rates will allow more people to return to work faster, strengthening the local population and economy. Along with lowering the infection rate in the immediate area due to more diagnosis, the surrounding areas would be expected to see a decline in the incidence of infection as well since a portion of the disease pathway has been removed.

7.4.4 Political Ramifications

Equal access to medical treatment has become an increasingly political position in recent history and this device attempts to increase the population of individuals that can seek this treatment in a cost-efficient way. This device competes with rapid diagnostic tests so a shift in the market could occur if this device becomes marketable. Overall, lowering the incidence of malaria is not a divisive issue so this device should not cause any significant ramifications.

7.4.5 Ethical Concerns

In some cultures, there is a taboo against the collection and use of blood from an individual. This device does not require significant volumes of blood, but does require intravenous collection. This can pose an ethical concern of how to approach a patient that should be tested for malaria but objects to blood collection [90].

7.4.6 Health and Safety Issues

The device designed does not directly contact patients in anyway, so the danger of the device to those being examined is minimal beyond the risks with gathering a blood sample for testing. If the personnel gathering samples are well trained, the device does not compromise the safety of the patients.

Dangers to the operator of the device are also minimal, but there are risks. The powerful earth magnet that runs alongside the chamber could become a risk if near other magnetic pieces. A warning should be placed upon the finished device to alert users of the risk. However, the magnet does pose serious threat to individuals who have any medical device such as a pacemaker, as it could interfere with the functioning of these devices. Therefore, individuals operating the device must be informed of the risk, and anyone with a device that could cause an issue should not be permitted to operate the device. The device requires the user to interact

indirectly with malarial blood that is contained in a dull tip syringe, so appropriate blood handling techniques and safety must be observed when operating the device. During operation, the device moves very slowly but if left unattended for too long, leakage of infected blood could occur so the device but be kept under observation while in use. Sterilization removes and kills cell present in the chamber, but must be thoroughly cleaned between tests to ensure it is sterile and must be sterilized before discarded to ensure parasitized blood is no longer present. These concerns are not unique to this device and with an appropriate use manual, no personal safety should be compromised.

7.4.7 Manufacturability

The device itself is designed so that once a negative mold has been created it can be used to make as many chambers as needed. Manufacturing the chamber requires a process with tolerances of 1/1000th of an inch, however, most machine shops are able to meet this standard. Molding the chamber at the moment relies on PDMS which is simple to create and only relies on a simple vacuum chamber to evacuate the air in the mixture and an oven to accelerate curing time. Additionally, the syringe pump will need some experience in construction but is made from parts that are generally easy to acquire. Manufacture of the device relies heavily on the access to a method of producing the negative mold and components to the PDMS and syringe pump, with those, it is easy to produce and assemble.

7.4.8 Sustainability

With a syringe pump driven without electricity, the device has a very minimal impact on the renewable resources of the places it is used. This detection method does not generate as much waste as RDTs, which are completely disposed of after use. Once chambers are manufactured, no electrical input is used. No part of the device has harmful effects on the environment even if disposed of improperly. Most parts of the device including the syringe pump and PDMS channel are very durable and reusable. One objective of the device was durability, which was met by the materials used so the device is sustainable in the hot and humid conditions where it should be used.

Chapter 8: Conclusions and Recommendations

8.1 Conclusions

The device created is a microfluidics magnet chamber for detection of malaria and meets the primary objectives of being accurate and low cost as well as the secondary objectives of being durable, easy to use, and quick to return a diagnosis. The secondary objective of being minimally invasive was not improved upon compared to current detection methods that also require small blood samples, making it comparable to microscopy and rapid diagnostic tests for this objective.

Blood testing has shown reliable and accurate detection in both healthy and infected samples allowing for proper diagnosis of patients, which has potential to be used in low resources areas that do not have extensive diagnostic equipment. Accuracy levels were above of that set by the World Health Organization for rapid diagnostic tests. The simplistic nature of the device allows for fast and easy diagnosis without the need for extensive training. The overall cost of the device without the syringe pump is around fifty cents, which meets the standard set to make the device low cost.

The manufacturing of the device in PDMS was achieved due to its accessibility and ease of use due to the resources available throughout the project. The creation of a negative mold allows for repeated manufacturing of device chambers that are consistently the same size allowing for repeatability in testing. The larger area entry chamber and glass cover allows for insertion of a needle tip without the creation of air pockets that would inhibit fluid flow down the chamber. A width in the scale of micrometers allows for the separation of individual red blood cells to distinguish healthy from infected cells.

This device design required a method to identify volumes of blood too small to be observed by the human eye. Experimentation with luminol has shown its sensitivity to blood in volumes lower than needed for this project, making it an ideal choice for the identification of blood in the chambers. It provides an accurate way to identify the presence of blood cells without requiring more expensive equipment such as a microscope.

The gravity-driven syringe pump has shown potential to create the constant flow rate that is needed for the functionality of the microfluidics chambers. One initialized the pump can maintain a constant rotation of the drive screw that drives the depression plate at a constant displacement and provided a consistent flow rate of blood samples at a speed that allows for full separation of the healthy and infected blood cells. This part of the device meets the objectives of being inexpensive and accurate, but has some user complexity problems due to its starting mechanism requiring rapid application and removal of force.

8.2 Recommendations

One option for improving this device is to find a stabilizing agent that will allow luminol to be stored as one solution rather than in two separate parts that need to be mixed before each use. This will allow for less material being wasted as with the current solution, once mixed the solution is only reactive for a few hours and therefore, may go to waste if many tests are not

being performed concurrently. Additionally, longer studies on the durability of luminol in hot and humid climates should be conducted to ensure that the luminol will remain reactive for longer periods of time than could be tested during this project.

The syringe pump mechanism is one of the aspects of the device that has the highest user complexity, due to the highly specific force requirements needed to start the drive so that it flows at the desired rate. Further investigation could be put into a new kickoff mechanism for the device, an entirely new design for a 1µl/min pump, or a new way to provide energy to an electric pump that could get a higher level of accuracy as well as perform other functions for the lab. To this end, if the users have access to a more advanced syringe drive than the one provided, preference is given to use that pump instead. Any of these ways of addressing some of the user complexity inherent with creating a controlled flow rate would be recommended.

One major issue encountered in experimentation is the blood sample clotting in the syringe so that fluid flow down the chamber is halted when the clot blocks flow from the syringe. This should be investigated further to reduce the number of null tests through improved anticoagulants. Additionally, warmer temperatures than what experiments were performed at may also help prevent clotting as blood samples were tested after taken from a 4C refrigerator.

More flow testing of the chamber is also necessary to obtain statistically relevant results on the ability to determine infection. More tests should be conducted using both healthy and infected blood samples to determine rates of false positives and false negatives. A normal parasitemia curve should be tested as well, to determine the limits as to what percentage parasitemia can be detected with the device.

The chamber itself should be improved upon by way of using a different material for the casting process. Currently PDMS is being used as it is easy to procure and make on campus, however, its components are not readily accessible everywhere, and therefore, other materials should be investigated for easier use and lower cost. Additionally, PDMS was damaged from repeated uses leading to cracks in the needle port and subsequent fluid leakage. Changing the material used to something more durable or altering the entry port will help reduce issues with fluid flow. Furthermore, the actual manufacture of the chamber should be elaborated and improved upon. Presently, the negative mold is machined from a standard block of aluminum stock and results in a chamber of appropriate dimensions and functionality. However, in the process of machining the chamber, there are many end mills used resulting in an apparent and potentially damaging surface finish on the mold. This should be addressed in future iterations of the design and improved upon whenever possible.

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Appendix

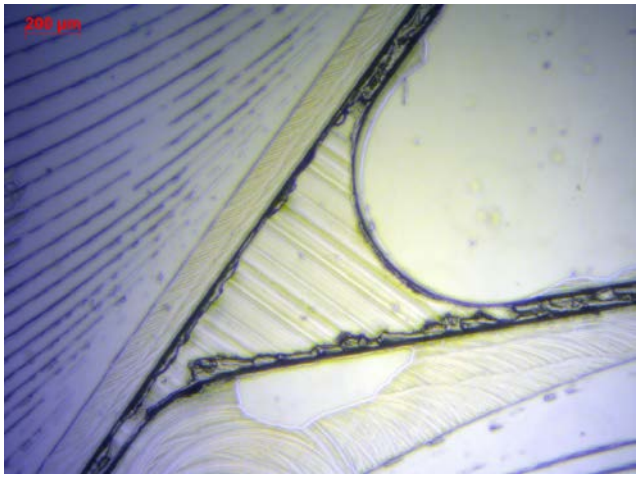
Appendix A: Expense Tracking Sheet

The budgeted amount for this project was 600 USD after a lab fee. Materials acquired were monitored through an expense sheet to monitor budget. Since the device was intended to be low cost and many materials used were accessible at no cost throughout the duration of the project, most of the budgeted money was not spent.

Item	Estimate Cost
Magnet	11.49
Nickel Wire	6.39
Needles	6.2
Luminol	22.76
Luminol 2	30.68
Glass	2.26
Screws	4.39
bearings	12.78
Weights	3.12
Total Spent	102.71
Total Left in Budget	499.93

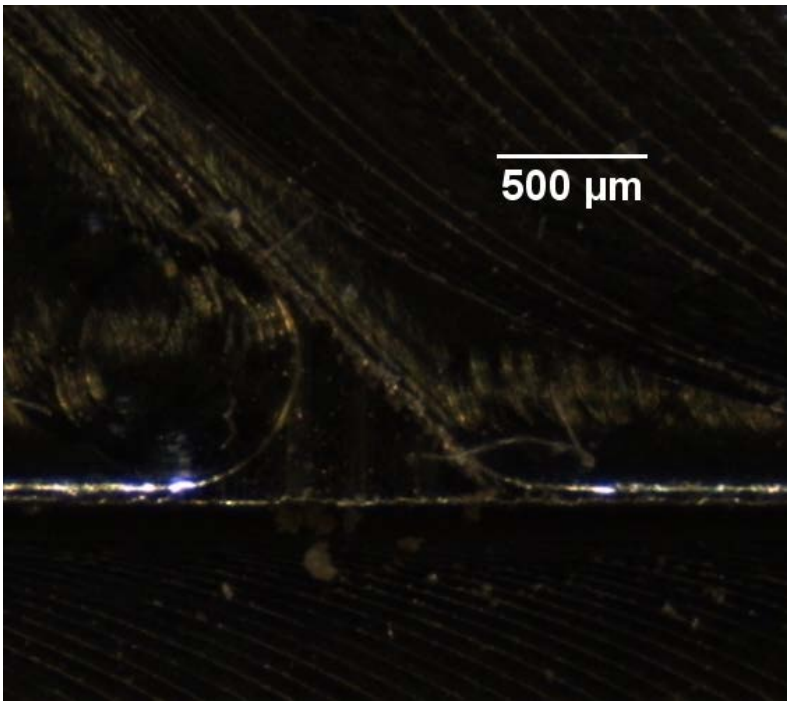
Appendix B: Chamber Images

Due to manufacturing limitations, the branching part of the chamber was a significantly larger size than the rest of the chamber. An image of the chamber at the branching point is shown below. The branch was much wider than either channel due to manufacturing limitations.



Chamber length was validated using ImageJ using a hemocytometer at the same magnification

as the set length.



Appendix C: Example Standard Operating Procedure

Purpose and Scope: To describe the procedure for setting up and operating the microfluidics magnetic malaria diagnosis device.

Background: Malaria infected red blood cells have altered magnetic properties and therefore, can be separated from healthy red blood cells through application of a magnetic field. Presence or absence of infected cells is used as a marker for diagnosis.

Supplies, Materials, and Equipment

- PDMS microfluidics chamber
- neodymium magnet
- nickel wire
- 20 gauge needle
- 1ml syringe
- saline
- syringe pump

Procedure

1. Ensure clean and tidy workspace with enough space for at least on clinic technician to work.
2. Gather the necessary materials in the testing area.
3. Use disposable gloves when handling human blood.

4. Collect 1ml of blood from patient based on the World Health Organization guidelines on drawing blood: best practices in phlebotomy.
5. Add saline to blood solution so that it is in a 1 to 4 dilution of blood to saline to make flow solution.
6. Fix 20 gauge needle to 1 ml syringe, pull up flow solution into syringe and remove all air bubbles.
7. Fit syringe into syringe pump and align PDMS chamber next to syringe and fit needle into PDMS chamber where entry port is
8. Press down on the chamber to remove air bubbles.
9. Align nickel wire along length of chamber and place magnet also along length of chamber so that the wire is between the magnet and flow chamber. Magnet and wire should be on side of chamber with lower branched collection chamber.
10. Set syringe pump to one microliter per minute if using an electric pump. If using a mechanical pump, apply enough force per calculations to set flow rate to one microliter per minute.
11. Allow test to run for fifteen to twenty minutes continuously monitoring chamber for fluid leakage. Flow can be ended when liquid is visible at the end of each chamber.
12. Once test is ended, stop syringe pump and remove syringe from chamber.
13. Mix approximately ten flakes of sodium perborate with 1ml of luminol solution and mix thoroughly.
14. Add luminol to each well and cover chamber or take to a dark place for observation.
15. Check each well for blue chemiluminescence indicating cell presence.
16. Chemiluminescence observed in each well indicates positive diagnosis for malaria, chemiluminescence present in only in top flow chamber indicates negative diagnosis and no presence of malaria, and chemiluminescence in only lower chamber voids tests.
17. Once test is complete, dispose of needle and syringe in proper biohazard containers.
18. Add PDMS chamber to boiling water for five minutes ensuring chamber is completely submerged.
19. Add luminol mixture down the length of chamber to ensure sterilization. Observe in dark conditions for blue glow/chemiluminescence. If no luminescence is observed, add chamber back into boiling water for two minutes to remove luminol. If luminescence is observed, add chamber back into boiling water for five minutes and repeat luminol test until all luminescence is gone indicating no more cell presence.

Reference

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