Designing an Aptamer mediated diagnosis kit for malaria

A thesis submitted to

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by

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Certificate

This is to certify that this dissertation entitled '**Designing an aptamer mediated diagnosis kit for malaria**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Animesh Anand** at Indian Institute of Science Education and Research under the supervision of **Prof. Saikrishnan Kayrat, Professor, Department of Biology, IISER Pune** during the academic year 2022-23.

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Declaration

I hereby declare that the matter embodied in the report entitled **'Designing an aptamer mediated diagnosis kit for malaria**' are the results of the work carried out by me at Curem Biotech LLP, c/o AIC IISER Pune SEED Foundation, Indian Institute of Science Education and Research, Pune, under the supervision of **Prof. Saikrishnan Kayarat** and the same has not been submitted elsewhere for any other degree.

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Abstract

Malaria is one of the world's deadliest diseases, with India being a prominent hotspot for malaria. According to WHO, 77% of the cases are reported from Southeast Asia. Malaria therapy must begin with an accurate and timely diagnosis. However, expensive precise detection methods and insufficient materials and personnel are the most challenging barriers to eradicating malaria.

Malaria diagnosis methods currently available are microscopy-based and nucleic acid amplification-based, which require a laboratory and trained individuals. Antibody-based rapid diagnosis kits are a popular method nowadays due to their rapid diagnosis and the non-requirement of trained individuals. These RDTs are getting highly unreliable due to the emergence of novel *plasmodium* mutants, which go unrecognized by antibody-based assays. My thesis revolves around developing an accurate, specific sensitive and cost-effective RDT compared to current malaria RDTs. The RDT Kit is based on Aptamer detection of *plasmodial* proteins.

Aptamers are single stretches of oligonucleotides that bind to the target molecule with high specificity. They are often referred to as molecular recognition elements and have gained considerable attention in recent years for their potential applications in medical diagnosis, therapeutics, and biosensors. The detection method relies on a colour change reaction that gives accurate results time-efficiently without needing trained personnel.

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Chapter 1 Introduction

Malaria

Malaria is a severe infectious disease caused by a malarial parasite*, Plasmodium* sp. The transmission of malaria occurs by the vector female *Anopheles* mosquito. *P. falciparum* is the most lethal form causing malaria. Other malaria species, *P. vivax, P. ovale, P. malariae*, and *P. knowlesi*, can cause acute, severe disease but do not have a high death rate. Malaria is a major global health problem, particularly in tropical and subtropical regions. In 2019, an estimated 229 million malaria cases were reported worldwide, with over 4 lakh deaths.(Steketee et al, 2021), Due to the heterogeneous and unequal distribution of malaria in India, pregnant women and people who live in the forest are disproportionately affected. In particular, over 8% of the so-called tribal people of the nation are responsible for 47% of the deaths and 46% of all malaria cases. (Lee et al, 2014) Malaria symptoms can vary but usually include fever, headache, chills, and body aches. These symptoms typically begin 10-15 days after being bitten by an infected mosquito. In severe cases, malaria can cause anemia, seizures, and organ failure and can be fatal if left untreated.(Mwangi et al, 2005)

The *Plasmodium* parasite has a life cycle involving two hosts: female *Anopheles* mosquito and human. When an infected mosquito bites a human, the parasite's sporozoite form enters the bloodstream. The sporozoites then move to the liver, where they proliferate and mature into merozoites. Merozoites are released from the liver and enter the bloodstream, targeting and infecting red blood cells. Merozoites multiply fast in red blood cells, causing the cells to burst and release a greater number of merozoites into the bloodstream. Malaria symptoms, such as fever, chills, and anemia, are caused by this cycle of invasion, growth, and disintegration of red blood cells. (Mwangi et al, 2005) During a blood meal, certain merozoites ingested by mosquito differentiate into male and female gametocytes. Gametocytes in the mosquito grow into gametes, which may fuse to generate a zygote. The zygote then matures into an ookinete, which subsequently penetrates the mosquito's midgut wall and grows into an oocyst. The parasite divides numerous times within the oocyst, producing thousands of sporozoites. When the oocyst ruptures, the sporozoites are released into the mosquito's body cavity. The sporozoites then move to the mosquito's salivary glands, where they can infect the human host during a later blood meal.(Aly et al, 2009)

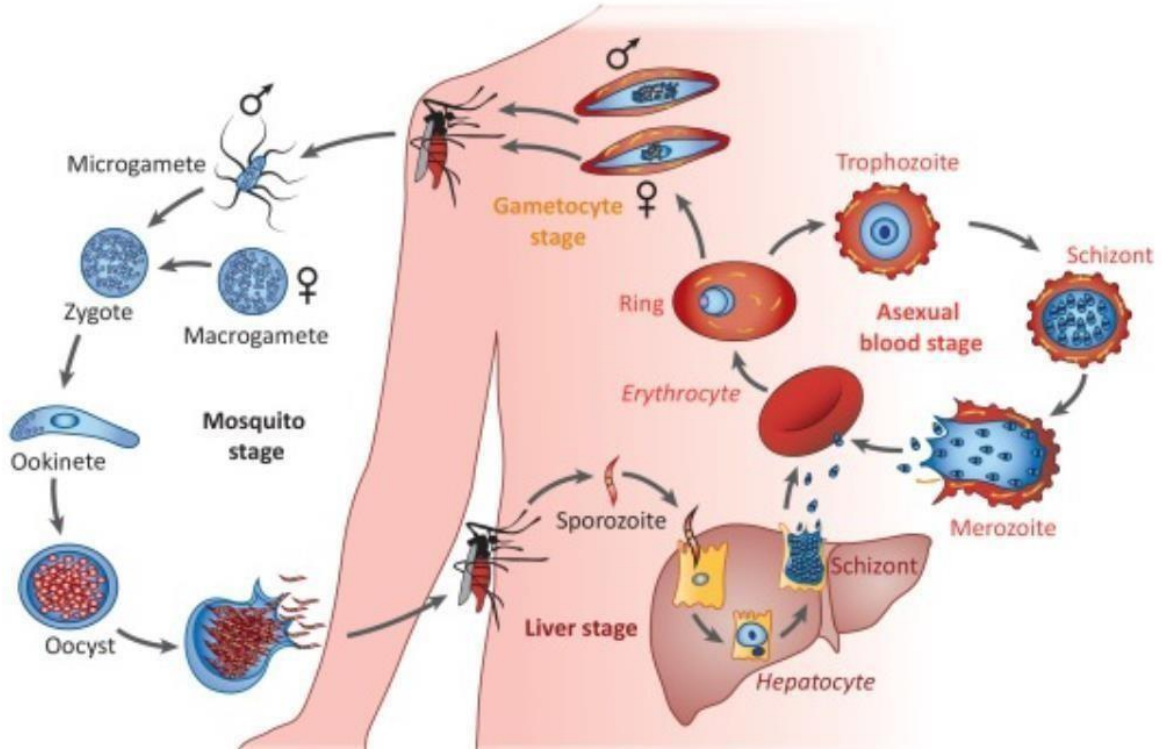


Figure 1: *Plasmodium* parasite life cycle in two hosts. (Maier et al, 2019) *Plasmodium's* sexual phase occurs in Anopheles mosquitos, which consume gametocytes from infected human blood. Once the gametes fuse, zygote formation occurs, followed by ookinete formation. Ookinete develops in an oocyst, which contains several sporozoites. The oocyst migrates to the salivary glands of the mosquito. The sporozoites then infect another human host by mosquito bite, infecting hepatocytes and eventually blood cells, where the asexual stage continues.

Malaria can be prevented and treated. Prevention methods include using mosquito nets and insect repellents and removing stagnant water where mosquitoes breed. Treatment usually involves using anti-malarial drugs, which kill the parasite in the blood. Malaria is most prevalent in sub-Saharan Africa, where it is the leading cause of disease and death, particularly among young children. However, the disease is common in Asia, Latin America and the Middle East. Efforts to eradicate malaria continued for many years with some success. Using insecticide-treated bed nets and anti-malarial drugs has helped reduce malaria cases and deaths. (Rahi & Sharma, 2021) However, much more needs to be done to eradicate the disease. In addition to traditional methods of fighting malaria, scientists are exploring new approaches, such as genetically engineered mosquitoes resistant to the malaria parasite and developing a malaria vaccine. Malaria is a complex disease that requires a multifaceted approach to control and eradicate it. While progress has been made, there is still work to be done to protect people worldwide from this devastating disease.

One of the ways to prevent the spreading of malaria is early diagnosis of malaria. (Nahlen et al, 2005) The methods available today are clinical diagnosis, laboratory diagnosis and molecular diagnosis of malaria.

Malaria diagnosis systems available:

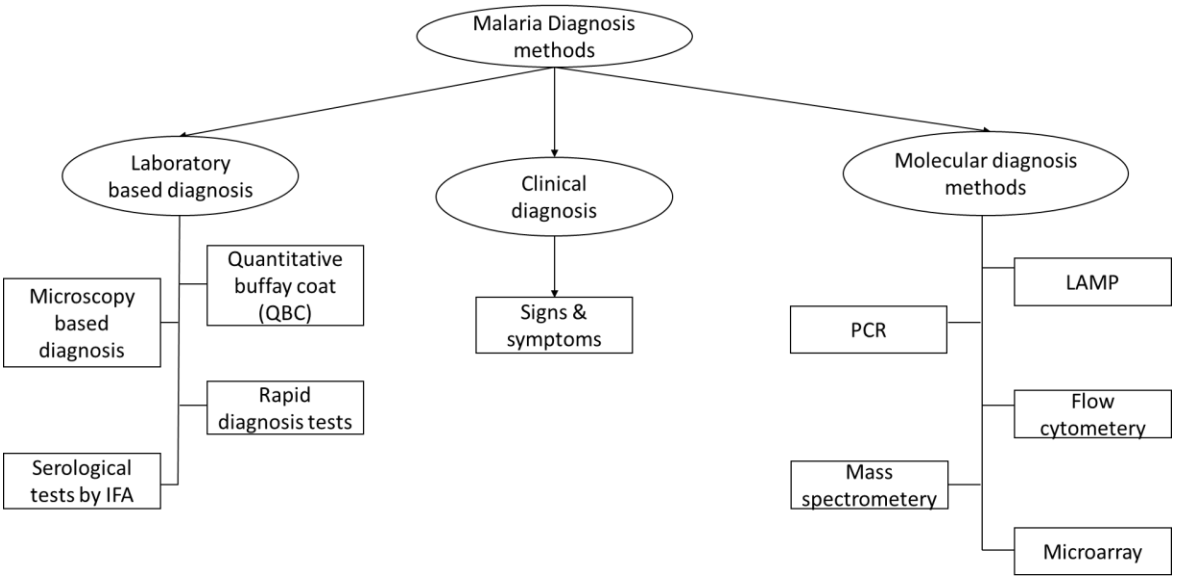


Figure 2: Schematic representation of current diagnosis methods available. The clinical diagnosis is referred to diagnosis by doctors by observing signs and symptoms. Recently, many target non-virulent proteins from *plasmodium* have been identified, which are used for detection in molecular and laboratory diagnosis systems.

Clinical diagnosis of malaria: It is the most widely practiced and the least expensive method for diagnosis. The diagnosis is based on observations of the individual's signs and symptoms, including fever, headache, nausea, dizziness, diarrhea, chills, etc. (Mwangi et al., 2005). Many of these symptoms are common in many other diseases; thus, it decreases the specificity of the malarial diagnosis. Also, they do not include the asymptomatic patients since no signs and symptoms are observed. This can lead to the indiscriminate usage of anti-malarial drugs, which can affect in quality of treatment and care, especially in the cases of small children. (Havlik et al, 2005)

Laboratory diagnosis methods: These are the recently developed methods for malaria diagnosis. It uses new laboratory diagnosis techniques to perform an early and accurate malaria diagnosis. It includes conventional techniques such as microscope-based observation of parasites in blood samples, concentration techniques such as quantitative buffay coat (QBC) and rapid diagnosis test (RDT) kits and molecular techniques which target specific compounds or components of cells for detection of parasites. (Mens et al, 2006; Mlambo et al, 2008)

- Microscopy-based diagnosis: Microscopy is a widely used diagnostic technique for detecting the malaria parasite in blood samples. A small amount of blood is collected from the patient and smeared onto a microscope slide to perform a microscopy-based diagnosis of malaria. The slide is then stained with a specific stain, most commonly Giemsa stain, which helps to visualize the malaria parasite. (Joseph et al, 2019) The stained slide is examined under a microscope by a skilled technician, who searches for the presence of the malaria parasite in the patient's blood. (Joseph et al, 2019)

Microscopy-based diagnosis of malaria has several advantages. It is inexpensive and widely available, making it an ideal tool for resource-limited settings. It is also highly specific and sensitive, enabling accurate malaria diagnosis, even in low-level parasitemia. However, there are some limitations to the microscopy-based diagnosis of malaria. The technique requires skilled personnel to correctly prepare and interpret the blood smear, which may not always be available in all settings. It can also not differentiate between different species of malaria parasites, which may require further testing with other diagnostic methods. (Bharti et al, 2007)

- Molecular diagnosis of malaria: These are the recently developed methods. It uses the new laboratory diagnostic techniques which target parasitic genome detection via nucleic acid amplification, for example, PCR, LAMP and QBC; thus, it can detect the parasite at a meagre amount, thus at the early stage of infection. (Erdman & Kain, 2008) Other techniques, such as flow cytometry, microarray and much more, use hemozoin, a pigment found in blood post-malarial infection. Thus, these methods of targeting a specific molecule for detection increase the specificity and sensitivity to the greatest extent. (Pornsilapatip et al, 1990)

However, since these techniques can only be performed in laboratories with machines which requires some specific conditions and place to work, it becomes a massive obstacle while conducting diagnosis for a large population, especially in rural areas where there are lesser facilities, resources and manpower since for operating and conducting these diagnostic test also requires trained professionals. It makes these diagnosis test less affordable to the rural population where malaria is more prevalent. (Tangpukdee et al, 2009)

All the above techniques of them have their pros and cons. The laboratory techniques are accurate for diagnosis but less affordable, and in places lacking resources, they cannot be used even though they show rapid accurate results. Other techniques are more affordable but less sensitive, thus more chances of mistreatment. Currently, the most recent, rapid, affordable diagnosis method is through checking the diagnosis by Rapid diagnosis test (RDT) kits designed for detecting a specific protein in malaria. (Banchongaksorn et al, 1996)

Rapid diagnosis Tests:

Rapid diagnostic tests (RDTs) have several advantages over other diagnostic methods, such as microscopy and polymerase chain reaction (PCR). RDTs offer a convenient, reliable, and cost-effective method for diagnosing malaria, particularly in resource-limited settings.

- Quick and easy to use: RDTs require minimal training and can be performed by non-expert health workers. They provide faster results, allowing timely diagnosis and treatment. (Moody, 2002)
- No special equipment needed: Unlike microscopy and PCR, RDTs do not require sophisticated equipment or electricity, making them ideal for use in low-resource settings where such resources may be limited.
- Highly sensitive and specific: RDTs are highly accurate and can detect low levels of malaria parasites in blood samples with high sensitivity and specificity. (Evans et al, 2018)

- Reduced risk of human error: RDTs are less prone to human error compared to microscopy, where the interpretation of slides can be subjective and influenced by the experience and skill of the technician.
- Reduced risk of transmission: RDTs require only a finger prick for blood collection, which minimizes the risk of transmission of other blood-borne infections, such as HIV and hepatitis B and C.

Based on the detection of the specific analyte, there are various techniques used in RDT kits are:

- Immunoassays: These tests detect the presence of an antigen in a sample. The most common types of immunoassays used in rapid diagnosis test kits are lateral flow assays, also known as immune-chromatographic assays, which use gold or latex particles to indicate the presence of a specific antigen visually.(Ratsimbasoa et al, 2008)
- Enzymatic assays: These tests detect the presence of specific enzymes in a sample, which may indicate the presence of a particular disease or condition. (Kaja et al, 2015)
- Biosensors: These tests use a sensor that detects the presence of a specific target molecule, such as a protein or nucleic acid. The sensor produces an electrical signal, which is measured and interpreted to determine the presence or absence of the target molecule. (Singh, 2019)

For malaria diagnosis, the analytes commonly being targeted for diagnosis are glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), Histidine rich proteins -II (HRP-II)(Kyabayinze et al., 2008), etc. These proteins are not part of var genes of *plasmodium* and are conserved in all *plasmodium* strains and are expressed in high levels during all blood infection stages, making them good candidates for detection. (Joseph et al, 2019) The RDTs used specific antigen-antibody interaction to capture these proteins. However, the recent testing of EDTs has reported many false positive cases for other cases like RF-positive non-malaria patients, and there is an increase in false negative cases in patients with new *plasmodium* strain infection without Histidine rich proteins. (Lee et al, 2014) The Panel detection score (PDS) for the common RDTs like SD-Bioline, Falcivax, para screen device, etc., were susceptible and accurate in 2000. According to WHO testing round 8 held in 2018, the PDS score value has decreased for each of them below 75% and thus is unreliable.(Steketee et al, 2021) They were designed specifically for the detection of HRP-II and *p*LDH.
The results were explained as due to the loss of HRP-II in the newer strains of *Plasmodium* and due to the non-specific binding of Antibodies designed for detecting *plasmodial* proteins. (Cheng et al, 2014) In subtropical regions like South Asian countries where the average temperature is higher, it causes antibodies' degradation and thus causes detection errors. An alternative to antibody-based detection can be used using aptamers-based biosensors to overcome these problems.

Aptamers:

Aptamers are short, single-stranded nucleic acids or peptides that can bind to specific molecules with high specificity.(Cheung et al, 2018) They are often referred to as molecular recognition elements and have gained considerable attention in recent years for their potential applications in fields such as medical diagnosis, therapeutics, and biosensors.
In medical diagnostics, aptamers are used as molecular probes for the detection of specific biomolecules, such as proteins, nucleic acids, and small molecules. They can be incorporated into various platforms, such as biosensors and lateral flow assays, to provide rapid and sensitive detection of disease biomarkers. (Yüce et al, 2015)
In therapeutics, aptamers are used as drugs or drug delivery vehicles due to their high binding affinity and specificity for target molecules. They can be used to target cancer cells, viruses, and bacteria, among other targets, and have shown promising results in preclinical and clinical trials. (Yüce et al, 2015)
In biosensors, aptamers are used as recognition elements to detect analytes in complex biological samples, such as blood, urine, and saliva. They can be integrated into various biosensor platforms, such as surface plasmon resonance and quartz crystal microbalance, to provide label-free and real-time detection of analytes. (Singh, 2019)

Properties of Aptamers:

- Aptamers are made up of nucleic acids or peptides that are relatively easy to synthesize or modify, and their binding affinity and specificity can be optimized through in vitro selection methods.
- They have a high binding affinity for their target molecules, often in the nanomolar to picomolar range, and can distinguish between structurally similar molecules with high specificity.
- In addition, they have low immunogenicity and can be easily modified to improve their pharmacokinetic properties, such as half-life and biodegradation.(Zhu et al, 2015)

Aptamer Selection:

It involves an iterative process of *in vitro* selection from a large pool of random oligonucleotides or peptides (10^{15} to 10^{17} oligonucleotide pool), known as a library. The library is incubated with the protein, and the aptamers that bind with high affinity are separated from the non-binding ones. The selected aptamers are then amplified and subjected to additional selection rounds (17 to 20 rounds) until a desired level of binding affinity and specificity is achieved. Several in vitro selection methods have been developed, including systematic evolution of ligands by exponential enrichment (SELEX) for nucleic acid aptamers and peptide display techniques for peptide aptamers. (Joseph et al, 2019; Tuerk & Gold, 1990)

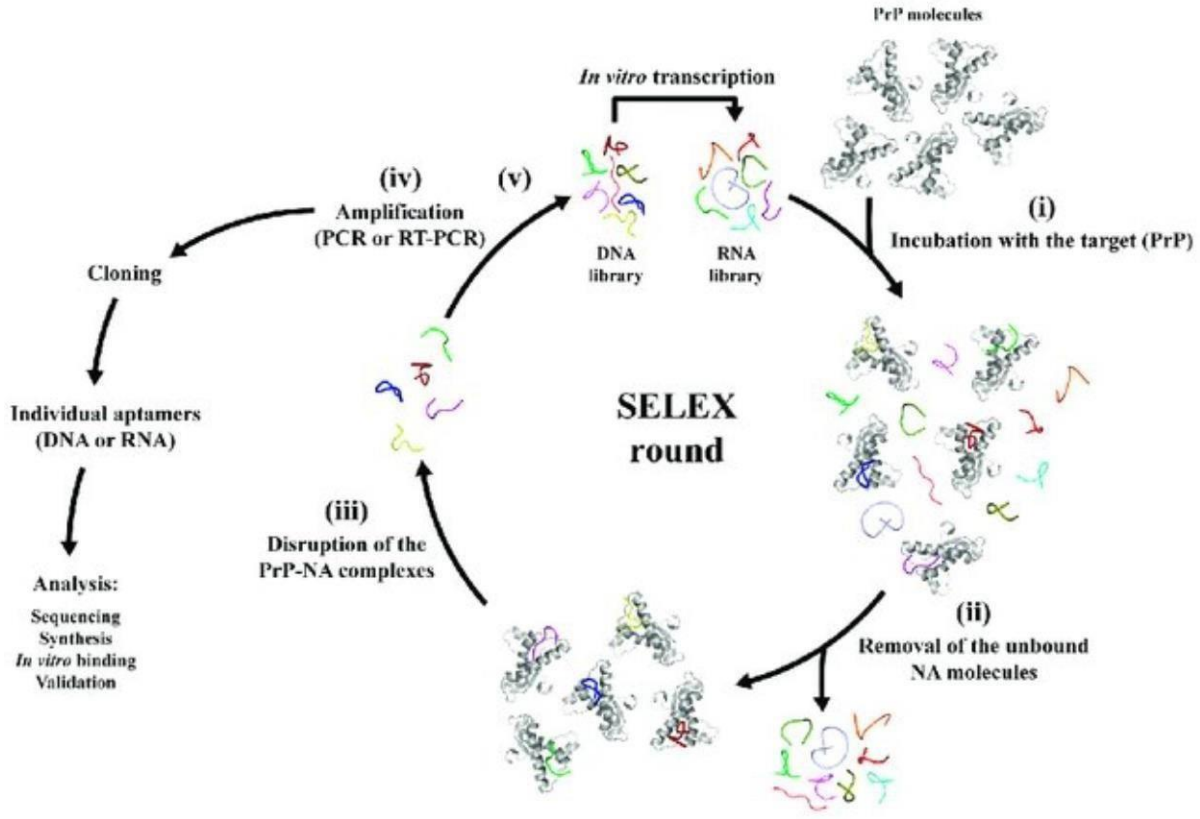


Figure 3: Systematic evolution of ligands by exponential enrichment. (Macedo & Cordeiro, 2017) From an extensive DNA/RNA library incubated with the target protein, several oligonucleotides which bind with the target with high affinity are selected, cloned and sequenced. This cycle continues up to 17- 20 times to get more aptamers.

To eradicate malaria in India, the malarial diagnosis is especially required for the rural regions where monetary and human resource malaria is still prevalent due to the unavailability of facilities. For this, I aim to make rapid, accurate, sensitive, specific and cost-effective RDT kits which can be made available to people quickly. There are aptamers generated by SELEX which bind to *Plasmodium* proteins such as pL1(Choi & Ban, 2016), 2008s(Cheung et al, 2013), pL11 and many more, which bind Lactate dehydrogenase of various species of *Plasmodium* and Pf R1, PfR3, PfR6 binds to HMGB-1 protein. (Joseph et al, 2019)

Lactate dehydrogenase (LDH):

Lactate dehydrogenase is an important enzyme which comes in the class of oxidoreductases. The function of this enzyme is to catalyze reversible conversion reactions of pyruvate and lactate by utilizing NADH and NAD+ as co-factors. LDH is critical for maintaining the energy balance in cells under different physiological conditions.(Farhana & Lappin, 2022) In normal conditions, the energy generation occurs by the glycogenesis pathway followed by the TCA cycle, which maximizes the energy generation of 38 ATP. However, in anaerobic conditions, the end product of glycolysis, i.e., pyruvic acid, is used by lactate dehydrogenase and is converted to lactate.(Forkasiewicz et al, 2020) In tissues with high energy demands, such as skeletal muscle, LDH is primarily involved in the production of ATP through

anaerobic respiration under conditions of low oxygen availability, such as during intense exercise. (Farhana & Lappin, 2022)
Plasmodium lactate dehydrogenase (pLDH) is an enzyme found in *Plasmodium* species, the parasites that cause malaria. This enzyme plays a crucial role in the parasite's metabolism, as it catalyzes the conversion of pyruvate to lactate, an essential step in producing energy for the parasite. (Kaja et al, 2015)
The pLDH enzyme is a tetramer composed of four identical subunits, each containing a binding site for the coenzyme NADH. (Dunn et al, 1996) During the catalytic cycle, pLDH binds pyruvate and NADH, which undergoes a series of redox reactions that ultimately form lactate and NAD+. The release of lactate provides energy for the parasite's survival and growth.
pLDH has become an important target for developing new anti-malarial drugs, as it is essential for the parasite's survival and is not found in human cells. (Penna-Coutinho et al, 2011)
In recent studies, the structure of *pf*LDH revealed two sites different from mammalian LDH, which can be targeted for diagnosis and drug treatment. The antigenic loop is present from residue 205-221, and the substrate-specific loop is present from residues 97 to 108. The aptamers selected for diagnosis also bind to substrate-specific loop, which differs and thus helps in the specificity and sensitivity of RDT. (Dunn et al, 1996)

Aptamer immobilization through N-hydroxysuccinimide (NHS) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) coupling:

The conjugation of NHS and EDC is a standard mechanism used in protein bio-conjugation and cross-linking. NHS is an amine-reactive compound that can react with primary amines on proteins, while EDC is a carbodiimide that can activate carboxylic acid groups on a protein.

The coupling reaction usually involves activating carboxylic acid groups on the protein with EDC. This activation creates an unstable O-acylzone intermediate that can rapidly hydrolyze to form an unstable intermediate that can react with primary amines in the NHS. The reaction between the activated protein and the NHS forms an amide bond that binds the two molecules together. The NHS/EDC coupling mechanism is commonly used in protein immobilization applications, such as the covalent attachment of proteins to solid surfaces or the cross-linking of proteins to form stable complexes. The coupling reaction usually occurs in an aqueous (MES buffer) with a slightly acidic pH of 4.5 - 6.5, optimizing NHS and EDC's reactivity. (Billiet et al, 2013)

One of the advantages of the NHS/EDC coupling mechanism is that it is selective and specific, as it affects the primary amino groups of proteins, usually found in lysine residues. This specificity allows for precise targeting and control of the conjugation reaction, resulting in stable and homogeneous protein conjugates. (Wang et al, 2011)Another advantage of the NHS/EDC coupling mechanism is that it is gentle and does not require harsh reaction conditions such as high temperatures or harsh chemicals. This mild reaction state helps preserve protein structure and function and minimizes the possibility of protein denaturation or degradation (Vashist, 2012).

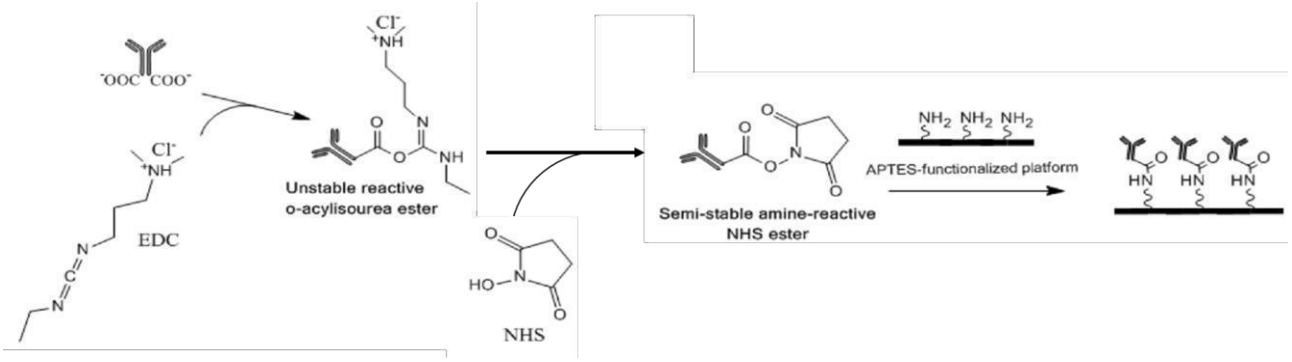


Figure 4: Coupling reaction of NHS/EDC to immobilize biotinylated aptamers on a surface (Vashist, 2012) EDC first activates carboxyl groups, producing an intermediate called O-acylisourea that is reactive with amines and spontaneously interacts with primary amines of biotin to produce an amide bond and an isourea by-product.

Chapter 2 Materials and Methods

PfLDH gene was integrated into the pET 28a (+) vector with Kanamycin resistance were ordered from Genscript.

Transformation by electroporation:

- An aliquot of *Escherichia coli* NEB turbo competent cells stored at -80ºC was thawed on ice.
- 100ng of plasmid DNA was added to the cells. Mix the mixture gently by flicking.
- Add 100µl of autoclaved miliq water and transfer the mixture to a prechilled cuvette for electroporation.
- Incubate the cuvette on ice for 20-25 minutes.
- The electroporation was done by BIO-RAD GenePulser Xcell at 2.5kV for 4 seconds.
- Then LB media was added to the mixture, which was incubated at 37ºC for 20 minutes at 300rpm (rotation per minute).
- The mixture was then spread on an LB agar plate containing a 20µg/ml kanamycin concentration, and the plates were placed for overnight incubation at 37ºC.
- The plates were then stored at 4ºC until further use.

Transformation by heat-shock method:

- An aliquot of *Escherichia coli* BL21 DE3 competent cells stored at -80ºC was thawed on ice.
- 100ng of plasmid DNA was added to the bacteria cells. Mix the mixture gently by flicking.
- Incubate the mixture on ice for 10 minutes, followed by 3 minutes of heating at 37ºC.
- Then the immediate transfer of mixture to ice and incubate for 2 minutes.
- Add 150µl of LB media to revive cells and incubate at 37ºC for 15-20 minutes.
- The mixture was then spread on an LB agar plate with a 20µg/ml kanamycin concentration.
- The plates were kept for incubation overnight at 37ºC.
- Until further use, the plates were stored at 4ºC.

Plasmid isolation through mini-prep kit:

10ml cultures were inoculated from transformed *E. coli* NEB turbo cultures and grown in LB media with a kanamycin concentration of 20µg/ml overnight at 37^{o}C. Plasmid isolation was done with QIAgen mini prep kit. The plasmid isolation step followed were:

- The culture was spun at 8000rpm, and the media was extracted.
- The cells in the pellet were resuspended in 250µl of buffer P1 and then transferred to a microcentrifuge tube.
- Add 250µl of buffer P2 to the mixture and mix gently by inverting the tube four to six times. The solution will become clear.
- Make sure that cell lysis does not go on for five minutes.
- Add 350µl of buffer N3 and mi gently by inverting the tube.
- The solution was centrifuged at room temperature for 10 minutes at 13,000 rpm.
- Add 800µl of supernatant and spin it for 1 minute again.
- Wash the column by adding 750µl of PE buffer and spin at 13,000 rpm for 1 minute. Discard the flow through. Respin the column and make sure all the flow through has been cleared.
- Wash the column in EB buffer and elute the plasmid DNA.
- Check the concentration of plasmid DNA on Nanodrop and then store it at -20^{o}C until further use.

Restriction digestion:

pET 28a (+) containing *pf*LDH was double digested using restriction enzymes XhoI and NcoI to check the presence of a gene in the plasmid,

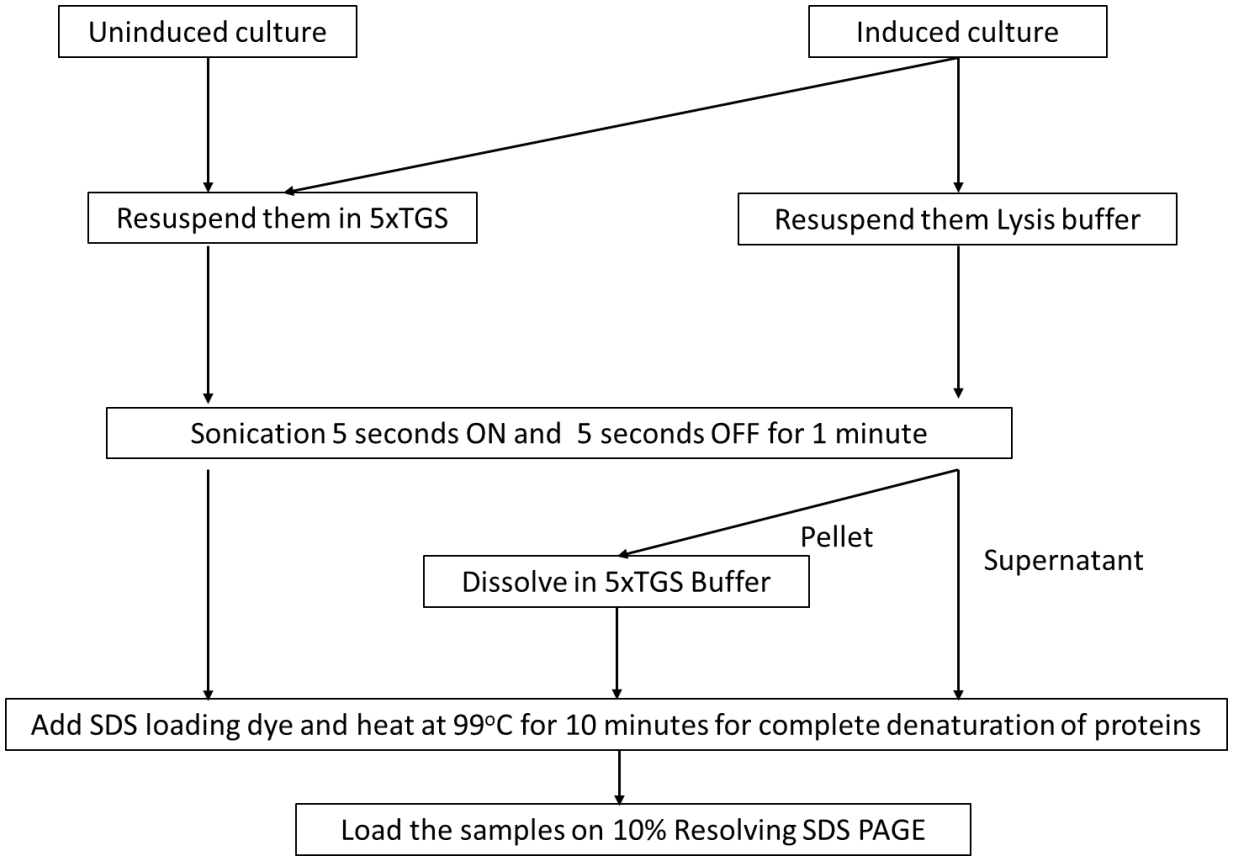
Reaction mixture:

Reagents	**Reaction (**µl)	**Control(**µl)
10x NEB Buffer 3.1	1	1
RE enzymes	0.8	0
DNA (~100ng)	2.5	2.5
Miliq H_2O	5.7	6.5

- Incubate the mixture at 37^{O}C for digestion for 1 hour.
- Stop the reaction by adding 6x SDS
- Load the samples on 1% agarose gel and check the fragment under UV light.

Protein Expression test:

A swab of colonies from transformed *E. coli* BL21 DE3 cells from the plate was inoculated in 10ml LB media containing kanamycin (concentration - 20µg/ml) in two different cultures, one with IPTG (induced) and one without IPTG (concentration - 0.5mM) induction (uninduced) overnight at 37°C.



The cell pellet was resuspended in TGS to dissolve all the proteins and check the expression. The protein solubility in the lysis buffer was checked by checking the protein band in the supernatant or pellet to proceed with protein purification.

Table 1: 10% resolving gel mix for SDS PAGE

S No.	Reagents	Volume (ml)
1.	MiliQ water	4
2.	AB mix(30%)	3.3
3.	1.5M Tris (pH=8.8)	2.5
4.	10% SDS	0.1
5.	10% APS	0.1
6.	TEMED	0.004

Table 2: 5% stacking gel mix for SDS PAGE

S No.	Reagents	Volume (ml)
1.	MiliQ water	1.4
2.	AB mix(30%)	0.33
3.	1.5M Tris (pH=8.8)	0.25
4.	10% SDS	0.02
5.	10% APS	0.02
6.	TEMED	0.002

Table 3: SDS PAGE Destaining Agent

S No.	Reagents	Volume (ml)
1.	MiliQ water	35
2.	Ethanol	10
3.	Glacial acetic acid	5

SDS PAGE Staining Agent: Destaining agent + coomassie dye

Table 4: Lysis buffer composition

S No.	Reagents	Concentration
1.	NaCl	500(mM)
2.	$MgCl_2$	5(mM)
3.	Tris (pH=8)	50(mM)
4.	Imidiazole	15(mM)
5.	Glycerol	10%

Table 5: Loading Buffer (Buffer A) composition for affinity chromatography (Ni-NTA)

S No.	Reagents	Concentration
1.	Tris (pH=8)	50mM
2.	NaCl	500mM
3.	Imidiazole	15mM

Table 6: Elution buffer (Buffer B) composition for affinity chromatography (Ni-NTA)

S No.	Reagents	Concentration
1.	Tris (pH=8)	50mM
2.	NaCl	500mM
3.	Imidiazole	500mM

Table 7: Buffer composition for size exclusion chromatography (Superdex 200)

S No.	Reagents	Concentration
1.	Tris (pH=8)	50mM
2.	NaCl	100mM
3.	DTT	1mM

Protein purification of *pf*LDH:

- 10ml Primary cultures of transformed *E. coli* BL21 DE3 strain containing *pf-LDH* was grown in Kanamycin (20µg/ml) containing medium overnight at 37ºC.
- From the primary culture, 1 litre of secondary culture (kanamycin: 20µg/ml) was inoculated and grown at 37^{O}C for 8 hours until OD reached 0.6.
- The culture was induced by adding IPTG (concentration: 0.5mM) and grown for 4 hours at 37^{O}C.
- The cultures were centrifuged at 5000rpm at 4 degrees, and the pellet was collected, frozen, and stored at -80^{O}C until further use.
- The pellet was resuspended in lysis buffer and mixed gently to achieve a homogeneous mixture.
- The mixture was then sonicated in the 'Sonics Vibra cell' sonicator for 1 second ON and 3 seconds OFF, at an amplitude of 60%.
- The lysed cells were centrifuged at 13,000 rpm for 5 minutes at 4^{O}C, and extract the supernatant.
- The protein purification was done using AKTA prime plus FPLC and Ni-NTA column. A column wash was given for 40 minutes with autoclaved miliq water, followed by buffer A prior use.
- The supernatant was then loaded through the Ni-NTA column along with buffer A.
- The protein samples were collected at a gradient of 5% Buffer B, 10% buffer B, 20% buffer B, 40% buffer B, 60% buffer B and 100% buffer B.
- 10 ml of each of the solutions was collected.

- The fractions with the peak of the chromatogram of abs 280nm were collected and were loaded along with SDS loading dye after denaturing at 99^{O}C for 10 minutes.
- The samples were loaded on a 10% resolving gel and checked for the desired protein at the size.
- Make sure that a single band is present. In case of multiple bands at different sizes, proceed for Size exclusion chromatography using superdex 200 column.
- The fractions (in the case without contamination) containing the desired protein were concentrated with 10kDa centricon at 5000rpm at 4^{O}C and flash frozen in small aliquots and stored at -80^{O}C until further use.
- The fraction containing the desired protein and contaminants was collected and concentrated by centricon with ten kDa at 5000rpm at 4^{O}C and then loaded on superdex 200 in BIO-RAD NGC chromatography system along with SEC buffer.
- The fractions on the chromatogram of Abs280nm with the desired peak were collected.
- The fractions collected were then loaded on 10% resolving SDS PAGE to check the desired protein without contamination.
- In case of getting the pure protein, collect and concentrate the fractions with 10kDa centricon at 5000rpm at 4^{O}C.
- Small aliquots of the collected protein were made, flash frozen, and stored at -80^{O}C until further use.

*Pf*LDH activity check:

Lactate dehydrogenase is responsible for converting pyruvate to lactate conversion by using H^+ ions from NADH. The activity of *pf*LDH was checked by measuring the decreasing concentration of NADH with increasing time. The absorbance for NADH was measured at 340nm.

For the concentration measurement from absorbance values, the absorbance of increasing concentration of NADH was measured, and the standards were used to calculate the concentration of NADH throughout the reaction.

Table 8: Reaction buffer composition for *pf*LDH activity:

S. No.	Reagents	Concentration(mM)
1.	Tris (pH=8)	50
2.	NaCl	100
3.	$MgCl_2$	10

NADH standards were made with NADH concentrations of 0µM, 25 µM, 50 µM, 100 µM, 200 µM, 400 µM, 600 µM. For the reaction, pyruvate concentration used as 1mM and protein concentration varying from 0nM to 100nM was used, and a decrease in NADH absorbance was measured at 340nm.

Aptamer protein binding assay:

Preparation of Aptamer: biotinylated aptamers were ordered from Synbio technologies. The aptamers were hydrolyzed with nuclease-free water and incubated for 15 to 20 minutes at room temperature at 250rpm. An aliquot aptamer of 1 µM was prepared and heated at 90^{O}C for five minutes for complete denaturation, then refolding at room temperature for 30 minutes.

100nM of Aptamer was incubated with protein in a reaction buffer for one hour on ice. Then ST loading buffer was added to samples and loaded 6% Native PAGE ran at 4^{O}C at 180 volts. The native PAGE was stained by SYBR GOLD dye for 30 minutes followed by 3 to 4 washes with miliQ then the Image was taken at BIO-RAD Geldoc Go Imaging system .

Table 9: 6% Native PAGE composition

S No.	Reagents	Volume (ml)
1.	MiliQ water	5.9
2.	AB mix(30%)	2
3.	5xTBE buffer	2
4.	10% APS	0.1
5.	TEMED	0.004

Calorimetric change assay:

Lactate dehydrogenase converts pyruvate to lactate and vice versa by using H^+ from NADH, forming NAD+. Here, we will be using the reverse reaction, i.e., conversion of lactate to pyruvate leading to the formation of NADH from NAD+, which is used by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) dye to form formazan crystals. Thus, over time the colour of the solution changes from yellow to blue.

The reaction mixture contained 1mM lactate, 600 µM of NAD+, ten µM of Phenazine methosulfate, and 250 µg/ml MTT in reaction buffer, which works as a transporter taking H^+ to MTT dye for the formation of formazan crystals.

After adding different concentrations from 0nM to 100nM of proteins, the mixture was incubated for 30 minutes in the dark at 37^{o}C. The reaction was stopped by adding 40 µl of 0.5% Trifluoroacetic acid. The mixture was centrifuged at 13,000 rpm for 10 minutes, and the supernatant was discarded. The formazan crystals in the pellet were dissolved in 100% DMSO, and the absorbance was measured at 518nm.

The conc. of NADH formed during the reaction was calculated by measuring the absorbance value for formazan formed by adding 0 µM to 800 µM NADH.

On paper, the concentration of substrate lactate, NAD+, MTT and PMS were increased. Two mixtures were prepared.

A) 50ul of Lactate(0.7M) + NAD+ (0.4M) + Tris (0.2M)
B) 50ul of MTT Dye (2.5mM) + PMS (0.1mM)

Before reaction both the solutions were mixed and 30ul of the solution was applied on paper and incubated for 30 minutes.

Aptamer immobilization on Paper:

The immobilization of biotinylated aptamers p11, pL1, 2008s was done via streptavidin- biotin Interactions. 2.5 ul of 400mM EDC and 100mM NHS (prepared in 100mM MES) was added to paper surface and incubated for 1-2 hours at 4^{o}C. Then treated with washes of 30ul of 100mM MES buffer and left to dry for 30 minutes at RT. 2ul of 100ug/ml streptavidin was added to the surface spot where aptamers would bind and the paper was left to incubate for 1 hour. The washes with PBS-T buffer were given to remove unbound streptavidin and paper was dried for 20 mins. to bloc addition succinimidyl residues from EDC/NHS activation, 5ul of 10% BSA was added to streptavidin containing Zone. The paper was incubated for 30 minutes then given a as with 10ul PBS-T for three times. The excess fluid was drained out and paper was left to dry for 1 hour. 2ul of 1uM aptamers were added on surface at streptavidin added spot and incubated for 1 hour at room temperature. Before assays, 30ul aliquot of Reaction mixture as added to the surface and incubated for 10 minutes. Then proceeded with the reaction.

Chapter 3 Results

Protein Purification of *pfLDH:*

Restriction digestion for confirmation of gene in the plasmid:

After the transformation of *E. coli* NEB turbo cells by pET 28a plasmid vector having gene of interest. Plasmid was isolated from the cultured transformed cells, the verification of the presence of the plasmid and the respective gene size were performed by restriction digestion. The *pf*LDH gene in the pET plasmid vector is in between XhoI & NcoI sites. The band size for uncut plasmid, double digest for *pf*-LDH came at the designated areas. Due to the supercoiled nature of uncut plasmid, the uncut plasmid runs faster than the digested plasmid, which is linear DNA. Hence, even with greater size, the uncut plasmid band lies lower than its actual size.

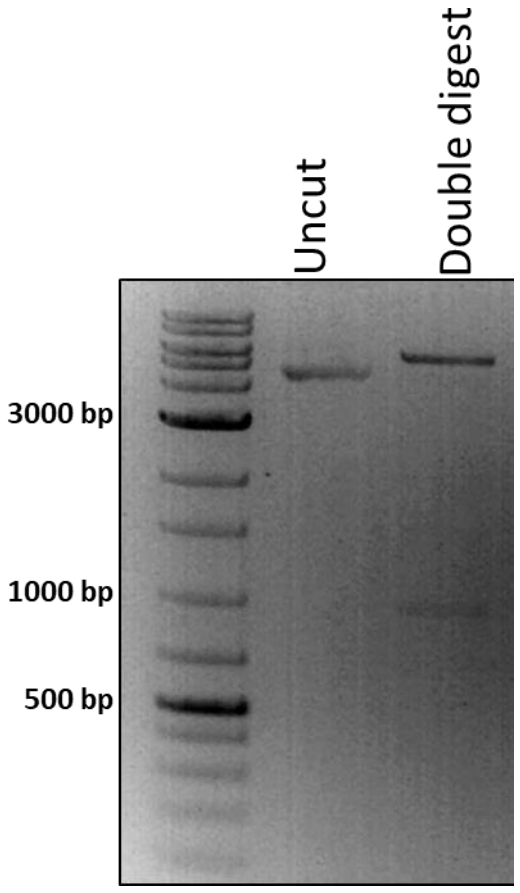


Figure 5: Restriction digestion of pET28a containing *pf*LDH. The fragment size for plasmid is 5369bp, the gene size *pf*-LDH is 977bp. The double digest shows both fragment of *pf*-LDH and pET+ *pf*-LDH (6346bp).

Protein expression and solubility check:

The BL21 DE3 strain of *E. coli* was transformed by pET 28a vector with the *pf*LDH gene for gene expression. The cultured cells, induced and uninduced after being grown, were pellet down and dissolved in TGS buffer and Lysis buffer. The samples were then sonicated for cell lysis and then loaded after heating the samples for denaturation. The *pf*LDH protein size is 34.1 kDa, and the darker band below 37kDa band in the induced total culture shows the expression of the protein. The band at the same position in the supernatant shows that the protein is soluble in Lysis buffer.

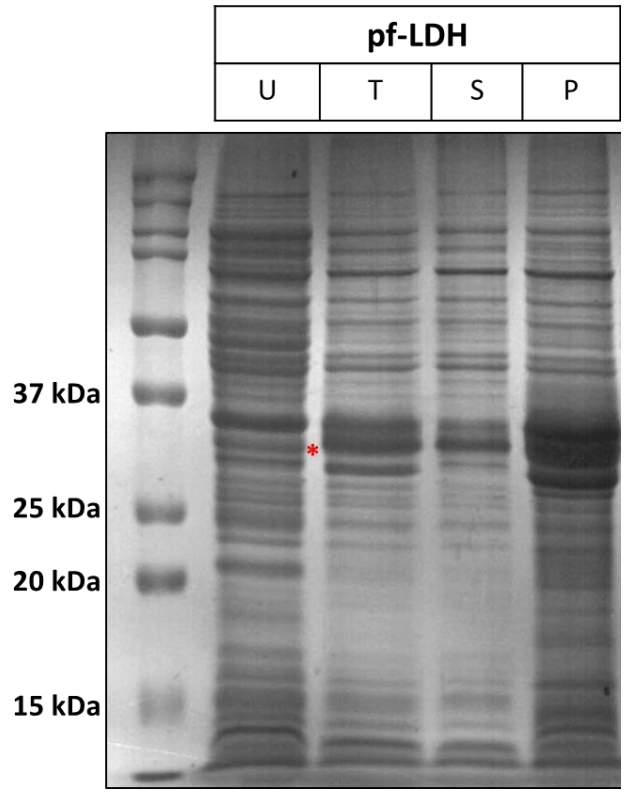


Figure 6: SDS PAGE run to check the solubility of *pf*-LDH. The size of *pf*-LDH is 34.1 kDa. There is a darker band in IPTG-induced cells (T) compared to uninduced cells (U) between 25 kDa and 37 kDa for *pf*-LDH followed by a darker band in the supernatant(S) compared to pellet (P). (Red asterisk shows the protein of interest to be purified.)

Protein Purification:

The purification of *pf*LDH from cell lysate supernatant using Ni-NTA-based chromatography. The protein was loaded alongside buffer A and eluted at a gradient of 0% buffer B, 10% buffer B, 20% buffer B,40% buffer B, 60% buffer B, and 100% buffer B.

The eluted fractions were loaded on an SDS PAGE, and alongside the protein, multiple bands were seen near 75 kDa and above 100 kDa. The fractions were concentrated and passed through the superdex 200 columns for size exclusion chromatography to remove the contaminants with higher molecular weight. A single prominent peak was observed in the SEC

chromatogram at 280nm. The fractions containing the protein eluted in the peak were collected and loaded on SDS PAGE, which showed the same bands as Ni-NTA fractions. The fractions with lesser contaminants were pooled, concentrated and used for further assays.

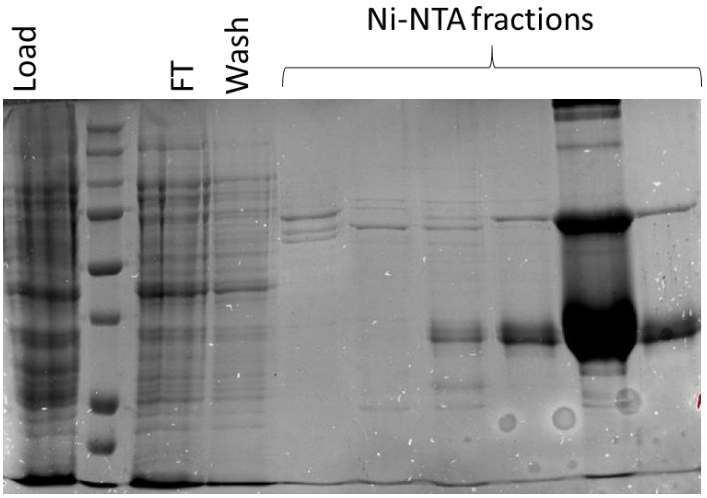
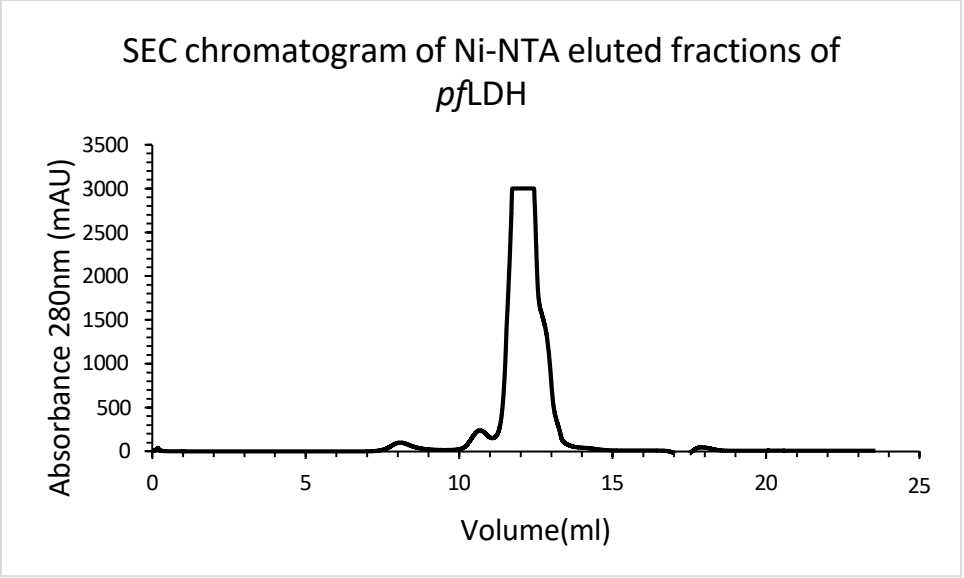


Figure 7: SDS PAGE with Ni-NTA eluted fractions. Protein was eluted at a gradient of 0% buffer B, 10% buffer B, 20% buffer B,40% buffer B, 60% buffer B, and 100% buffer B. The fractions here contain protein eluted in 20% buffer B, 40% buffer B and 60% buffer B.

A)



B)

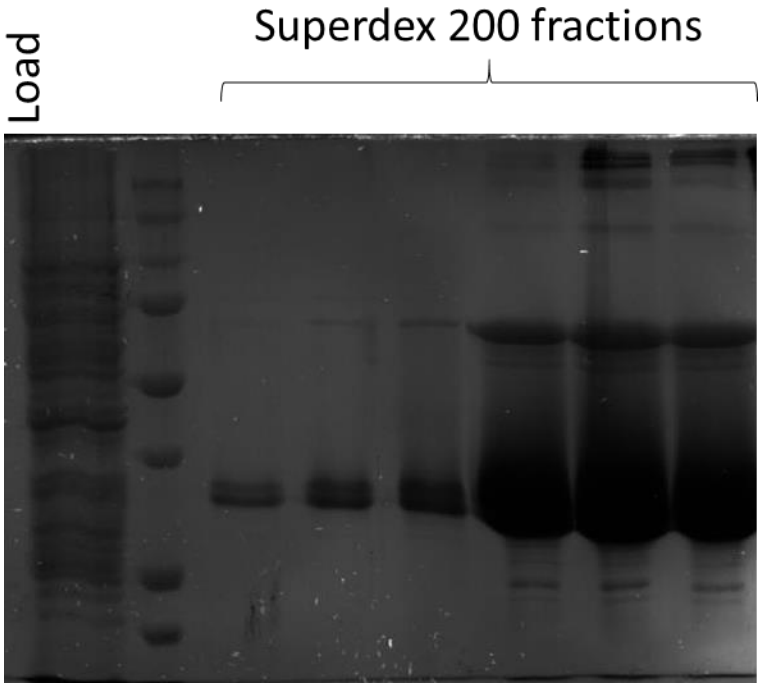


Figure 8: (A)Chromatogram and (B)SDS PAGE of superdex200 eluted fractions. The saturated peak coming at 11-12ml (Lane3&4), 12-13ml (Lane5&6), and 13-14 ml (Lane7&8) region of chromatogram at 280nm was collected and loaded on the SDS page**.**

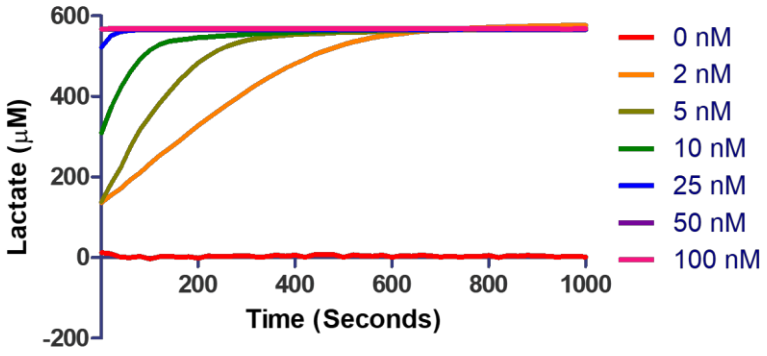


Figure 10: Confirmation of *pf*-LDH activity by lactate formation: Lactate dehydrogenase (LDH) enzyme works for converting pyruvate to lactate and vice versa and using hydrogen ions from NADH and converting it to NAD+. NADH shows fluorescence at 340nm, and its rate of decrease of fluorescence increases with increasing LDH concentration. Thus the rate of lactate formation is higher with a higher LDH concentration

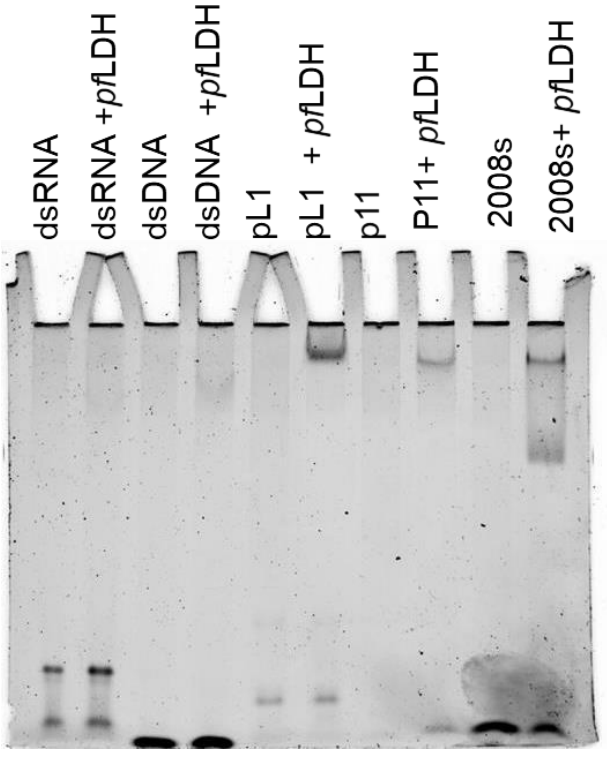
Protein activity check:

*pf*LDH is responsible for the conversion of pyruvate to lactate and vice versa. Different concentrations of protein were added with pyruvate as a substrate and NADH as a co-factor. As the reaction started, the amount of NADH started decreasing and thus the amount of lactate formed increased upto the point until the reaction saturated. As the concentration of protein added was increased in the reaction, the slope of curve increased concluding that the protein is active.

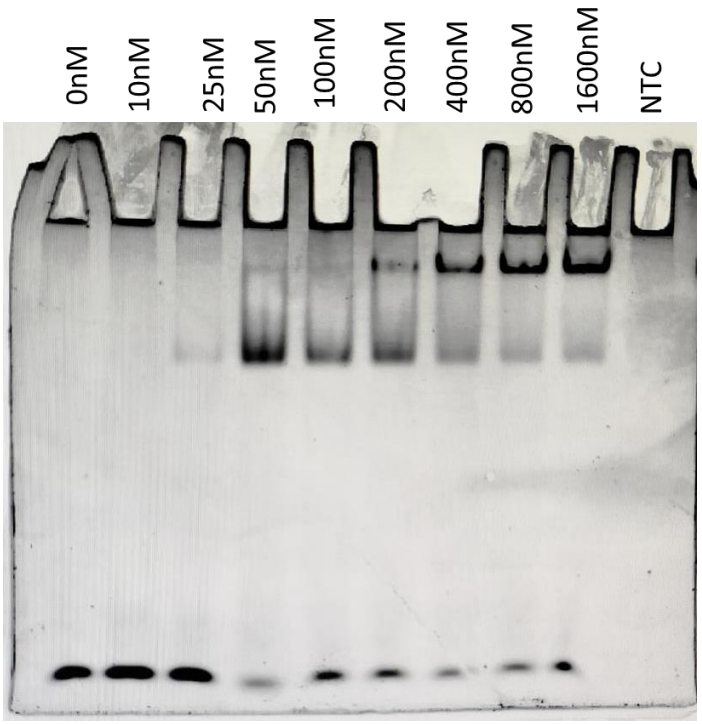
Binding specificity with 2008s Aptamer:

To check the binding of *pf*LDH with 2008s, it was incubated with various nucleic acids and other aptamers. The EMSA gel for the protein binding showed the complex formed by the binding of 2008s-*pf*LDH and none of the bands were seen in other wells. The aptamer binding was tested with a varied conc. Of *pf*LDH with 2008s and at low concentration of 25nM of protein was observed. For p11 it was 20nM and pL1 minimal binding was showed for 40nM.

A)



B)



C)

D)

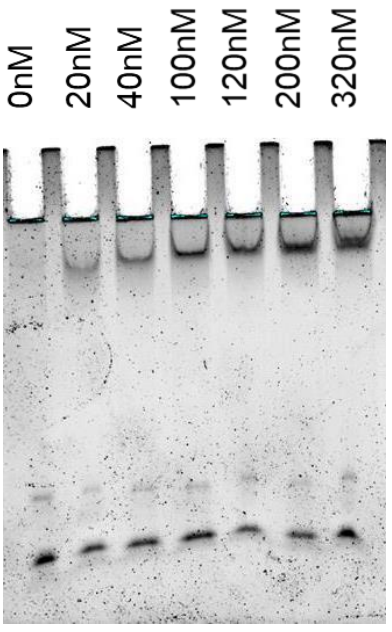


Figure 10: EMSA of *pf*LDH binding to nucleic acids (A) EMSA of Different nucleic acids binding to *pf*LDH showing aptamers specificity towards *pf*LDH. **(B)** EMSA of 2008s binding to various conc. of *pf*LDH. **(C)** EMSA of pL1 binding to various conc. of *pf*LDH. **(D)** EMSA of p11 binding to various conc. Of *pf*LDH.

Colorimetric assay to detect *pf*LDH:

The reverse reaction of *pf*LDH activity, i.e. conversion of lactate to pyruvate, was used for this reaction. The generation of NADH is a crucial step in this reaction. The NADH formed from NAD+ is required to convert MTT dye to form formazan crystals that are blue in colour.

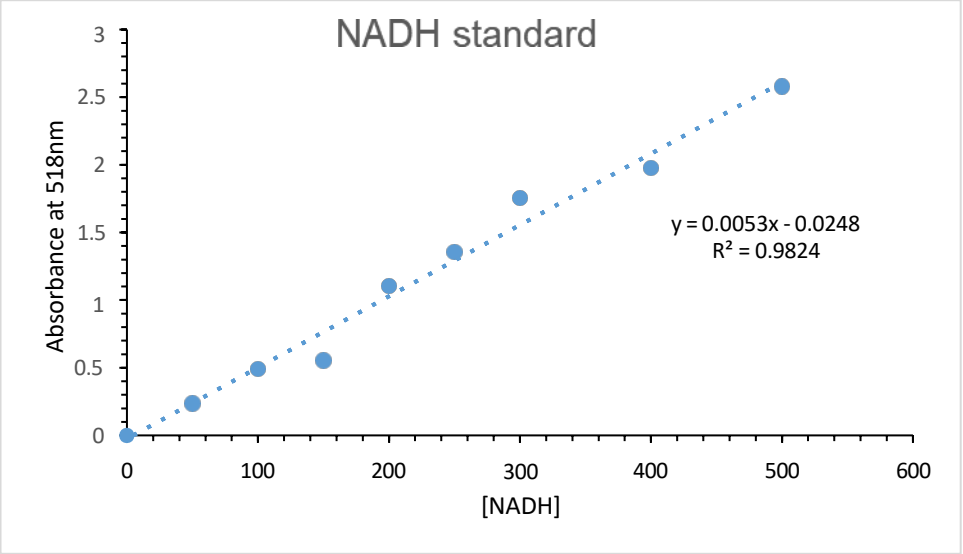
The substrate lactate concentration and co-factor NAD+ concentration were kept higher to avoid making them a limiting factor of the reaction. The reaction follows the conversion of lactate to pyruvate by lactate dehydrogenase, captured by Aptamer, leading to the generation of NADH by utilizing NAD+. This NADH is used by phenazine methosulphate, an electron transporter, to transfer hydrogen ions to MTT dye for the formation of formazan.

The assay was done with increasing concentration of *pf*LDH, and the reaction mixture was incubated for 30 minutes, as the protein conc. The formazan formed also increased, which was detected at 518nm.

Effect on colorimetric assay by protein-aptamer binding:

An equal concentration of unbound and aptamer-bound protein was added to the reaction mixture to verify the effect of Aptamer binding to protein. The reaction was incubated for a fixed period of 30 minutes, and at different time points, the amount of NADH formed was checked by checking the absorbance of formazan formed at 518nm. The values for the absorbance of formazan between Aptamer bound protein and unbound protein had no significant difference.

A)



B)

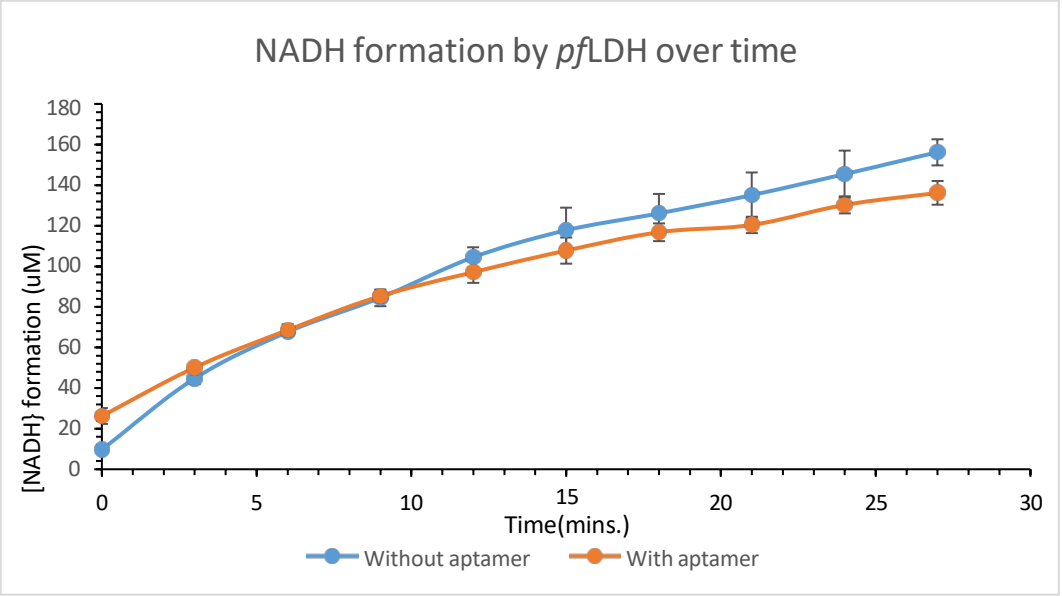


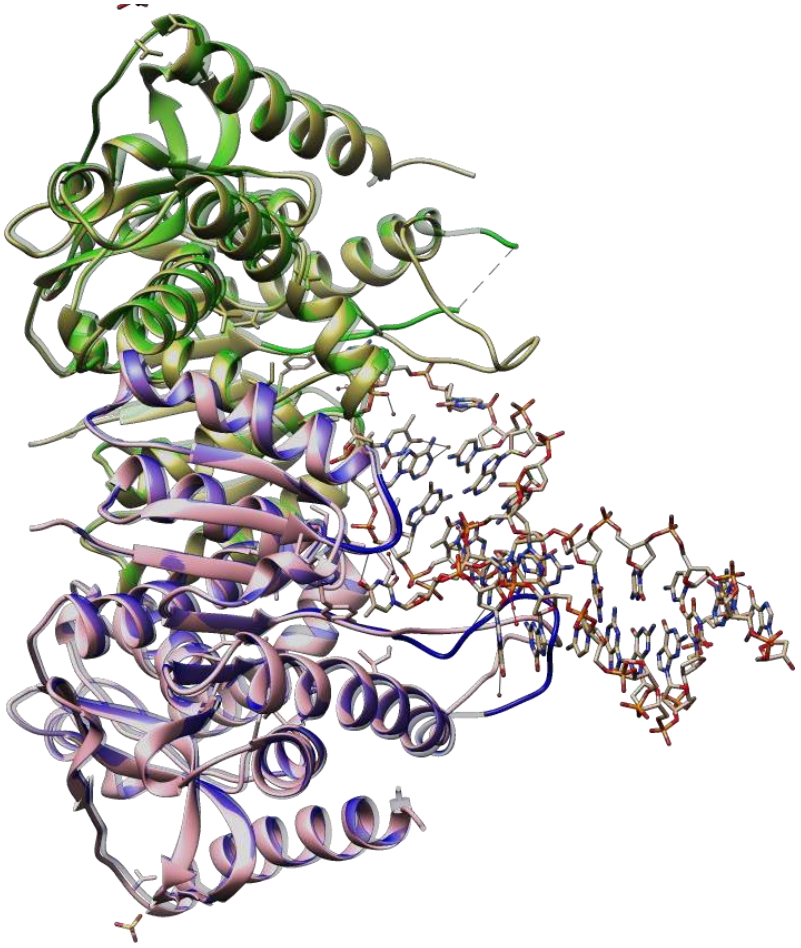
Figure 11: Colorimetric Assay Time-dependent reaction by checking the NADH formation by unbound and Aptamer bound *pf*LDH by measuring formazan absorbance 518nm.

Structure studies of 2008s and *pf*LDH binding:

The structure of *pf*LDH bound to 2008s (PDB ID: 3ZH2) was analyzed in chimera software to check the interaction between 2008s and *pf*LDH. The nucleotide bases showing interactions are G7, G8, T9, G11 and A22. Other nucleotides, A16, A18, and G19, show indirect stabilization via water residues. The backbone of A22 also shows linkage with the substrate specificity loop.

The *plasmodium vivax* LDH (PDB IB: 2AA3) was superimposed with the *pf*LDH structure. The pairwise alignment showed that the sequences are similar, but on superimposing the structures, there was a structural difference in the extended substrate-specific loop, which is the reason for the specific binding of 2008s for *pf*LDH only. In other LDH, for example, LDH from mammals like humans, dogs and pigs, this substrate-specific loop is not extended and thus cannot be captured by Aptamer.

A)



B)

S.No.	Amino Acid residue	Aptamer residue	Bond distance
1	ASP 35.A OD1	DT 9.E N3	2.982
2	ILE 36.A N	DT 9.E O4	3.099
3	ASP 90.A OD2	DG 8.E N2	3.065
4	ALA 85.A O	DA 22.E N6	2.810
5	ASP 90.A OD1	DG 7.E N1	2.456
6	LYS 84.A NZ	DA 22.E N1	3.131
7	LYS 88.A N	DG 11.E N7	2.912
8	SER 89.A OG	DG 11.E O6	2.653
9	SER 89.A N	DG 11.E O6	2.591
10	LYS 88.A NZ	DA 22.E OP2	2.869

Figure 12: Superposed structure of pvLDH on *pf*LDH-2008s bound structure and the H bond details. The green and pink coloured chains are of *pf*LDH, and the blue and pale yellow coloured chains are of *pv*LDH.

Chapter 4 : Discussion

Malaria is a disease caused by *plasmodium sp.,* parasite. It is a significant public health concern, especially in developing countries like India. One of the prevention strategies followed is early diagnosis of malaria. In developing countries, the affected population majorly consists of the rural population, where the reach of accurate laboratory-based diagnosis methods is unreachable due to a lack of resources and trained professionals. Hence, the use of rapid diagnostic test kits to detect malaria rapidly and accurately is of paramount importance.. However, diagnostic device accuracy is declining due to the emergence of novel parasites lacking the target proteins for diagnosis.

The primary purpose of this project was to create a reliable alternative to the present day RDT diagnosis method. Aptamers-based diagnosis is superior to antibody-based diagnosis in various ways some of which are its high accuracy, low costs of manufacturing, and easily modifiable short sequences. The detection kit targets the *plasmodium falciparum* lactate dehydrogenase enzyme. The detection is checked by colour change due to the reaction from the targeted enzyme. The colorimetric-based detection is based on the reversible conversion of pyruvate to lactate and lactate to pyruvate. Here, the unfavourable reverse reaction of lactate conversion to pyruvate occurs, generating NADH from NAD+. This NADH is used by MTT dye to form formazan crystals, which leads to changing colour from yellow to blue. This colour change can be detected through the naked eye and thus is used to detect LDH.

The docking studies of the structure of *pf*LDH with 2008s Aptamer (PDB ID: 3ZH2) showed that 2008s Aptamer specifically recognizes the substrate specificity loop present. Other than H-bonds, none of the other interactions were found. The H- bond interactions observed were between peptide-nitrogen bases and sugar-phosphate backbone-peptides on superimposing *plasmodium vivax* lactate dehydrogenase with *pf*LDH. The aptamer 2008s showed lesser binding with substrate loop from *pv*LDH and minimal interaction, which concludes the explanation for the specificity of the 2008s for *pf*LDH, also shown by Cheung et al., 2018. However, the reason behind the structural difference in the loop, even with the same sequence of proteins in the loops is still being studied.

The *pf*LDH protein was incorporated with 6x His tag, and the Ni-NTA affinity-based purification method was followed for purifying the protein. In the SDS-PAGE of Ni-NTA-based eluted fractions containing the protein of interest collected, there were more bands of different sizes in all the fractions. To minimize the contaminations, Size exclusion chromatography was performed. The SEC chromatogram showed a single sharp peak. The samples from fractions collected and loaded on SDS PAGE showed the same bands as from Ni-NTA fractions. The pI value of *pf*LDH is 7.12; hence the Ion exchange chromatography-based purification method is ineffective.

The protein activity was checked by pyruvate to lactate conversion and measuring the decreasing NADH fluorescence. The higher the concentration of *pf*LDH added to

the reaction, the faster the reaction reached saturation, showing that the purified *pf*LDH protein is active.

In *pf*LDH tetramer, the NADH binding site differs from the aptamer binding site. Hence, no effect was seen in the catalytic activity of *pf*LDH and aptamer-bound *pf*LDH. The conc. of Aptamer required for protein is two Aptamer: 1 tetramer, the amount of Aptamer used was 10:1 leaving the DNA band at the lowest region after using all the protein for making complex, thus leaving no tetramer unbound.

The current incubation time for the colour based detection by RDT for 100nM of *pf*LDH is 30 minutes. This reversible reaction of lactate to pyruvate and vice versa, at lower pH, the pyruvate to lactate formation is favoured, and there are more chances of the formation Pyruvate-NAD+ complex, which inhibits the interconversion. This complex formation is not favourable as the reaction buffer's pH increases, and the reaction is more favourable at higher pH.

Chapter 5 Future Perspectives

Aptamer based technology has a vast range of applications ranging from diagnosis and enzyme capture assays to being used as drugs which can binds to a specific molecule to either inhibit the protein or to inactivate the protein. The plasmodial parasite apart from LDH contains a variety of enzymes which are active during the infection in human host and are essential for the survival of parasite. The aptamers can be used to target these enzymes and the binding can be used for detection or inhibit the normal life cycle of plasmodium which can become a huge milestone in eradication of malaria. Due to low cost of production and easy modification in aptamers, it is possible to get rapid solutions for new variants of malarial parasite.

Some of the immediate objectives for the current project are: to be fulfilled for current project are:

- Integrating the aptamer technology for detecting pfLDH onto a microfluidic paper based device
- Optimizing the minimal concentration of pfLDH that can cause a color change
- Conducting tests with malarial blood samples
- Optimize the binding of other aptamers and the plasmodial proteins to get alternative to the current targeted enzyme.
- Modification of aptamers being used to increase the binding affinity to increase the sensitivity of the tests.

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