UNIVERSITY OF WISCONSIN-MADISON

MICROFLUIDIC POINT OF CARE DEVICE FOR MALARIA DIAGNOSIS IN ETHIOPIA

PRELIMINARY REPORT

BME 200-300

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ABSTRACT

Malaria is one of the most severe parasitic diseases in the world with and estimated 438,000 deaths annually. Although malaria can be treated, the disease is extremely devastating in developing countries, primarily due to lack of timely and accurate diagnosis. Developing countries, such as Ethiopia, have little to no resources or laboratory infrastructure sites, worsening the effect of infection and increasing the economic burden to diagnose and treat the disease. Although malaria prevalence has decrease in the last decade, due to increased research interest, the current point of care devices used in rural areas today could greatly improve on specificity, availability, and cost. The creation of an innovative microfluidic point of care device for earlier and more easily accessible malaria diagnosis could save thousands of lives each year. This design aims to utilize the differing magnetic characteristics of infected red blood cells in its approach to concentrate infected red blood cells for easier detection and diagnosis. After separation of infected red blood cells, a lateral-flow immunoassay method will be paired with gold nanoparticles to detect and differentiate between the four major strains of the malarial parasite. The integrated separation and detection designs mentioned are ideal for enrichment of infected red blood cells for the purpose of diagnosing all strains of the disease at the earliest possible time point.

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INTRODUCTION

Project Motivation:

Infectious diseases continue to plague the developing world as there is a basic lack of diagnostic testing to identify these devastating diseases [1]. Many harmful diseases are treatable but continue to spread due to the lack of a timely diagnosis. One such disease that continues to be one of the main causes of death in the world today is malaria. Nearly half of the world's population, around 3.2 billion people, live in areas at risk of contracting malaria [2]. Today, over 200 million cases of malaria are reported each year, with an estimated 438,000 malaria related deaths. Though malaria incidence and mortality have both decreased significantly in the last 15 years, this disease continues to be a global burden and requires immediate attention. A specific, effective, cheap, and easily administered malaria diagnostic test could save thousands of lives and prevent millions of misdiagnosed treatments per year. According to the World Health Organization, diagnostic devices for developing countries should be affordable, sensitive, specific, user-friendly, rapid, equipment free and delivered to those with the greatest need.

Background Information:

Malaria is caused by a genus of parasites known as *Plasmodium* with four main species: *P. falciparum, P. malariae, P. ovale,* and *P. vivax.* As the most prevalent, *P. faliciparum* and *P. vivax* possess the greatest threat [3]. The malaria life cycle involves two stages within two different organisms, the *Anopheles* mosquito and humans. In mosquitos, the sporogenic cycle occurs, where it matures inside the salivary glands until the mosquito inoculates the sporozoites into the human host. From there the sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites, fully mature parasites, into the bloodstream. The parasite then undergoes asexual proliferation in the erythrocytes, continually infecting and rupturing red blood cells (RBC), which then are responsible for the clinical manifestations of the disease. Once RBCs are infected (iRBC), they express specific antigens indicative of the disease, such as histidine-rich protein 2 (HRP2) or pan-malaria antigen. The cells also exhibit different cell membrane properties, increase in electrical conductivity, and increased magnetic properties. Figure 1 below depics the malaria parasite life cycle.

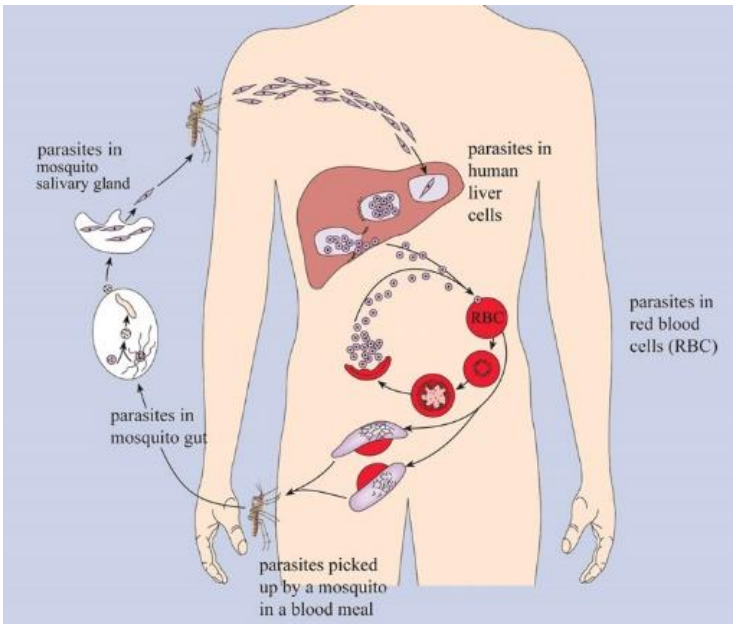


Figure 1: Diagram of malaria parasite life cycle

The main method, and gold standard, of malaria detection involves a blood smear visualization of the actual parasite in iRBCs through a microscope. The most common detection is a Giemsa stain, which colors the nuclei of the parasites. Though this method is cheap and extremely effective, it requires a microscope and a trained technician [1]. An alternative and expanding field of research focused on malaria diagnosis today encompasses the development of point of care (POC) testing devices [4]. This approach utilizes both nanotechnology and microfluidics in the development of portable, compact and effective micro platforms for disease testing and diagnosis. These devices make near patient setting possible, without the use of clinics, and giving timely diagnostic information. Though saliva and excrement can also be used in diagnostic tools, blood is universally used as the main input method. Two main types of POC devices in use today include dipsticks and lateral flow tests. Dipsticks detect the presence of simple compounds whereas lateral flow tests rely on immunoassays to detect specific analytes in the specimen via antibody-antigen chemistry.

The most accurate POC microfluidic malaria diagnostic device available today is BinaxNOW® by Alere [5]. The paper based test utilizes an immunochromatographic assay for the qualitative detection of *Plasmodium* antigens found in iRBCs of individuals with malaria. It is used for the diagnosis of human malaria, being a rapid, easily administered and interpreted test, as well as 93.5-99.7% sensitivity for detection of all four strains of malaria. The main issues regarding this test include the extremely high cost ($40 a test), inability to distinguish between the four strains of malaria, need for negative result confirmation via smear microscopy, and the

requirement for parasite levels to reach 5,000 parasites/µL, a number already associated with severe malaria with clinically perceived symptoms.

Due to these shortcomings and the current need for POC testing, the design team was tasked with developing a microfluidic device to diagnose malaria in rural areas in a highly specific, time efficient, cheap, and replicable manner.

Specifications:

The focused area of this study is Ethiopia, Africa, as the client is from Jimma University. Ethiopia is a developing country, with limited resources, unreliable internet, little to no laboratory infrastructure/personnel, and predominantly rural areas. Thus, the device must be able to be fabricated under these conditions. The device also must be 95% accurate, return results within an hour, small in size, under $5 a test, and able to distinguish between the four strains of malaria, to diagnose the disease.

For more information, related to the design specifications, reference the Product Design Specification (PDS), located in appendix A.

Client Information:

The client is Dr. Timothy Kwa, a professor at Jimma University. Dr. Kwa received his PhD from UC-Davis in Biomedical Engineering and his research interests include improving healthcare through early diagnosis technology.

PRELIMINARY DESIGNS

The design of the device consists of two major components. The first aspect of the design is meant to separate iRBCs from normal RBCs based on unique physical characteristics that iRBCs exhibit, while the second aspect is meant to detect if the cells are indeed infected. The separation component operates by concentrating the iRBCs for easier detection. The second component of the design operates by detecting the malarial parasite from the concentrated sample of iRBCs. Preliminary designs for both the separation and detection methods are shown below.

Separation Designs

A) Separation: Design Idea One – Cell Deformability

This separation design is based on changes in cell deformability caused to the host RBCs upon infection with the malarial parasite. Parasitic proteins are secreted within the iRBC to make its membrane more adhesive, as well as stiffer. The increased stiffness along with a reduction in the surface area to volume ratio contributes to a significant decrease in cell deformability, especially in late stage iRBCs. Normal RBCs, however, remain highly deformable. It has been demonstrated that the stiffer iRBCs can separate towards the sidewalls of a long and straight

channel microfluidic device [6]. The diagram below illustrates the preliminary cell deformability design (Figure 2). It has an inlet, where a blood sample is loaded via a syringe. The blood cells then flow down the 3-centimeter-long channel (15um wide), which forces the iRBCs to the sidewalls. Finally, the iRBCs can be concentrated in separate outlet from the RBCs via the use of smaller side channels on the outside walls. The innermost outlet would thus mostly contain RBCs, and the outer would contain mostly iRBCs.

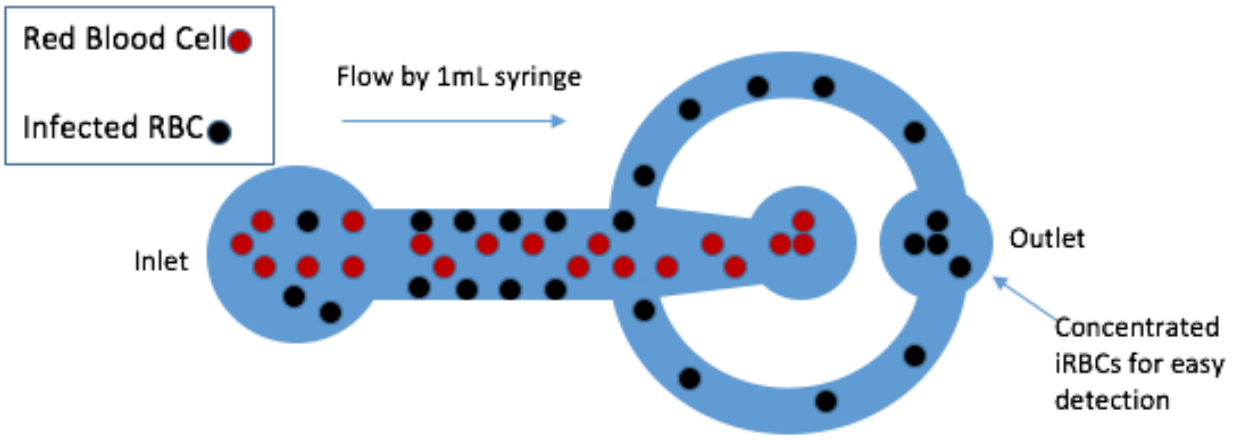


Figure 2. This microfluidic device acts in separation of iRBCs from RBCs on the basis of cell deformability characteristics.

B) Separation: Design Idea Two – Magnetism

This separation design is based on changes in magnetic properties of the host RBCs upon infection with the malarial parasite. When parasites infect a cell, they metabolize the hemoglobin of RBCs in order to survive and proliferate. This process releases heme as a toxic byproduct. Heme is then converted into a non-toxic secondary byproduct known as hemozoin. Hemozoin is a weakly paramagnetic crystallite, which is produced during all life stages by all four strains of the disease. Normal RBCs are diamagnetic, meaning that when exposed to a strong magnetic field they will align in the opposite directions. Utilizing the different magnetic properties of iRBCs and normal RBCs, the two types can be separated in a highly specific manner in a microfluidic device exposed to properly aligned magnetic fields. Shown below is a preliminary design for magnetic separation of iRBCs involving neodymium magnets (Figure 3) [7][8].

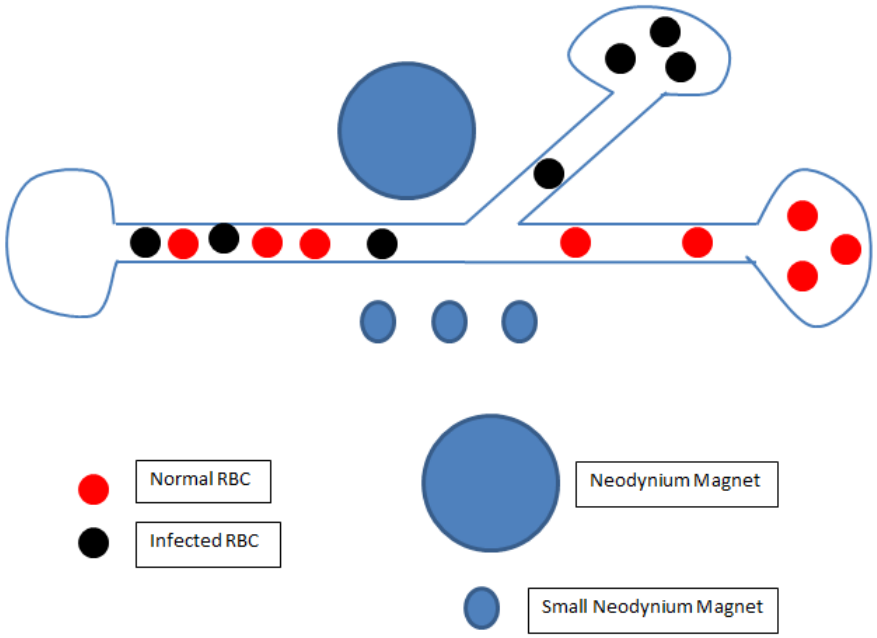


Figure 3. This microfluidic device acts in separation of iRBCs from RBCs on the basis of paramagnetic characteristics due to hemozoin.

C) Separation: Design Idea Three – Electrical

This separation design is based on changes in electrical properties caused to the host RBC upon infection with the malarial parasite. The electrical conductivity of iRBCs is significantly higher than normal RBCs, which allows for electrical separation of these cells. Dielectrophoretic (DEP) forces are non-uniform electric fields that can be created by cells when subject to direct or alternating current. The Differing DEP properties of iRBCs and RBCs contributes to their unique conductivities [8]. These properties can be utilized to separate iRBCs and RBCs in a microfluidic device exposed to properly placed electrical currents. The diagram below illustrates the concept of electrical stimulation in a microfluidic device for the function of separating RBCs (Figure 4).

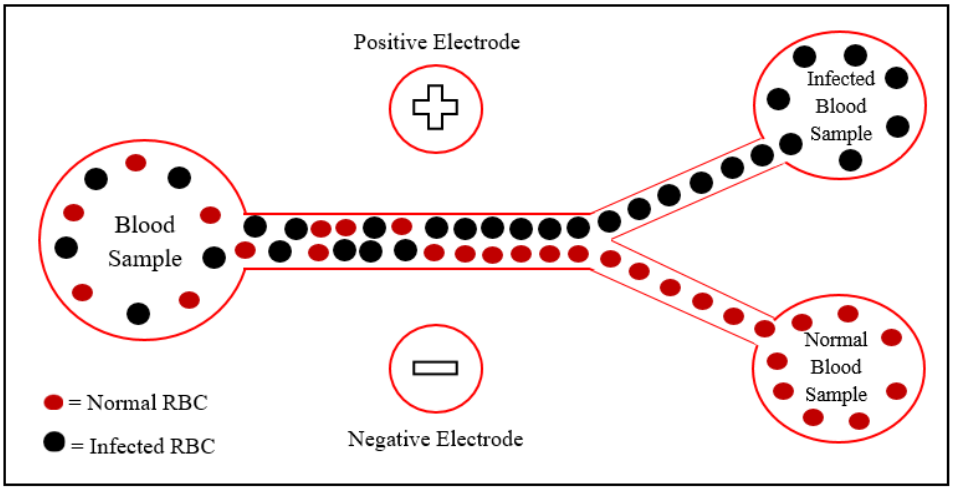


Figure 4. This microfluidic device acts in separation of iRBCs from RBCs on the basis of electrical conductivity characteristics.

Detection Methods:

D) Detection 1: Design Idea One – BinaxNOW

BinaxNOW is a currently available diagnostic tool for malaria as described in the introduction section. Its major pitfall is that it requires 5,000 parasites/uL in blood samples for detection. The separation techniques described above would act in concentrating the parasites for easier and more successful detection. This assay takes 15 minutes to run. When combining BinaxNOW with one of the separation techniques, this tool could become more successful in diagnosing malaria [10]

E) Detection 2: Design Idea Two - Polystyrene Beads

This method of malaria detection is based on an antigen-antibody interaction whereby polystyrene beads are conjugated with an antibody and interact with iRBC antigens specific to that antibody. The beads and iRBCs would then form a visible aggregate in the well in a process known as immunoagglutination. The dried beads could be coated in the well and rehydrated upon use. The aggregation would occur within 2 minutes and would be visible without a microscope [10]. The diagram shown below depicts the polystyrene bead detection design (Figure 5).

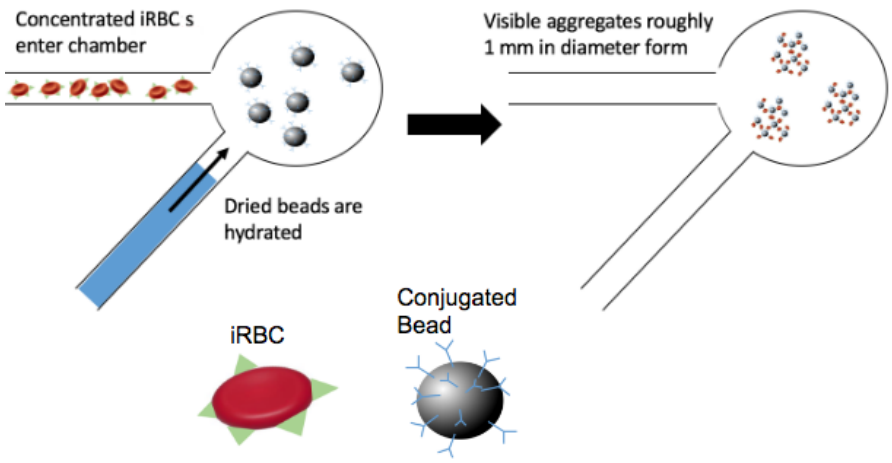


Figure 5. This detection method occurs through immunoagglutination involving polystyrene beads and iRBCs.

F) Detection 3: Design Idea Three – Gold Nanoparticles

This method of malaria detection is a lateral flow immunoassay (LFIA) whereby iRBCs are detected using unique antigen-antibody interactions. The concentrated blood sample would be placed on the sample pad and then would run down the nitrocellulose paper to pass through the conjugated gold nanoparticle pad. Gold nanoparticles, would be conjugated with an antibody specific to iRBC antigens and would change the color of iRBCs. The same antibodies present on the gold nanoparticles would also be immobilized on the detection pad. Thus, colored iRBCs would cause a colored line at this junction to signify detection of the specific disease. A conjugated gold nanoparticle for each strain of malaria could be placed in the conjugate pad. Thus, the actual design would contain four separate detection lines for each strain of the disease in addition to the antihuman control line. The antihuman antibody control line would always be detected, if properly functioning, and is present to demonstrate antibody integrity [9]. An image of the gold nanoparticle detection method is outline below (Figure 6).

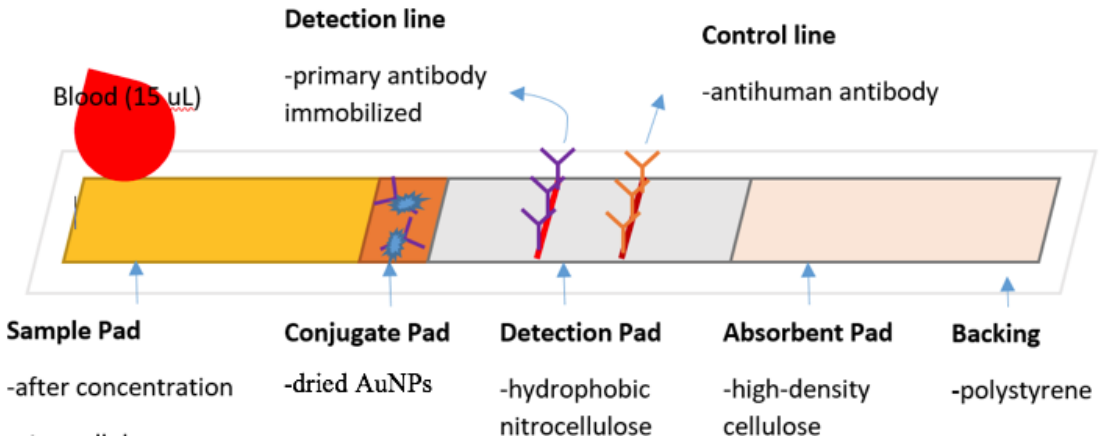


Figure 6. This lateral flow immunoassay detection method occurs through the use of conjugated gold nanoparticles, which color iRBCs prior to immobilization on the detection pad.

Figure 7 below shows the design matrix evaluation of each separation and detection design. The criteria chosen by the group encompassed the clients main design specifications as well as the most relevant categories used for point of care devices today.

PRELIMINARY DESIGN EVALUATION:

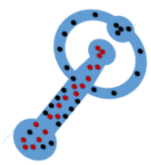
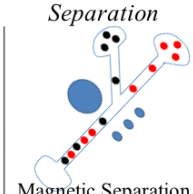
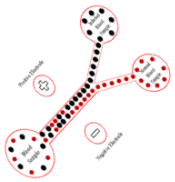
Design			*Separation*						*Detection*					
Criteria (weight)	 Cell Deformation		 Magnetic Separation		 Electric Separation		 BinaxNOW		 PS Beads		 GNPs	
Sensitivity (25)	3	15	5	25	4	20	5	25	4	20	5	25
Equipment Free/Usable in Field/Intuitive (20)	5	20	4	16	3	12	3	12	4	16	5	20
Userfriendly (20)	5	20	5	20	3	12	4	16	4	16	5	20
Time (10)	2	4	4	8	4	8	4	8	5	10	4	8
Cost (10)	5	10	4	8	3	6	1	2	5	10	3	6
Ease of Fabrication (10)	4	8	4	8	3	6	5	10	3	6	2	4
Versatility (type of Malaria or other diseases) (5)	4	4	2	2	2	2	3	3	1	1	5	5
Total	81		**87**		66		76		79		**88**	

Figure 7: Decision matrix with criteria in the left column and each design following in two groups of three. The first three designs are separation technique. The second three designs are detection techniques.

Design Matrix Evaluation

To evaluate the preliminary designs, they were separated into two categories (figure 6): separation techniques (including cell deformation, magnetic, and electric separation) and detection methods (BinaxNOW, polystyrene beads, and gold nanoparticles). It is important to evaluate the designs separately so competing designs can be compared. The client stressed the importance of improving upon the accuracy of current point of care (POC) devices over everything else so sensitivity got the highest weight. Another major demand the client had was to produce a device that can be used in rural areas with minimal equipment by untrained technicians. He also stated that the price and speed of the device should compete with currently available rapid diagnostic tests (RDTs). Lastly he stressed that the device should be able to separately detect different strains of malaria for proper treatment of the disease.

The separation techniques all aim to concentrate the iRBCs before analysis. For the separation techniques, sensitivity refers to how specific the separation of iRBCs is; the magnetic separation method scored highest because the magnetic properties of cells are specific to the diseased state. Research shows that the cell deformation method would separate any cells with different surface properties than uninfected red blood cells, where diseases such as diabetes would interfere with results [11]. To be classified as equipment free and usable in the field, the device must be able to function without a trained technician and should not require a large amount of resources for each test. The cell deformation design scored highest because it would consist solely of PDMS that could be manufactured in bulk off site and transported in. The electric separation method scored lowest because it would require batteries and a base with electrodes to be transported to the clinic. To be user friendly the method must be able to concentrate cells without training, so the electric separation method does not pass this test because tuning the electrodes for optimal separation would take constant supervision. Cell deformation scored the lowest for time because the small channels require a low flow rate of liquid that is not as fast as capillary action in the other designs. Lastly, for ease of fabrication, the device must be able to be fabricated and assembled on site. The magnetic separation only had one downfall which was figuring out the placement of the magnets for optimal cell separation. The cell deformation design needed a very thin channel to be made out of PDMS, which can pose fabrication problems. The best separation method was deemed to be the magnetic design; it did the best in four out of six of the criteria and second best in the other two.

The detection methods were evaluated with the same criteria as the separation techniques. Sensitivity is where BinaxNOW is the top performer, this device is over 99% sensitive so it is the gold standard in paper based tests. Gold nanoparticles have the possibility to be just as sensitive so they got the same rating, and for ease of use, this design got the top ranking because it can be read in 15 min by looking for colored bands. The polystyrene bead design is the top performer in time and cost because the test takes two minutes to finish and is made from the cheapest and easiest to assemble materials (except for the preassembled BinaxNOW). The major drawback of adapting BinaxNOW to the microfluidic device is the cost of about $40 per test. The gold nanoparticles were the chosen detection method to provide the versatility to detect for all four strains of malaria on one test strip. One challenge of this design is the complexity of fabrication, however, this could be simplified by converting a printer to lay antibodies on nitrocellulose.

Proposed Final Design:

Through the use of our design matrix we decided that the best design combination is the magnetic separation combined with a lateral flow immunoassay using colored gold nanoparticles. This design is a sensitive and easy to use system that can be operated without any training and limited equipment. This system is also cheap enough to compete with current RDTs and has the ability to detect all four strains of malaria.

FABRICATION/DEVELOPMENT PROCESS:

Proposed Materials:

The proposed final design would consist of two integrated designs: the magnetic separation portion and the gold nanoparticle detection portion. The separation portion of the design utilizes a polydimethylsiloxane (PDMS) channel, loading wells, and collection wells cast from a master mold made of any generic material shaped with the proper dimensions for the microfluidic channel. It also contains a large neodymium magnet capable of producing strong magnetic fields and multiple other smaller neodymium magnets or ferromagnetic wires capable of orienting the magnetic field in the proper manner for separation of iRBCs from RBCs (Figure 3). The detection portion of the design would be immediately attached to the iRBC collection well either permanently or temporarily. The detection portion of the design utilizes the method of lateral flow immunoassay (LFIA) and conjugated gold nanoparticles (AuNPs). Separated blood containing iRBCs would be placed on nitrocellulose paper. This paper would be attached to a pad containing dried AuNPs conjugated with malaria antigen detecting primary antibodies for each of the four strains. Multiple lines of primary antibodies that are antigen specific to differing malarial antigens would be printed into separate lines following the conjugate pad. An absorbent pad and backing made of high density cellulose will follow for excess liquid runoff (Figure 6). Many more details regarding the specifics of materials to purchase need to be defined by the design team in order to move forward with these plans.

Proposed Methods:

In order to produce the separation portion of the design, many calculations regarding the placement and strength of the magnetic field need to be completed. A master mold will be machined so that the many PDMS molds of the device can be replicated. PDMS will be poured over the master mold, removed, and then bonded to a surface such as glass for use [12]. In order to produce the detection portion of the design, many specific protocols for the conjugation of AuNPs with antibodies are outlined for the team's usage [13]. In order to attach the lines of primary antibodies to the LFIA pad, an inkjet printer can be used with cellulose paper as a medium. Many protocols for the printing of primary antibodies to cellulose paper are outlined for the team's usage [14].

Proposed Testing:

When a prototype is produced by the design team, testing of blood with the malaria parasite will be impossible at this stage. In order to test the design, different proof of concept assays need to be implemented. The main aspects of the design that need to be tested are the

design's ability to successfully separate micro-paramagnetic particles from a fluid within the microfluidic channel and to detect antigens using LFIA methods. Because the design team does not have the ability to test using blood infected with the malaria parasite, creative testing methods must be outlined in order to test these aspects of the design. For the paramagnetic particle test, it could be feasible to model the properties of hemozoin in iRBCs with small paramagnetic beads suspended in a solution of similar viscosity to blood. For the antigen detection testing, it would be possible to choose an antigen that is more generic and less harmful, such as some animal blood antigen. The design team could produce an LFIA model that represents the same process of detection, but simply incorporates different antigen-antibody interactions as a proof of concept. The team could also test the efficiency of conjugating the AuNPs with antibodies from scratch as opposed to attempting to order them premade. This would help to prove that the LFIA/AuNP detection method is feasible for detection of malaria.

DISCUSSION

The final design is the combination of the best idea from the separation group and the detection group. These ideas scored the highest in our ranking system, and by integrating separation and detection into a low cost test, this device is novel to the market for RDTs. If this device can be accurate to over 95%, it would make a huge impact on the diagnosis of malaria in resource limited settings. This would save thousands of lives and open up possibilities for POC clinics to rapidly diagnose and treat malaria without equipment or training.

To effectively market this device, it needs a low false positive and false negative rate to diagnose patients correctly. The misdiagnosis of a patient can be fatal, so ensuring the device is effective before implementation is crucial. Another consideration is the possibility of blood-borne pathogen transfer from the device to the patient or technician. This is a huge concern for blood diagnostic devices, so the final design will have a disposal plan to ensure safety. Upon further research, it was found that the antibodies used in currently available RDTs degrade under thermal stress and high humidity. In Ethiopia these conditions are common, so this is a concern that would need to be addressed before the device is marketed.

Fabrication of the device in Ethiopia may be difficult, however, the design follows the constraints provided by our client. Therefore, consistent fabrication should be achievable. Production of PDMS molds is prone to variability, so proper quality control will be vital to ensuring consistent results. Another source of error is the placement of magnets in the base separation device. This needs to be optimized for the design by someone with experience in modeling magnetic fields.

The next steps in the design process are directed at finalizing the fabrication process. Some of the main concerns with the LFIA procedure are the complexity of the printed antibody lines and the conjugated gold nanoparticles. Optimally these processes will be simplified for the

final design. Another area of research to look to is how to combine the magnetic separation and LFIA devices into a single unit for rapid detection. Lastly, finding a way to test the final device is challenging but the proof of concept testing depicted above could be used to model these approaches.

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APPENDIX

Product Design Specification

Function: To create a point of care microfluidic device functional in developing countries that can successfully concentrate and diagnose all four strains of malaria via blood chemistry analysis.

Client Requirements:

- 95% accurate
- Result within one hour
- Battery powered with a battery life of up to 3 hours
- Device needs to be approximately the size of a laptop or smaller
- No more than $5 per test
- Able to diagnose malaria and distinguish between the strains Plasmodium falciparum and Plasmodium vivax

Design Requirements:

1. Physical and Operational Characteristics

A. *Performance requirements*: The device should accurately diagnose malaria in remote conditions without the use of electricity or advanced laboratory equipment. The device needs to be disposable, ideally give results within an hour and can be used with minimal training.
B. *Safety*: The device should put the user at a minimal risk for accidental malaria infection via puncture and blood-borne infection.
C. *Accuracy and Reliability*: Greater than 95% accuracy in detecting all four strains of malaria in a blood sample.
D. *Life in Service*: The device should be easily discarded after each use to reduce the possibility of transmitting a blood-borne pathogen.
E. *Shelf Life*: The device should be thermostable and last for up to one year if stored properly.
F. *Operating Environment*: The device needs to be able to function in outdoor environments experiencing large temperature variations, depending on the season. The device will be used in rural areas, so it should be water and dust resistant.
G. *Ergonomics*: The device should be easy to recreate without advanced technical knowledge and with minimal laboratory facility requirements.
H. *Size*: The size of the entire device should be approximately the size of a laptop or smaller.
I. *Weight*: The weight should be minimal as to increase the ease of which it can be transported to onsite, point of care, locations.
J. *Materials*: The device must be made with safe and sanitary materials.

K. *Aesthetics, Appearance, and Finish:* The device should be durable and resistant to normal use by the lab in Ethiopia. Also, it should be able to be shipped in a ready to use form.

2. Production Characteristics

A. *Quantity*: The creation of at least one functioning device that is able to detect for malaria. The design should be able to be mass produced.
B. *Target Product Cost*: A test should cost a maximum of $5.

3. Miscellaneous

A. *Standards and Specifications*: The device should be greater than 95% accurate, cost less than $5 per unit, be battery powered (if applicable), and smaller than a laptop. Protocols need to be followed for disposal of the device, so that no blood-borne pathogens are transferred. The design and fabrication should be repeatable, and the device should perform consistently.
B. *Customer*: This product will be used by technicians in rural areas of Ethiopia to test patients for malaria. The entire diagnosis should take less than an hour, so that treatment can be provided quickly, if needed. The faster the disease is diagnosed and treated, the less fatal the disease becomes.
C. *Patient-related concerns*: After usage, the device should be able to be easily disposed of in order to not contaminate other patients.
D. *Competition*: The device cannot infringe on any existing patents or copyrights.
a. Binax Now- only brand of malaria rapid diagnostic test approved for use in the United States. Pack of 12 tests sells for $396.20.
b. 86 different malaria rapid diagnostic tests are available from 28 different manufacturers. Cost is $0.50 to $1.50 per test, these have no quality control standards and are the currently available method for testing